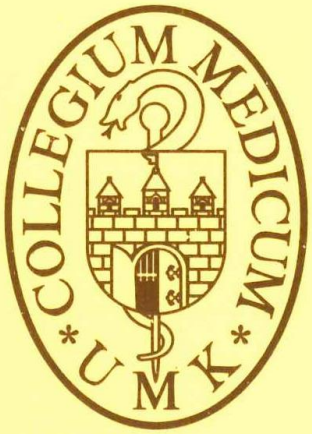
UNIWERSYTET MIKOŁAJA KOPERNIKA
w TORUNIU
COLLEGIUM MEDICUM im. LUDWIKA RYDYGIERA w BYDGOSZCZY

KAROL BIAŁKOWSKI

Antymutagenne białko MTH1 - rola biologiczna, regulacja aktywności enzymatycznej oraz znaczenie w procesie nowotworzenia

ROZPRAWA HABILITACYJNA



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czy chcę czy nie chcę
patrząc wstecz
na zadeptane ścieżki
dostrzegam
zwykłą
l u d z k ą r z e cz ..?
to wierność serc
to drwin uśmieszki

mój okręt porwał szkwał
odpłynął
umknął w świat
a czterokonnych kwadryg
cwał
pozrywał cugle
zostawił bat

więc mam tym batem
teraz wybatożyć
ironii głupawy los
spodchmurykapelusza
wzrok nasrożyć
obojętności świata
odparować cios..?

czy chcę czy nie chcę
późno już
wyznaczać górnolotne cele
ja niepokorny..?
spokorniały
cóż
i nie żal nic
i żal tak wiele

wiem jedno
że już umiem siebie ostrzec
przed powszechnością zdrad
spodchmurykapelusza
miłość dostrzec
gdyż ta najtrwalszy
pozostawia ślad

Czesław Niemen

Pracę tę dedykuję Janowi Pawłowi Drugiemu, który miał odwagę poświecić swoje życie odkrywaniu prawd absolutnych.

Panom Profesorom, Ryszardowi Olińskiemu i Kazimierzowi Kasprzakowi, składam serdeczne podziękowania za wszechstronną pomoc, obdarzenie mnie wielkim zaufaniem i swobodą twórczą, która pozwoliła mi osiągnąć „naukową samodzielność" na długo przed powstaniem tej rozprawy.

Od autora

Publikacje naukowe rzadko stwarzają sposobność do dzielenia się spostrzeżeniami natury osobistej. Tym skwapliwiej korzystam z unikalnej okazji, jaką daje szczególna forma rozprawy habilitacyjnej, by w skrócie opowiedzieć jak doszło do jej powstania.

Praca ta stanowi publikacyjny zapis części moich eksperymentalnych zmagań zmierzających do rozwikłania tajemnic fascynującego białka MTH1, jego fizjologicznej roli, znaczenia w procesie nowotworzenia oraz regulacji jego aktywności enzymatycznej. Jej powstanie było możliwe dzięki skojarzeniu mojej fascynacji tematem ze szczęśliwymi zbiegami okoliczności, które pozwoliły mi spotkać na zawodowej drodze ludzi mądrych, serdecznych i tolerancyjnych.

Moje zainteresowania enzymologiczne są w całości zasługą niedoścignionego Mistrza, Pana Doktora Antoniego Leźnickiego z Zakładu Biochemii Uniwersytetu Mikołaja Kopernika w Toruniu, który nadając mi magisterskie szlify w latach 1989-1992, udzielił mi niezapomnianej lekcji naukowej charyzmy, rzetelności, kreatywności i uczciwości. Gwoli ścisłości, lekcja ta ciągle trwa i nie spodziewam się, abym chciał z niej dobrowolnie zrezygnować.

Jako samodzielny eksperymentator rozwinąłem skrzydła w Katedrze Biochemii Klinicznej, Akademii Medycznej w Bydgoszczy, w której miałem szczęście zostać zaangażowany w 1992 roku przez Pana Profesora Ryszarda Olińskiego. Jest to miejsce szczególne, w którym obłudzie, nieszczerości, próżności i marazmowi nie udało się zakorzenić dzięki wielkiemu wysiłkowi całego zespołu. Do dnia dzisiejszego stanowi ono dla mnie azyl, który pozwala na przynajmniej częściowe odizolowanie się od chaosu i mizerii świata zewnętrznego, oferując na osłodę nieskrępowaną swobodę twórczą i rodzinną atmosferę. Atmosfery takiej nie udałoby się nigdy wykreować bez wyjątkowej osobowości Pana Profesora Olińskiego. Właśnie w tym miejscu, żeglując 11 lat temu po oceanie wolnorodnikowej literatury biomedycznej, natknąłem się na przełomowe publikacje japońskich badaczy poświęcone antymutagennej funkcji bakteryjnego białka MutT i jego ludzkiego homologu, nazwanego MTH1. Jako człowiek uprzednio zarażony enzymologiczną pasją natychmiast zapałałem wielką ochotą, by poświęcić kolejne lata mojego życia zawodowego temu właśnie enzymowi. Białko to, jako strażnik wierności kopiowania informacji genetycznej, wydało mi się niezmiernie istotne dla najważniejszych procesów

biologicznych, które stanowią esencję życia. Nie mniej stymulujący był dla mnie fakt, iż światowa literatura dotycząca rodziny genów *MutT* i kodowanych przezeń enzymów zamykała się podówczas skromną liczbą 25 publikacji, pomimo że po raz pierwszy istnienie antymutagennego genu *MutT* postulowano już w roku 1954. Pierwsze kroki, koncentrujące się głównie na opracowaniu własnej metodologii badań nad enzymem, wykonałem w połowie lat dziewięćdziesiątych.

Po obronie pracy doktorskiej w lutym 1997 roku łaskawy los pozwolił mi spędzić trzy lata pod skrzydłami Pana Profesora Kazimierza Kasprzaka, kierownika Sekcji Metali w Laboratorium Karcynogenezy Porównawczej, Narodowego Instytutu Raka we Frederick (Maryland, USA). Połączyła nas wspólna pasja, jaką jest odkrywanie tajemnic białka MTH1. Pasja ta stała się podstawą bardzo owocnej współpracy, która trwa nieprzerwanie od roku 1997. Większość eksperymentów stanowiących zrąb tej rozprawy mogła zostać przeprowadzona w prezentowanym kształcie dzięki doskonałym warunkom, jakich było mi dane doświadczyć w okresie pracy w Narodowym Instytucie Raka.

Po dziesięciu latach pracy eksperymentalnej oraz wczytywania się w osiągnięcia innych badaczy, ilość pytań i nierozwiązanych zagadek dotyczących białka MTH1 wzrosła w mojej głowie nieproporcjonalnie do ilości poznanych odpowiedzi. Spodziewałem się tego i pocieszam się, że stan taki jest jedynym przewidywalnym rezultatem prowadzenia rzetelnej działalności naukowej.

W skład jednotematycznego cyklu publikacji stanowiącego niniejszą rozprawę habilitacyjną włączono następujące artykuły:

Kolejność i numeracja artykułów nie są chronologiczne, ponieważ zostały podporządkowane strukturze logicznej rozprawy.

1. Karol Białkowski, Ryszard Oliński (1997) Metabolizm fosforanowych pochodnych 8-oksy-2'-deoksyguanozyny, *Postępy Biochemii* **43**: 199-208.
2. Karol Białkowski, Kazimierz S. Kasprzak (1998) A novel assay of 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity, *Nucleic Acids Research* **26**: 3194-3201.
3. Karol Białkowski, Aneta Białkowska, Lucy M. Anderson, Kazimierz S. Kasprzak (1999) Higher activity of 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) coincides with lower background levels of 8-oxo-2'-deoxyguanosine in DNA of fetal compared with maternal mouse organs, *Free Radical Biology & Medicine* **27**: 90-94.
4. Karol Białkowski, Aneta Białkowska, Kazimierz S. Kasprzak (1999) Cadmium(II), unlike nickel(II), inhibits 8-oxo-dGTPase activity and increases 8-oxo-dG level in DNA of the rat testis, a target organ for cadmium(II) carcinogenesis, *Carcinogenesis* **20**: 1621-1624.
5. Kazimierz S. Kasprzak, Karol Białkowski (2000) Inhibition of antimutagenic enzymes, 8-oxo-dGTPases, by carcinogenic metals. Recent developments, *Journal of Inorganic Biochemistry* **79**: 231-236.
6. Karol Białkowski, Kazimierz S. Kasprzak (2003) Inhibition of 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity of the antimutagenic human MTH1 protein by nucleoside 5'-diphosphates, *Free Radical Biology & Medicine* **35**: 595-602.
7. Karol Białkowski, Kazimierz S. Kasprzak (2000) Activity of the antimutagenic enzyme, 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase (8-oxo-dGTPase), in cultured Chinese hamster ovary cells: Effects of cell cycle, proliferation rate, and population density, *Free Radical Biology & Medicine* **28**: 337-344.
8. Karol Białkowski, Kazimierz S. Kasprzak (2004) Cellular 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase activity of human and mouse MTH1 proteins does not depend on the proliferation rate, *Free Radical Biology & Medicine* **37**: 1534-1541.
9. Ryszard Oliński, Daniel Gackowski, Rafał Różalski, Marek Foksiński, Karol Białkowski (2003) Oxidative DNA damage in cancer patients: a cause or a consequence of the disease development?, *Mutation Research* **531**: 177-190.

Spis treści

I. WSTĘP

Rozprawa ta dotyka jednego z najistotniejszych, immanentnych fenomenów życia na Ziemi, a mianowicie zdolności do wiernego przenoszenia w czasie genetycznej informacji o budowie i funkcjonowaniu organizmów. Wierność kopiowania informacji genetycznej jest podstawą zachowania tożsamości gatunkowej organizmów oraz prawidłowego funkcjonowania osobników kolejnych generacji. Z drugiej strony wiemy jednak, że absolutna dokładność w kopiowaniu tej informacji nie jest zjawiskiem ani możliwym do osiągnięcia, ani pożądanym z punktu widzenia życia jako takiego, którego nadrzędnym *„celem biologicznym”* wydaje się być samo trwanie. Spróbujmy sobie wyobrazić praktyczne konsekwencje funkcjonowania dwu skrajnych, przeciwstawnych modeli powielania informacji genetycznej, z których jeden zasadzałby się na absolutnej wierności replikacji DNA, drugi zaś na znacznym upośledzeniu tejże. Model pierwszy uniemożliwiałby zjawisko ewolucji, procesów specjacji, mikroewolucji przystosowawczej oraz wewnątrzgatunkowej zmienności osobniczej, ponieważ każdy żywy organizm potomny (jedno- czy wielokomórkowy) musiałby stanowić idealnie wierną kopię osobnika poprzedniej generacji. Siłą rzeczy na nic zdałyby się również powszechne w przyrodzie procesy płciowe, jako metoda na kreowanie wewnątrzgatunkowej zmienności osobniczej. Mało tego, intuicja podpowiada nam, iż w takich warunkach powstanie ogromnej różnorodności form życia na ziemi byłoby z gruntu niemożliwe.

Konsekwencje funkcjonowania drugiego teoretycznego modelu powielania informacji genetycznej byłyby również bardzo drastyczne. Z filogenetycznego punktu widzenia stabilne istnienie gatunków organizmów żywych w przyrodzie byłoby raczej wykluczone. W warunkach niskiej stabilności informacji genetycznej niemożliwy byłby nawet prawidłowy rozwój ontogenetyczny organizmów wielokomórkowych, który w przypadku kręgowców wymaga zajścia wielu bilionów podziałów komórkowych począwszy od chwili pierwszego podziału zapłodnionej komórki jajowej aż do chwili naturalnej śmierci organizmu. Funkcjonowanie przyrody ożywionej na naszej planecie przypomina więc kroczenie po cienkiej linie, odzwierciedlającej wąskie granice tolerancji w zakresie wierności przenoszenia informacji genetycznej w nieustającej sztafecie komórek macierzystych i potomnych.

Jeśli porównamy ten proces do benedyktyńskiej pracy przepisywania ksiąg w czasach średniowiecznych, łatwo zrozumiemy jak wiele czynników

może wpływać na podobieństwo kopii i oryginału. Na przykład: czytelność samego oryginału, jakość użytego atramentu, pióra i papieru, zdolność rozumienia tekstu przez przepisującego, kondycja jego narządu wzroku, jego zdolności psychomotoryczne, stopień koncentracji… I tak można by je mnożyć niemal w nieskończoność, poczynając od powyższych, najbardziej oczywistych czynników, a kończąc na tak iluzorycznych, jak chociażby „niezamierzony" błąd literowy. Nie inaczej rzeczy mają się w przypadku kopiowania informacji genetycznej, nieodzownie poprzedzającej każdy podział komórkowy. Tutaj również zasadniczą rolę odgrywa stan matrycowego DNA, który jest nieustannie uszkadzany przez ogromny wachlarz czynników chemicznych i fizycznych przedostających się do komórki ze środowiska, jak i tych powstających w komórce. Oczywistą rolę w utrzymywaniu wierności kopiowania DNA ma efektywność wszystkich systemów naprawy DNA, które utrzymując matrycę w dobrej kondycji zapobiegają powstawaniu wszelkiego typu mutacji punktowych i aberracji chromosomowych. Bardzo ważną rolę odgrywa również dostępność substratów dla syntezy DNA w komórce, trifosforanów deoksynukleozydów, wzajemne stosunki ich stężeń oraz ich nienaruszona struktura chemiczna, o czym będzie mowa dalej. W sposób najoczywistszy na wierność replikacji DNA wpływają polimerazy DNA, enzymy katalizujące syntezę komplementarnych nici DNA na bazie nici matrycowych oraz zdolność polimeraz (oraz szeregu innych wyspecjalizowanych białek) do korekcji ewentualnych błędnych par nukleotydowych powstałych w procesie replikacji. Autor tej rozprawy nie ma ambicji usystematyzowanego przedstawienia ogromu czynników wpływających na wierność przenoszenia informacji genetycznej. Ilość danych eksperymentalnych zgromadzonych przez ludzkość w tym zakresie już dawno przekroczyła możliwości percepcyjne pojedynczego człowieka.

Choć ograniczona stabilność informacji genetycznej niesionej przez genom jest źródłem ewolucyjnych zdolności przystosowawczych przyrody ożywionej, niesie jednak ze sobą wielkie zagrożenia dla życia poszczególnych osobników. Niestabilność ta jest bowiem odpowiedzialna nie tylko za genotypowe i fenotypowe zróżnicowanie osobników tego samego gatunku, ale również za choroby o podłożu genetycznym, w tym nowotworowe. Transformacja nowotworowa komórki jest nieodłącznie związana z akumulacją mutacji w genach strategicznie ważnych dla utrzymania komórki w rygorach narzucanych jej przez organizm jako całość. Ponieważ walka ze zmorą chorób nowotworowych stała się bodaj największym medycznym wyzwaniem naszych czasów, naturalny wydaje się burzliwy rozwój badań naukowych nad

molekularnymi mechanizmami utrzymywania integralności genomu w komórce. Jedną z grup czynników chemicznych wywołujących niestabilność genetyczną są reaktywne formy tlenu, które poprzez oksydacyjne reakcje wolnorodnikowe uszkadzają DNA, prowadząc m.in. do modyfikacji chemicznej zasad azotowych oraz pojedynczych i podwójnych pęknięć helisy DNA. Szereg modyfikacji DNA takiego typu powoduje powstawanie mutacji, do których dochodzi w procesie powielania informacji genetycznej (replikacji DNA), poprzedzającym każdy podział komórki. Uszkodzeniom oksydacyjnym podlegają nie tylko kwasy nukleinowe, ale również substraty do ich syntezy, 2'-deoksynukleozydo-5'-trifosforany, których zasady azotowe mogą również podlegać oksydacyjnym modyfikacjom. Niektóre z tak zmodyfikowanych nukleotydów wykazują właściwości mutagenne z powodu ich zdolności do nietypowego parowania z różnymi zasadami matrycowej nici DNA. Polimerazy DNA inkorporują uszkodzone oksydacyjnie nukleotydy do nowosyntetyzowanych nici DNA na podobieństwo koni trojańkich, które ujawniają swój zdradziecki potencjał podczas kolejnych rund replikacyjnych. Oczywiście organizmy żywe nie pozostają bezbronne wobec zagrożeń związanych z metabolizmem tlenowym, dysponując orężem w postaci skomplikowanego, wielopoziomowego systemu obrony antyoksydacyjnej. Jednym z licznych obrońców komórki przed zgubnym działaniem reaktywnych form tlenu jest bakteryjne białko enzymatyczne MutT, którego homologi funkcjonalne w komórkach eukariotycznych opatrzono skrótową nazwą MTH1. Cechą wspólną tych białek jest (poza pewną homologią sekwencji aminokwasowej i struktury przestrzennej) zasadnicza rola jaką zdają się pełnić w komórce, polegająca na degradacji uszkodzonych oksydacyjnie trifosforanów deoksynukleozydów purynowych. Dzięki jego aktywności enzymatycznej, komórkowa pula wolnych nukleotydów jest oczyszczana z mutagennych substratów dla syntezy DNA, co przyczynia się do utrzymywania niskiej częstości mutacji punktowych. Białko to jest więc jednym z licznych strażników wierności kopiowania informacji genetycznej. Właśnie biologicznej roli białka MTH1, regulacji jego enzymatycznej aktywności oraz jego znaczeniu w procesach nowotworowych poświęcona jest ta rozprawa.

Zanim jednak zapoznam Czytelnika ze szczegółowymi celami przeprowadzonych przeze mnie badań, pozostawiam Go sam na sam z lekturą pracy przeglądowej, która wprowadzi w zagadnienie na sposób bardziej naukowy i przedstawi fascynującą historię badań nad tym enzymem od roku 1954 do roku 1996, w którym stał się on niepodzielnie moją zawodową pasją.

1.

<u>Karol Białkowski</u>, Ryszard Oliński (1997) Metabolizm fosforanowych pochodnych 8-oksy-2'-deoksyguanozyny, *Postępy Biochemii* 43: 199-208.

Pracę tę poświęcam przyjacielowi ***Komandorowi Maciejowi Muszyńskiemu,*** *który popłynął w swój najdłuższy rejs.*

Metabolizm fosforanowych pochodnych 8-oksy-2'-deoksyguanozyny

Metabolism of 8-oxo-2'-deoxyguanosine phosphates

KAROL BIAŁKOWSKI[1],
RYSZARD OLIŃSKI[2]

Spis treści:

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Wykaz stosowanych skrótów: 8-oxodG — 8-oksy-2'-deoksyguanozyna; 8-oxodGMP — 8-oksy-2'-deoksyguanozyno-5'-monofosforan; 8-oxodGDP — 8-oksy-2'-deoksyguanozyno-5'-difosforan; 8-oxodGTP — 8-oksy-2'-deoksyguanozyno-5'-trifosforan; dG — deoksyguanozyna; CTP — cytydyno-5'-trifosforan; dCTP — deoksycytydyno-5'-trifosforan; UTP — urydyno-5'-trifosforan; dUTP — deoksyurydyno-5'-trifosforan; ATP — adenozyno-5'-trifosforan; ADP — adenozyno-5'-difosforan; dATP — deoksyadenozyno-5'-trifosforan; dTTP — tymidyno-5'-trifosforan; GTP — guanozyno-5'-trifosforan; GDP — guanozyno-5'-difosforan; RFT — reaktywne formy tlenu

I. Wstęp

Zagadnienia związane z destruktywnym wpływem reaktywnych form tlenu (RFT) na organizmy stały się w ostatnich latach obiektem szeroko zakrojonych i intensywnych badań. Omówienie zagadnień związanych z powstawaniem i reaktywnością różnych RFT *in vivo*, znajdzie czytelnik *Postępów Biochemii* w dwu pracach Liczmańskiego [1, 2]. Jednym z najniebezpieczniejszych efektów działania RFT w komórce są uszkodzenia DNA. Znaczenie oksydacyjnych uszkodzeń zasad DNA przez reaktywne formy tlenu oraz procesy naprawy tych uszkodzeń były już opisane na łamach *Postępów Biochemii* [3, 4].

8-Oksyguanina jest jednym z najłatwiej powstających i najgruntowniej przebadanych produktów modyfikacji zasad DNA przez reaktywne formy tlenu. Najlepiej rozpoznanym czynnikiem powodującym przekształcenie guaniny w 8-oksyguaninę jest rodnik hydroksylowy. Potencjalne mechanizmy takiej reakcji przedstawiono na rycinie 1. Pierwszy z tych mechanizmów zakłada bezpośrednie formowanie adduktu rodnika hydroksylowego z guaniną w pozycji C8, drugi zaś, jako etap początkowy zakłada oderwanie elektronu od cząsteczki guaniny pod wpływem rodnika hydroksylowego. Należy zaznaczyć, iż do początkowego oderwania elektronu z cząsteczki guaniny (Ryc. 1, Schemat 2) może dochodzić nie tylko pod wpływem rodnika hydroksylowego, ale także na skutek jonizacji spowodowanej oddziaływaniem kwantu promieniowania jonizującego lub na skutek kontaktu z równie silnym jak rodnik hydroksylowy utleniaczem, np. rodnikiem ferrylowym lub nadferrylowym [5, 6]. Pojawiły się także dane przemawiające za tym, iż takie oderwanie elektronu z częsteczki Gua może zachodzić pod wpływem fotouczulaczy w reakcji fotouczulania typu I [7-9].

Kolejnym czynnikiem zdolnym do wprowadzenia oksydacyjnej modyfikacji guaniny w pozycji C8 jest

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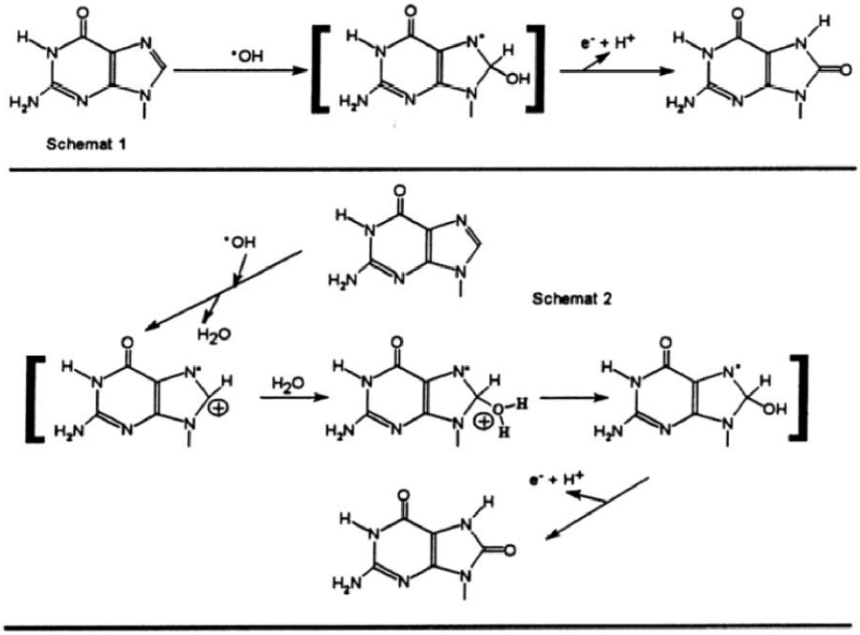
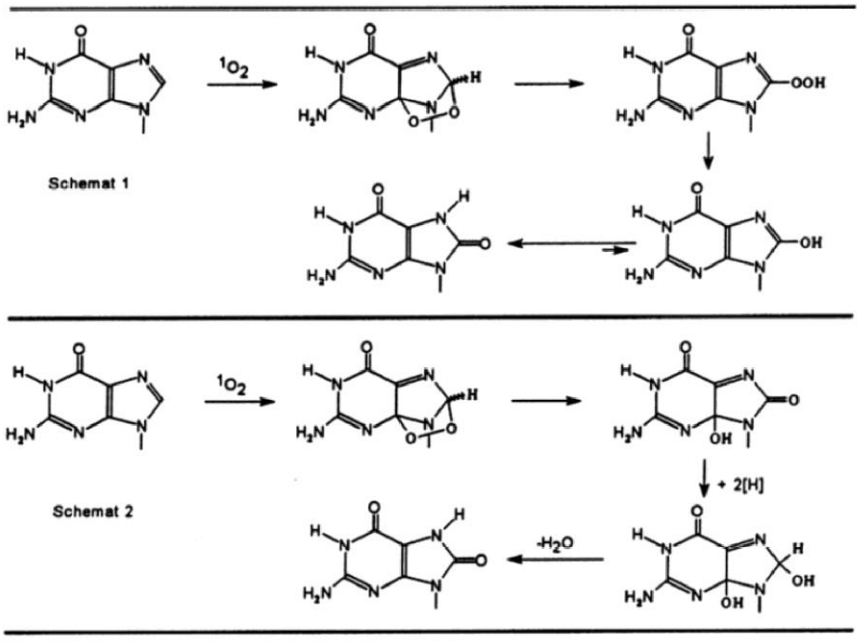


Ryc. 1. Potencjalne mechanizmy powstawania 8-oksyguaniny pod wpływem rodnika hydroksylowego.
Pierwszy z proponowanych mechanizmów (schemat 1) zakłada utworzenie przejściowego adduktu guaniny i rodnika hydroksylowego, który na skutek utlenienia przekształca się w 8-oksyguaninę. Drugi mechanizm (schemat 2) zakłada oderwanie elektronu od cząsteczki guaniny przez rodnik hydroksylowy z wytworzeniem cząsteczki wody i kationorodnika guaniny, który w późniejszym etapie reaguje z cząsteczką wody i ulega przekształceniu do 8-oksyguaniny w procesie utlenienia.

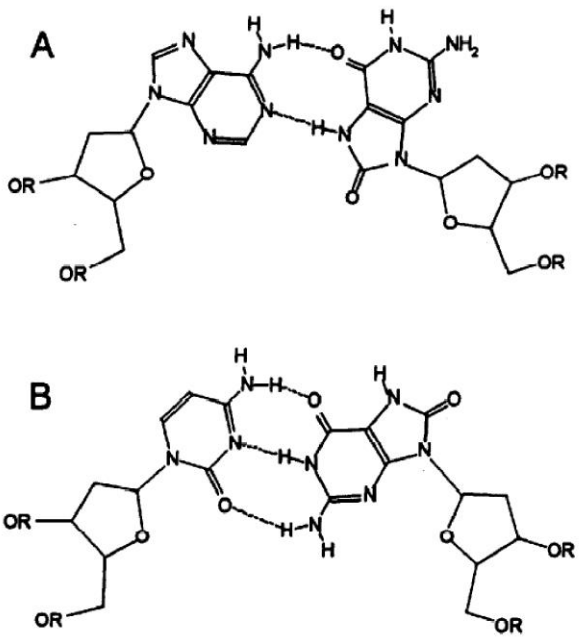
tlen singletowy — $^1O_2$ [10-15]. Najprawdopodobniej reakcja reszty guaniny z tlenem singletowym zachodzi z wytworzeniem przejściowego 4,8-endonadtlenku, który szybko ulega rozpadowi. Dwa możliwe szlaki prowadzące do powstania 8-oksyguaniny w reakcji z $^1O_2$ przedstawiono na rycinie 2. Niektórzy autorzy sugerują również możliwość odrywania elektronu od reszty guaniny przez tlen singletowy z wytworzeniem kationowego rodnika guaniny i anionorodnika ponadtlenkowego ($Gua + {}^1O_2 \rightarrow Gua^{\bullet} + O_2^{\bullet -}$) [16]. W takiej sytuacji oksydacja do 8-oksyguaniny zachodziłaby według schematu 2 przedstawionego na rycinie 1.

II. 8-Oksy-2'-deoksyguanozyno-5'-trifosforan — mutagenny substrat w procesie syntezy DNA

Oksydacyjna modyfikacja guaniny w pozycji C8 może zachodzić nie tylko w obrębie kwasów nukleinowych, ale równie dobrze może dotyczyć reszt guaniny wchodzących w skład wolnych nukleozydów i nukleotydów komórkowych. Wykazano, że produkt modyfikacji dGTP-8-oxodGTP jest substratem polimeraz DNA, inkorporujących 8-oxodGMP do nowosyntetyzowanych nici DNA [17, 18]. Taka inkorporacja zmodyfikowanego nuleotydu do DNA jest źródłem



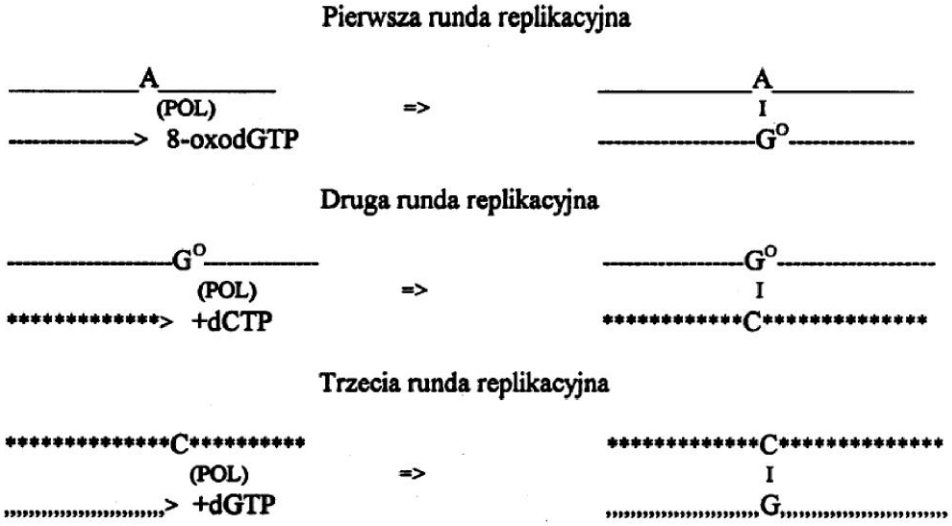
Ryc. 2. Proponowane mechanizmy modyfikacji guaniny przez tlen singletowy, prowadzącej do powstania 8-oksyguaniny.
Pierwszym produktem reakcji tlenu singletowego z guaniną jest nietrwały 4,8-endonadtlenek guaniny. Schemat 1 zakłada późniejsze przekształcenie endonadtlenku w 8-wodoronadtlenek guaniny, który ulega rozpadowi do 8-oksyguaniny. Schemat 2 zakłada przekształcenie 4,8-endonadtlenku guaniny do 4-hydroksy-8-oksyguaniny, która w procesie redukcji i odłączenia cząsteczki wody ulega przekształceniu do 8-oksyguaniny.



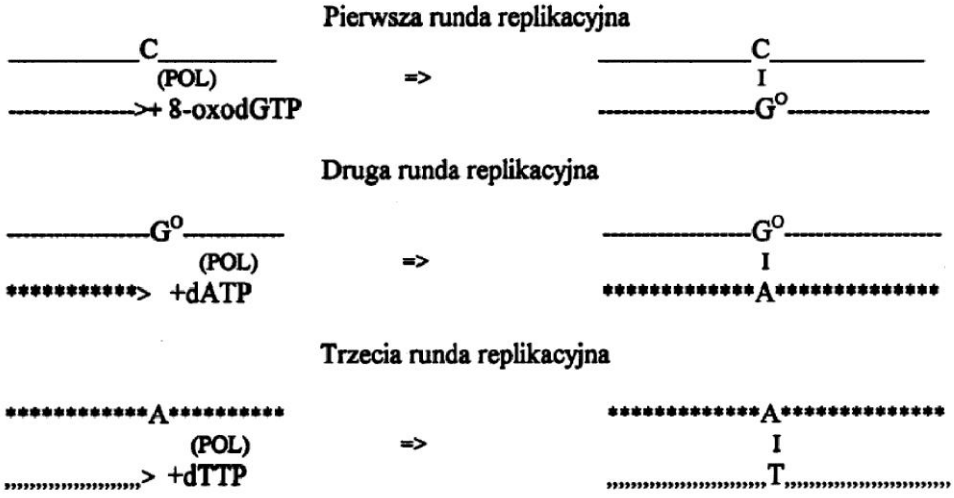
Ryc. 3. Struktura par A) 8-oxodG:dA i B) 8-oxodG:dC występujących w dwuniciowym B-DNA.

późniejszych mutacji, w efekcie możliwości parowania 8-oksyguaniny nie tylko z cytozyną, ale i z adeniną (Ryc. 3). Niezwykle sugestywny eksperyment demonstrujący tę groźną właściwość 8-oxodGTP został przeprowadzony przez C h e n g a i w s p. [17]. Wykorzystano dwuniciowy, kolisty DNA z polinukleotydową luką w jednej z nici. Lukę tę wypełniono używając polimerazy DNA I z *E. coli* oraz dTTP, dCTP, dATP i 8-oxodGTP zamiast dGTP. Tak przygotowanym plazmidem transformowano komórki *E. coli*, w których ulegał on replikacji w trakcie proliferacji komórek bakteryjnych. Po pewnym czasie analizowano ilość powstałych mutacji w obrębie uzupełnionej luki wprowadzonego plazmidu. Aż 16% zanalizowanych przypadków wykazywało obecność mutacji punktowych w obrębie tego fragmentu plazmidu. Transwersje typu AT ⇒ CG stanowiły 97% tych mutacji, a 3% transwersje GC ⇒ TA. Najbardziej prawdopodobnym wytłumaczeniem zaobserwowanych transwersji AT ⇒ CG jest inkorporacja 8-oxodGMP na przeciw adeniny zlokalizowanej w nici matrycowej. Proponowany mechanizm powstawania tej transwersji pod wpływem 8-oxodGTP przedstawiono na rycinie 4. Drugi rodzaj transwersji obserwowanych w tym eksperymencie (GC ⇒ TA), to taka sama mutacja, jaką powoduje pojawienie się 8-oksyguaniny w matrycy na skutek bezpośredniej modyfikacji oksydacyjnej DNA. Potencjalny mechanizm prowadzący do powstawania tego typu mutacji pod wpływem 8-oxodGTP jako substratu polimerazy został przedstawiony na rycinie 5.

P a v l o v i w s p. [19] wykazali, że 8-oxodGTP jest substratem także innych polimeraz DNA. Przeprowadzając eksperymenty według koncepcji opisanej przez C h e n g a i w s p. (lecz stosując jako substraty do syntezy równomolowe stężenia dTTP, dCTP, dATP, dGTP i 8-oxodGTP) dowiedli oni, że następujące polimerazy DNA wprowadzają 8-oxodGMP (z 8-oxodGTP) naprzeciwko adeniny wywołując transwersje typu AT ⇒ CG: polimeraza DNA I (*E coli*), termostabilna polimeraza DNA z *Thermus thermophilus*, polimeraza DNA faga T4, polimeraza DNA γ (mitochondrialna) z embrionów kurzych.



Ryc. 4. Prawdopodobny mechanizm powstawania transwersji AT = > CG pod wpływem 8-oxodGTP.
Z uwagi na zdolność błędnego parowania, 8-oxodGTP może zostać inkorporowany przez polimerazę do syntetyzowanej nici DNA (w postaci monofosforanu) na przeciw adeniny znajdującej się w nici matrycowej. Dochodzi do powstania dwuniciowego DNA posiadającego błędną parę adenina : 8-oksyguanina. Jeśli błąd ten nie zostanie skorygowany przez enzymy naprawy DNA, to w kolejnej rundzie replikacyjnej na przeciw matrycowej 8-oksyguaniny polimeraza DNA wprowadzić może dCMP do syntetyzowanej nici, co prowadzi do wytworzenia dwuniciowego DNA zawierającego błędną parę 8-oksyguanina : cytozyna. Cytozyna ta pojawia się więc w miejscu, gdzie oryginalnie w sekwencji znajdowała się adenina. Trzecia runda replikacyjna prowadzi więc do powstania cząsteczki dwuniciowego DNA, zawierającej mutację punktową (AT = > CG).
(POL) — polimeraza DNA, G^0 — reszta 8-oksyguaniny, C — reszta cytozyny, G — reszta guaniny, A — reszta adeniny.

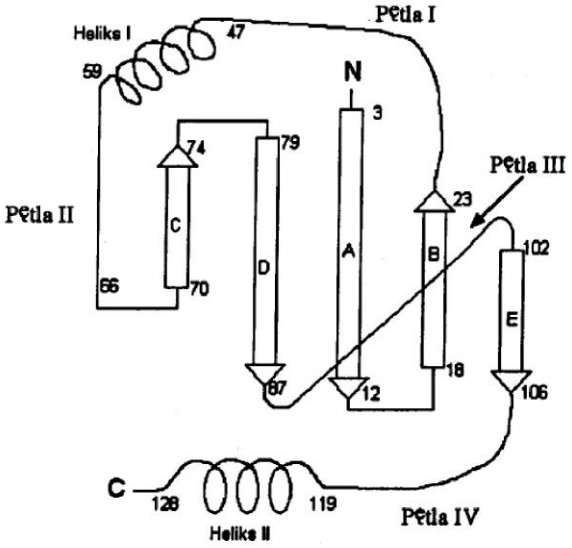


Ryc. 5. Prawdopodobny mechanizm powstawania transwersji GC = > TA pod wpływem 8-oxodGTP.
8-oxodGTP może zostać wprowadzony przez polimerazę (POL) do syntetyzowanej nici DNA na przeciw cytozyny znajdującej się w nici matrycowej. Prowadzi to do powstania dwuniciowego DNA zawierającego parę cytozyna : 8-oksyguanina. Jeżeli 8-oksyguanina nie zostanie usunięta przez enzymy naprawy DNA, to po replikacji nici zawierającej 8-oksyguaninę dojść może do powstania dwuniciowego DNA zawierającego błędną parę 8-oksyguanina : adenina. Adenina pojawia się więc w miejscu, gdzie pierwotnie w sekwencji znajdowała się guanina. Kolejny proces replikacji prowadzi do powstania dwuniciowej cząsteczki DNA obarczonej mutacją punktową (GC = > TA).

III. Enzymatyczny system obrony organizmów przed mutagennym wpływem oksydacji dGTP w pozycji C8: pirofosfohydrolaza 8-oxodGTP

W 1954 roku Treffers i wsp. otrzymali linię zmutowanych komórek *E.coli*, charakteryzującą się wysoką częstością mutacji spontanicznych w porównaniu do komórek dzikiej linii i postulowali istnienie genu odpowiedzialnego za tę cechę, który nazwali *mutT* [20]. W ten sposób odkryto pierwszy tzw. gen mutatorowy u *E. coli*. Dwanaście lat później Yanofksy i wsp. wykazali, iż wysoka niestabilność genetyczna linii mutT związana jest z podwyższeniem częstości występowania tylko jednego rodzaju mutacji punktowych, mianowicie transwersji typu AT ⇒ CG [21]. W celu zidentyfikowania biologicznych funkcji produktu genu mutT dzikiej linii, gen ten sklonowano, jego produkt białkowy nazwany białkiem MutT (129 reszt aminokwasowych, 14.9 kDa) oczyszczono do stanu homogenności i zidentyfikowano jako fosfohydrolazę ośmiu podstawowych trifosfonukleozydów występujących w komórkach (dCTP, GTP, dTTP, UTP, dCTP, CTP, dATP, ATP), o wyraźnej preferencji wobec dGTP [22]. Porównanie sekwencji zmutowanego i dzikiego genu mutT wykazało, iż mutacja tego genu polega na insercji tzw. elementu IS1 [23]. Białko MutT (produkt genu dzikiego) katalizuje reakcję hydrolizy trifosfonukleozydów do monofosfonukleozydów i pirofosforanu [24]. Niedługo potem pirofosfataza ta została wykrystalizowana [25], co dało początek wnikliwym badaniom struktury enzymu. Rok 1991 dostarczył wielu danych na temat właściwości katalitycznych odkrytej pirofosfazaty [24]. Wyznaczono optimum pH (pH 9.0) dla katalizowanej reakcji i wykazano jej całkowitą zależność od jonów magnezu (w obecności innych dwuwartościowych jonów metali, jak Mn^{2+}, Zn^{2+}, Ca^{2+}, aktywność nie przekraczała 15% aktywności uzyskiwanej z jonami Mg^{2+}). Gruntowna analiza specyficzności substratowej z użyciem wszystkich ośmiu kanonicznych trifosfonukleozydów oraz ich pochodnych wykazała, że najlepszymi substratami białka MutT są kolejno dGTP(K_m = 1.5 mM) > > GTP > dUTP > dCTP (2.2 mM). Badania te wskazały, że szczególnie istotną cechą dobrego substratu białka MutT jest obecność grupy 6-keto i 2-amino w pierścieniu purynowym oraz deoksyrybozа występująca w nukleotydzie. Wykazano ponadto, że wszystkie 8-bromopochodne trifosfonukleozydów purynowych (8-Br-dGTP, 8-Br-GTP, 8-Br-dATP, 8-Br-ATP) są zdecydowanie lepszymi substratami białka MutT niż odpowiednie związki wyjściowe. Na tym etapie badań sformułowano również pierwsze hipotezy dotyczące antymutagennego działania białka MutT. Istniały już pewne przesłanki na temat mechanizmu transwersji AT ⇒ CG powstających z dużą częstością w mutantach mutT *E. coli*. Przepuszczano, że transwersje te są efektem błędnego parowania (ang. *mismatch*) adeniny w nici matrycowej, z dGTP inkorporowanym do nici syntetyzowej (A : T → A ~ G → C : G). Wcześniejsze doniesienia sugerowały, że guanina sparowana z adeniną w obrębie dwuniciowego fragmentu polinukleotydowego przyjmuje konformację *syn* względem deoksyrybozy. Postulowano więc, że rolą białka MutT jest hydroliza tych cząsteczek dGTP, które przybrały konformację *syn*, by uniemożliwić ich wprowadzanie do DNA na przeciw adeniny. Hipotezę tę wspierał mocno wspomniany wcześniej fakt, iż

bromowanie purynowych trifosfonukleozydów w pozycji C8 czyni je lepszymi substratami białka MutT, a jak było wiadomo, bromowanie takie wymusza konformację *syn* w obrębie modyfikowanego nukleotydu. Rok 1992 nieoczekiwanie dostarczył rewelacyjnych odkryć w tym zakresie. M a k i i S e k i g u c h u (1992) donoszą o swoich badaniach nad antymutagennym działaniem białka MutT [18]. Badali oni inkorporację znakowanego dGTP do matrycy poli(dA)/oligo(dT) pod wpływem polimerazy III *E. coli* i potwierdzili wcześniejsze spostrzeżenia, że niewielkie ilości znakowanego dGMP są wprowadzane do nici syntetyzowanej na matrycy poli(dA). Enzym MutT dodany do medium, w którym zachodziła replikacja hamował całkowicie tę inkorporację. Wykonano analizę chromatograficzną nukleotydów wchodzących w skład zsyntetyzowanej nici i okazało się, że radioaktywność niesiona przez nią nie należy wcale do dGMP, a do związku zidentyfikowanego jako 8-oxodGMP. Otworzyło to nowy etap w badaniach nad białkami MutT i przyciągnęło uwagę wielu badaczy zajmujących się stresem oksydacyjnym, a szczególnie oksydacyjnymi uszkodzeniami DNA. Jak dowiedli Maki i Sekiguchu, MutT posiada bardzo wysokie powinowactwo do 8-oxodGTP (K_m = 0.4 μM!). Pozwala to sądzić, że jego rola polega na hydrolizowaniu tego nukleotydu w celu uchronienia komórki przed mutagennymi skutkami jego wprowadzenia do DNA. Dla porównania, oznaczone przez nich wartości K_m wobec innych nukleotydów wynosiły: dla dGTP — 1.2 mM, dTTP — 1.8 mM, dCTP — 1.7 mM, GTP 1.2 mM. 8-OxodGTP jako substrat posiada wszystkie preferowane przez MutT cechy: grupę 6-keto i 2-amino pierścienia purynowego, deoksyrybozę i konformację *syn* wokół wiązania N-glikozydowego, wymuszoną przez atom tlenu w pozycji C8. Badania ostatnich 3 lat prowadzone technikami krystalograficznymi, a przede wszystkim metodami wielowymiarowego jądrowego rezonansu magnetycznego doprowadziły do szczegółowego rozpoznania struktury drugo- (Ryc. 6) i trzeciorzędowej MutT [26-28] oraz budowy centrum aktywnego enzymu i jego oddziaływań z substratem [29, 31]. Trifosforany nukleozydów odziałują z centrum aktywnym enzymu w obecności dwu jonów magnezu. Jeden z tych jonów związany jest z atomami tlenu reszt fosforanowych β i γ nukleotydu. Drugi jon Mg^{2+} oddziałuje z grupami karboksylowymi reszt glutaminianu 56, 57, 98, amidową grupą karbonylową glicyny 38 oraz z cząsteczką wody, która najprawdopodobniej dokonuje nukleofilowego ataku na atom fosforu β, powodując rozerwanie wiązania fosfodiestrowego pomiędzy resztą fosforanową α i β nukleozydotrifosforanu. Odpowiednią orientację przestrzenną atakującej cząsteczki wody zapewnia najprawdopodobniej jej oddziaływanie z grupą karboksylową glutaminianu 53. Zasada azotowa oraz reszta rybozy lub deoksyrybozy wchodzące w skład substratu lokują się w hydrofobowej szczelinie, przy czym zasada azotowa wiąże się



Ryc. 6. Zarys struktury drugorzędowej białka MutT z *E. coli*
W obrębie białka MutT występują dwa rejony α-helikalne (Heliks I i II) oraz pięć pasm zorganizowanych w strukturę β oznaczonych strzałkami A-E. Pasma A i B oraz C i D połączone są ciasnymi skrętami, co umożliwia ich antyrównoległe ułożenie. Pozostałe wymienione struktury połączone są dłuższymi pętlami (I-IV) o długości od 13 do 24 aminokwasów. Numerami oznaczono orientacyjne położenie kolejnych aminokwasów łańcucha polipeptydowego (wzorowano na rysunku zamieszczonym w pracy A b e y g u n a w a r d a n a i w s p., 1993 [26]).

z leucyną 4, izoleucyną 6 i 80. Grupa ketonowa w pozycji C6 zasady azotowej odziałuje z grupą-NH_2 łańcucha bocznego asparaginy 119, co tłumaczy duże powinowactwo enzymu do nukleotydów zawierających guaninę lub jej pochodne. Reszta (deoksy)rybozy wchodząca w skład nukleotydu oddziałuje z leucyną 4, izoleucyną 6 oraz z łańcuchem bocznym lizyny 39.

W ślad za badaniami nad pirofosfatazą 8-oxodGTP (MutT) z *E. coli* rozpoczęły się badania nad homologicznymi enzymami z innych organizmów. W 1992 roku M o i w s p. wyizolowali z komórek ludzkich enzym o masie cząsteczkowej 18 kDa specyficznie hydrolizujący 8-oxodGTP do 8-oxodGMP (K_m = 8,5 μM), a słabiej dGTP (K_m = 870 μM) i dATP [32]. Podobnie jak MutT, pirofosfataza ta jest zależna od jonów magnezu. Rok później ustalono częściowo jej sekwencję aminokwasową, co pozwoliło na sklonowanie i ekspresję genu kodującego to białko w komórkach *E. coli* [33]. Gen ludzkiej pirofosfatazy 8-oxodGTP, zajmujący *locus* 7p22 [34], koduje białko składające się ze 156 aminokwasów (masa cząsteczkowa 17.9 kDa), a jego sekwencja nukleotydowa wykazuje duże podobieństwo do bakteryjnego genu mutT w centralnej części otwartej ramki odczytu. W języku aminokwasów oznacza to 15 identycznych aminokwasów w odcinku A37-A61 MutT i A36-A60 pirofosfatazy ludzkiej. Enzym ludzki uznano za homolog białka MutT i nazwano go hMTH1 (*Human MutT Homolog*).

W ostatnim czasie sklonowano homologi genu mutT z innych źródeł; tzw. białko MutX ze *Streptococ-*

cus pneumoniae wykazuje analogiczne aktywności enzymatyczne jak MutT, jest jednak homotrimerem o masie 54 kDa (masa cząsteczkowa monomeru — 18 kDa). Gen kodujący to białko wykazuje duże podobieństwo do genu mutT w odcinku kodującym aminokwasy A37-A60 [35, 36]. Podobną homologię struktury genu i funkcji biologicznych kodowanego białka wykazano w przypadkach genu i białka MutT z *Proteus vulgaris* (112 aminokwasów, 12.8 kDa) [37] i MutT z *Haemophilus influenzae* (136 aminokwasów, 15.6 kDa) [38]. Sklonowano również homologi genu mutT myszy i szczura i scharakteryzowano ich produkty białkowe jako pirofosfatazy 8-oxodGTP. Mysia pirofosfataza 8-oxodGTP, to białko o masie cząsteczkowej 17.9 kDa (156 aminokwasów) wykazująca 83% homologii sekwencji aminokwasowej z ludzką pirofosfatazą, a podobieństwo sekwencji nukleotydowej mysiego i ludzkiego genu sięga 81% [39]. Szczurza pirofosfataza 8-oxodGTP (masa cząsteczkowa 18 kDa) posiada 131 identycznych aminokwasów z enzymem ludzkim z ogólnej liczby 156 aminokwasów [40].

Pojawiły się także pierwsze dane na temat poziomu ekspresji genu kodującego pirofosfatazę 8-oxodGTP w różnych narządach wewnętrznych ssaków. Uzyskanie przeciwciał poliklonalnych do konserwatywnego ewolucyjnie fragmentu pirofosfatazy 8-oxodGTP umożliwiło ilościową analizę poziomu ekspresji tego enzymu w tkankach mysich [39]. Relatywnie najwyższy poziom białka pirofosfatazy wykazano w wątrobie i grasicy, niższy poziom występuje w innych narządach: jelicie grubym > jądrach > nerkach > śledzionie > żołądku > płucach > sercu > i mózgu. Zdecydowanie najwyższy poziom białka pirofosfatazy 8-oxodGTP stwierdzono jednak w mysich embrionalnych komórkach pnia, przewyższał on sześciokrotnie poziom obserwowany w wątrobie. Potwierdziło to wcześniejszą tezę, że poziom ekspresji genu kodującego tę pirofosfatazę jest najwyższy w komórkach o charakterze embionalnym (m.in. nowotworowych, z których po raz pierwszy wyizolowano ssaczy homolog MutT). Skonstruowanie znakowanej sondy opartej na antysensownym RNA pozwoliło na kolorymetryczną ocenę ilości mRNA ludzkiej pirofosfatazy w histologicznych skrawkach ludzkiej skóry i sutka [41]. Stwierdzono duże zróżnicowanie poziomu $mRNA_{hMTH1}$ w zależności od rodzaju komórek, spośród których największą jego ilość wykazywały komórki charakteryzujące się dużym tempem podziałów mitotycznych. Z kolei w tkankach szczurzych badano ilość transkryptu genu pirofosfatazy 8-oxodGTP (mRNA) i wykazano jego największą produkcję w tkance serca, śledziony, nerki i jąder [40].

Przeprowadzono również pierwsze badania dotyczące subkomórkowej lokalizacji ludzkiej pirofosfatazy 8-oxodGTP [42]. Ustalono, że enzym zlokalizowany jest w cytoplazmie i mitochondriach. Z całkowitej aktywności komórkowej pirofosfatazy 8-oxodGTP 4% zlokalizowano w mitochodriach. Aktywności specyficzne enzymu w przeliczeniu na mg białka są jednak identyczne w obu tych kompartmentach komórkowych. Jednocześnie nie stwierdzono jakichkolwiek różnic dotyczących cech fizyko-chemicznych enzymu mitochondrialnego i cytosolowego; kwestia transportu enzymu do mitochondrium pozostaje nadal niewyjaśniona.

Groźne skutki mutacji w obrębie genów z rodziny mutT zaczęły przyciągać uwagę badaczy procesów nowotworzenia. Warunkiem transformacji nowotworowej komórki jest powstanie mutacji w wielu genach. Mutacja genu — homologu mutT, prowadząca do braku w komórce aktywności pirofosfatazy 8-oxodGTP, mogłaby w sposób bardzo istotny przyspieszać tempo powstawania mutacji w innych genach, z uwagi na drastyczny wzrost częstości transwersji typu AT ⇒ CG.

W u i w s p. [43] badali sekwencję nukleotydową genu hMTH1 (homologu MutT) w genomie ludzkich komórek nowotworowych raka jelita grubego i porównywali je z sekwencjami nukleotydowymi genu zdrowych komórek tej samej tkanki. Przebadano 32 przypadki, lecz nie stwierdzono różnic pomiędzy sekwencjami nukleotydowymi genu w komórkach nowotworowych i odpowiadających im komórkach zdrowych. Przy okazji wykazano istnienie polimorfizmu genu hMTH1 w populacji ludzkiej. W dziewięciu przypadkach stwierdzono tranzycję G ⇒ A w obrębie kodonu 83, która prowadzi do zmiany waliny na metioninę w pirofosfatazie 8-oxodGTP (tranzycję taką odnotowali autorzy także w genach hMTH1 ludzi zdrowych). Rzadziej obserwowano tranzycję C ⇒ T w obrębie kodonu 119, która jednak nie prowadzi do zmiany aminokwasu w białkowym produkcie genu. O k a m o t o i w s p. [44] porównywali poziom $mRNA_{hMTH1}$ w ludzkiej tkance raka nerki i otaczającej ją tkance nie zmienionej nowotworowo. Stwierdzono zdecydowanie wyższy poziom transkrypcji genu hMTH1 w komórkach nowotworowych. W kontekście znaczenia — homologów genu *MutT* w procesach mutagenezy i karcinogenezy rozpoczęto poszukiwania potencjalnych inhibitorów pirofosfatazy 8-oxodGTP pośród znanych czynników rakotwórczych. Wykazano na przykład, iż jony niklu (II) — dobrze udokumentowany karcinogen — są niekompetycyjnym inhibitorem ($K_i = 0.9$ mM) bakteryjnej pirofosfatazy 8-oxodGTP z *E. coli*, co wykazano w doświadczeniach przeprowadzonych *in vitro*, z użyciem oczyszczonego preparatu enzymu [45].

III-1. Rodzina genów MutT

Oprócz przedstawionych powyżej genów kodujących pirofosfohydrolazy o dużej specyficzności wobec 8-oxodGTP, do tzw. rodziny genów i białek mutT/MutT zakwalifikowano również odkryte ostatnio geny kodujące enzymy o nieco innej specyficzności substratowej. Zaliczono do tej rodziny pirofosfatazę

dinukleotydową z *E. coli* [46], zależną od dwuwartościowych jonów metali i wykazującą wysoką specyficzność substratową wobec NADH (257 aminokwasów, 29.8 kDa, natywnie — homodimer). Katalizuje ona hydrolizę NADH do AMP i zredukowanego mononukleotydu nikotynamidoadeninowego. Do rodziny MutT zakwalifikowano również pirofosfohydrolazę diadenozyno-5',5'''-P^1,P^4-tetrafosforanu (16.8 kDa, 147 aminokwasów), hydrolizującą ten związek do AMP i ATP [47]. Zidentyfikowano także produkt genu orfl.9 *E. coli*, w którym doszukano się charakterystycznej dla rodziny MutT sekwencji aminokwasów [48]. Białko to charakteryzuje aktywność specyficzna hydrolazy GDP-mannozy (guanozyno-5'difosfo--α-D-mannozy) i GDP-glukozy (guanozyno-5'-difosfo-α-D-glukozy). Produktami reakcji przezeń katalizowanej jest GDP oraz wolny cukier. Aktywność jej jest całkowicie zależna od obecności dwuwartościowych jonów metali.

Wszystkie opisane dotychczas geny szeroko pojmowanej rodziny mutT kodują białka zawierające charakterystyczny motyw w sekwencji aminokwasów [46, 49]:
$GXU(X)_3ET(X)_6RE^+UXEE^*$, gdzie E oznacza kwas glutaminowy, G — glicynę, R — argininę, T — treoninę, U oznacza leucynę lub izoleucynę, lub walinę, a X — dowolny aminokwas.

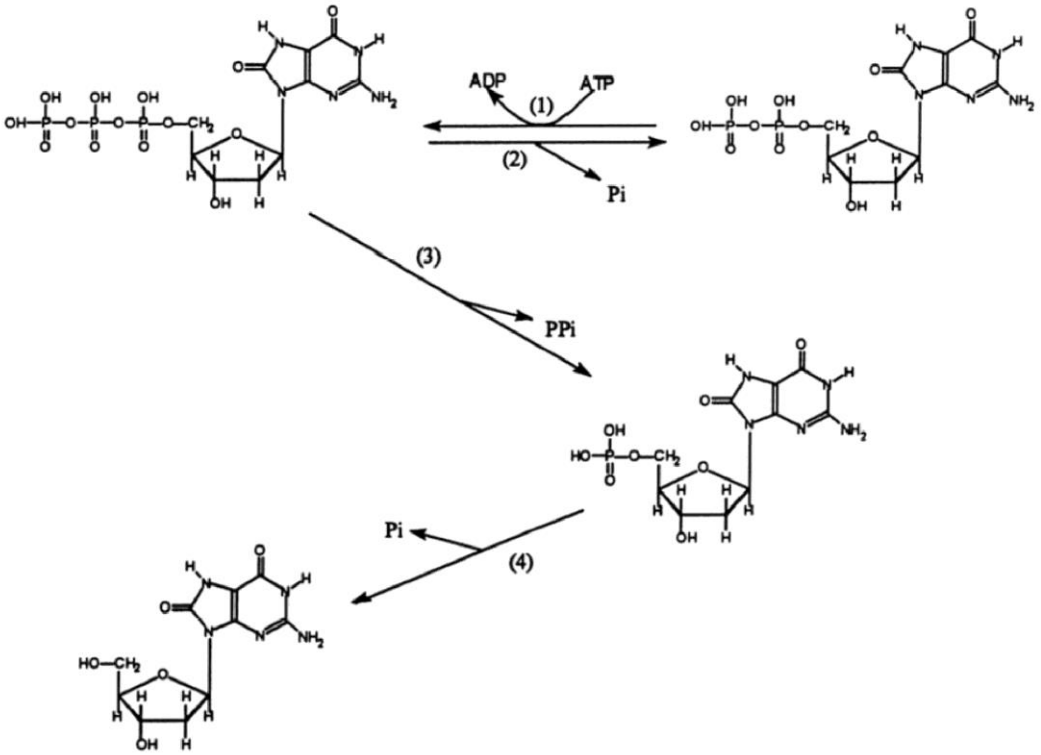
Uważa się, że konserwatywne aminokwasy tego unikalnego motywu odpowiadają za wiązanie nukleotydowego substratu w centrum aktywnym i hydrolizę pochodnych nukleotydowych. Funkcją skrajnych reszt kwasu glutaminowego konserwatywnej sekwencji (pozycja 56 i 57 w białku MutT z *E. coli*), oznaczonych gwiazdką, jest wiązanie jonu magnezu w centrum aktywnym [31, 50]. Aktywność enzymatyczna zmutowanego enzymu, zawierającego w pozycji 57 glutaminę zamiast kwasu glutaminowego jest 100 tys. razy niższa, niż aktywność nie zmutowanej wersji białka. Reszta kwasu glutaminowego oznaczona plusem oddziałuje z cząsteczką wody rozrywającej wiązanie fosfodiestrowe substratu.

IV. Metaboliczne losy fosforanów 8-oksydeoksyguanozyny

Scharakteryzowana powyżej pirofosfataza 8-oxodGTP nie jest jedynym enzymem zdolnym do hydrolizowania wiązań fosfodiestrowych tego zmodyfikowanego nukleotydu. We wszystkich dotychczas badanych organizmach, aktywności pirofosfatazy 8-oxodGTP towarzyszy aktywność fosfatazy 8-oxodGTP, która hydrolizuje ten substrat do 8-oxodGDP i ortofosforanu [18, 32, 39, 40 oraz nie opublikowane obserwacje własne]. Ponieważ jednak enzym ten hydrolizuje z większą wydajnością dGTP niż 8-oxodGTP [32], dotychczas nie przyciągnął on uwagi badaczy i pozostaje w cieniu bardzo popularnej pirofosfatazy. Wydaje się, że znaczenie metaboliczne tej aktywności jest jednak niesłusznie bagatelizowane. Poddanie bowiem 8-oxodGTP działaniu homogenatu szczurzej tkanki wątrobowej w pH 8.0 prowadzi do gwałtownej hydrolizy tego związku właśnie do 8-oxodGDP, a ilość powstałego w tym czasie 8-oxodGMP jest bardzo nieznaczna (nie opublikowane obserwacje własne). H a y a k a w a i w s p. [51] wykazali, że 8-oxodGDP jest substratem ludzkiej kinazy difosfonukleozydowej, enzymu o szerokiej specyficzności substratowej, który oprócz klasycznych difosforanów nukleozydów fosforyluje wydajnie 8-oxodGDP z udziałem ATP (8-oxodGDP + ATP → 8--oxodGTP + ADP). Ci sami autorzy podczyścili i wstępnie scharakteryzowali ludzką fosfatazę degradującą 8-oxodGMP do wolnego nukleozydu — 8-oxodG [51]. Enzym ten wadaje się być wysoce specyficzny dla 8-oxodGMP, ponieważ wydajność hydrolizy 8-oxodGMP jest daleko większa, niż w przypadku dGMP > dTMP > dAMP > > dCMP i GMP. Donoszą oni także, iż ludzka kinaza guanylanowa, która katalizuje zależną od ATP fosforylację dGMP do dGDP oraz GMP do GDP, nie wykazuje żadnej aktywności wobec 8-oxodGMP. W tym sensie pirofosfataza 8-oxodGTP jawi się jako jedyny enzym kierujący 8-oxodGTP nieodwracalnie na drogę katabolizmu. Zawiłości losów metabolicznych 8-oksypochodnych nukleotydów deoksyguanozynowych naszkicowano na rycinie 7.

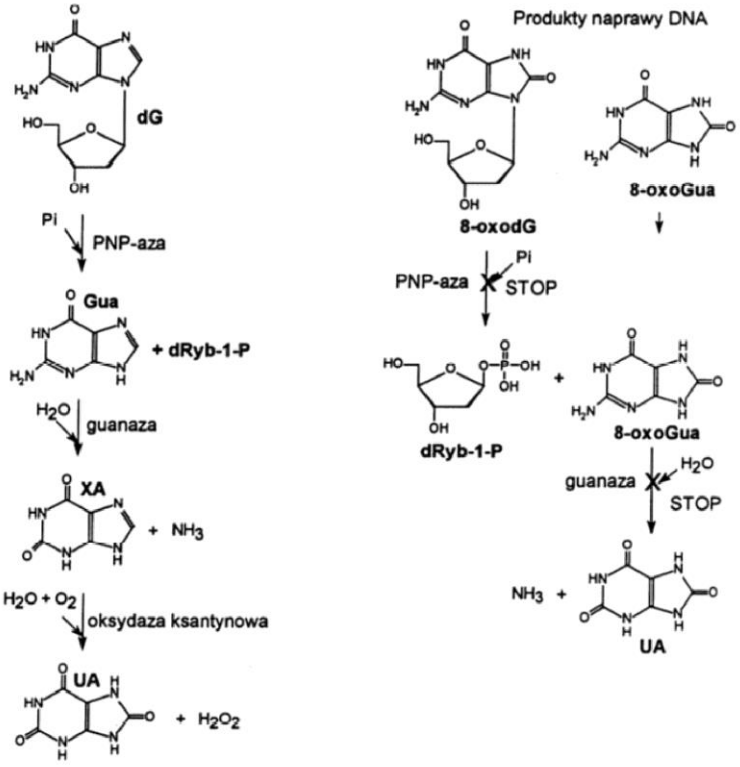
8-Oksy-2'-deoksyguanozyna (8-oxodG), która jest wydalana z moczem przez organizmy ssaków [52], jest powszechnie uznawana za marker oksydacyjnych uszkodzeń DNA i traktowana jako specyficzny produkt naprawy DNA, choć jak dotychczas nie opisano enzymu, który byłby zdolny do specyficznego wycinania tego uszkodzenia w postaci wolnego nukleozydu lub fragmentu oligonukleotydowego. Z uwagi na istnienie w układach biologicznych wysoce specyficznych enzymów defosforylujących pochodne fosforanowe 8-oxodG, należy brać pod uwagę możliwość, iż pewna część puli 8-oxodG wydalanego z moczem pochodzi z degradacji nukleotydów powstających na skutek oksydacyjnej modyfikacji fosforanów deoksyguanozyny. Do chwili obecnej jednak nie przedstawiono bezpośrednich dowodów na obecność którejkolwiek z pochodnych fosforanowych 8-oxodG w puli wolnych nukleotydów komórkowych, choć ich powstawanie jest wielce prawdopodobne.

Mocz ssaków zawiera także wolną 8-oksyguaninę [53], która jest produktem naprawy DNA powstającym na skutek działania kilku scharakteryzowanych enzymów usuwających tę zasadę z DNA. B e s s h o i w s p. zidentyfikowali w tkankach ludzkich enzymy o takiej aktywności [54]. Jeden z nich wykazuje jedynie aktywność N-glikozylazy i uwalnia 8-oxoGua pozostawiając miejsce apurynowe, drugi oprócz aktywności N-glikozylazowej przejawia także aktywność endonukleolityczną. Również ludzka N-metylopuryna — DNA glikozylaza wykazuje zdolność usuwania 8-oksyguaniny z DNA [55]. Enzymy reperujące ok-



Ryc. 7. Szlak przemian metabolicznych 8-oksy-2'-deoksyguanozyno-5'-trifosforanu.
Pi — ortofosforan, PPi — pirofosforan, ATP — adenozyno-5'-trifosforan, ADP — adenozyno-5'-difosforan, (1) — kinaza difosfonukleozydowa, (2) — fosfataza 8-oksy-2'-deoksyguanozyno-5'-trifosforanu, (3) — pirofosfataza 8-oksy-2'-deoksyguanozyno-5'-trifosforanu (białko hMTH1, homolog MutT), (4) — fosfataza 8-oksy-2'-deoksyguanozyno-5'-monofosforanu

sydacyjne uszkodzenia DNA omówiono niedawno na łamach *Postępów Biochemii* [4]. Najprawdopodob-



Ryc. 8. Szlak katabolizmu deoksyguanozyny (z lewej), z charakterystycznym dla naczelnych, ptaków, gadów i owadów końcowym produktem katabolizmu — kwasem moczowym. Potencjalne etapy katabolizmu 8-oksy-2'-deoksyguanozyny i 8-oksyguaniny, którym te cząsteczki nie podlegają (z prawej). Pi — ortofosforan, PNP-aza-fosforylaza nukleozydów purynowych, dRyb-1-P — deoksyrybozo-1-fosforan, Gua — guanina, 8-oxoGua — 8-oksyguanina, XA — ksantyna, UA — kwas moczowy.

niej, zarówno 8-oksyguanina jak i 8-oxodG nie podlegają dalszej degradacji w organizmach ssaków. Świadczą o tym następujące obserwacje:

1. Znakowana trytem 8-oxodG wstrzyknięta do krwiobiegu szczura wydalana jest z moczem nie podlegając degradacji [52].
2. 8-OxodG jest cząsteczką bardzo trwałą (w warunkach podwyższonej temperatury i niskiego pH wielokrotnie trwalszą od deoksyguanozyny) i nie podlega nieenzymatycznej dekompozycji w warunkach zbliżonych do fizjologicznych [56].
3. W doświadczeniach *in vitro* przeprowadzonych z użyciem oczyszczonych preparatów enzymów wykazano, że:

a) 8-oxodG nie jest substratem ludzkiej fosforylazy nukleozydów purynowych, katalizującej fosforolizę wiązania N-glikozydowego m.in. deoksyguanozyny (Ryc. 8) [57, 58].

b) 8-oksyguanina nie jest substratem guanazy z wątroby króliczej, katalizującej fizjologicznie dezaminację hydrolityczną guaniny do ksantyny. Innymi słowy, guanaza nie przekształca 8-oksyguaniny do kwasu moczowego (Ryc. 8) [57, 58].

V. Uwagi końcowe

DNA izolowany z komórek ludzkich zawiera niewielką ilość oksydacyjnie zmodyfikowanych zasad azotowych, wśród nich 8-oksyguaninę [59-62]. Można stąd wyciągnąć wniosek, że enzymy wycinające te zmodyfikowane zasady nie są w stanie usunąć wszystkich uszkodzeń tego typu. Niektóre z pochodnych zmodyfikowanych oksydacynie mogą tworzyć błędnie sparowane połączenia, co stanowi źródło mutacji i w konsekwencji może spowodować transformację nowotworową komórki [63]. Produkty oksydacyjnej modyfikacji zasad obecne w DNA zazwyczaj traktowane są jako wynik ataku reaktywnych form tlenu na cząsteczkę DNA. W bieżącej literaturze podkreśla się znaczenie możliwości mutagenezy powodowanej przez 8-oxodGTP. Należy jednak podkreślić, iż nie dostarczono jeszcze dowodów na obecność 8-oxodGTP *in vivo*, a tym bardziej nie oszacowano, jaka część ogólnej ilości 8-oksyguaniny obserwowanej w DNA komórkowym pochodzić może z 8-oxodGTP. Czy degradacja 8-oxodGTP to jedyna aktywność homologów MutT mająca wpływ na tempo mutagenezy spontanicznej? Zapewne rozwikłanie tych problemów stanie się w najbliższej przyszłości ważnym zadaniem dla badaczy tej materii.

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Piśmiennictwo

1. Liczmański AE (1988) *Post Biochem* **34**: 273-291
2. Liczmański AE (1988) *Post Biochem* **34**: 293-310
3. Tudek B (1993) *Post Biochem* **39(2)**: 91-99
4. Zastawny TH (1996) *Post Biochem* **42(1)**: 31-41
5. Von Sonntag C (1987) *The chemical basis of radiation biology*, Taylor & Francis, New York, 1987
6. Floyd RA (1990) *Carcinogenesis* **11**: 1447-1450
7. Kasai H, Yamaizumi Z, Yamamoto F, Bessho T, Nishimura S, Berger M, Cadet J (1992) *Nucleic Acids Symp Ser* 181-182
8. Nicotera TM, Munson BR, Fiel RJ (1994) *Photochem Photobiol* **60**: 295-300
9. Ito K, Inoue S, Yamamoto K, Kawanishi S (1993) *J Biol Chem* **268**: 13221-13227
10. Floyd RA, West MS, Eneff KL, Schneider JE (1990) *Free Radic Biol Med* **8**: 327-330
11. Schneider JE, Price S, Maidt L, Gutteridge JM, Floyd RA (1990) *Nucleic Acids Res* **18**: 631-635
12. Schneider JEJ, Phillips JR, Pye Q, Maidt ML, Price S, Floyd RA (1993) *Arch Biochem Biophys* **301**: 91-97
13. Sies H (1993) *Mutat Res* **299**: 183-191
14. Ravanat JL, Cadet (1995) *Chem Res Toxicol* **8**: 379-388
15. Shau C, Foote CS (1995) *J Am Chem Soc* **117**: 6439-6442
16. Boiteux S, Gajewski E, Laval J, Dizdaroglu M (1992) *Biochemistry* **31**: 106-110
17. Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA (1992) *J Biol Chem* **267**: 166-172
18. Maki H, Sekiguchu M (1992) *Nature* (Lond) **355**: 273-275
19. Pavlov YI, Minnick DT, Izuta S, Kunkel TA (1994) *Biochemistry* **33**: 4695-4701
20. Treffers HP, Spinelli V, Belser NO (1954) *Proc Natl Acad Sci USA* **40**: 1064-1071
21. Yanofsky C, Cox EC, Horn V (1966) *Proc Natl Acad Sci USA* **55**: 274-281
22. Bhatnagar SK, Bessman MJ (1988) *J Biol Chem* **263**: 8953-8957
23. Bhatnagar SK, Bullions LC, Lew G, Bessman MJ (1990) *J Bacteriol* **172**: 2802-2803
24. Bhatnagar SK, Bullions LC, Bessman MJ (1991) *J Biol Chem* **266**: 9050-9054
25. Bessman MJ, Bullions LC, Bhatnagar SK, Braden BC, Love WE (1991) *J Biol Chem* **266**: 9055-9056
26. Abeygunawardana C, Weber DJ, Frick DN, Bessman MJ, Mildvan AS (1993) *Biochemistry* **32**: 13071-13080
27. Weber DJ, Abeygunawardana C, Bessman MJ, Mildvan AS (1993) *Biochemistry* **32**: 13081-13088
28. Abeygunawardana C, Weber DJ, Gittis AG, Frick DN, Lin J, Miller AF, Bessman MJ, Mildvan AS (1995) *Biochemistry* **34**: 14997-15005
29. Frick DN, Weber DJ, Gillespie JR, Bessman MJ, Mildvan AS (1994) *J Biol Chem* **269**: 1794-1803
30. Frick DN, Weber DJ, Abeygunawardana C, Gittis AG, Bessman MJ, Mildvan AS (1995) *Biochemistry* **34**: 5577-5586
31. Lin J, abeygunawardana C, Frick DN, Bessman MJ, Mildvan AS (1997) *Biochemistry* **36**: 1199-1211
32. Mo JY, Maki H, Sekiguchi M (1992) *Proc Natl Acad Sci USA* **89**: 11021-11025
33. Sakumi K, Furuichi M, Tsuzuki T, Kakuma T, Kawabata S, Maki H, Sekiguchu M (1993) *J Biol Chem* **268**: 23524-23530
34. Furuichi M, Yoshida MC, Oda H, Tajiri T, Nakabeppu Y, Tsuzuki T, Sekiguchi M (1994) *Genomics* **24**: 485-490
35. Bullions LC, Mejean V, Claverys JP, Bessman MJ (1994) *J Biol Chem* **269**: 12339-12344
36. Mejean V, Salles C, Bullions LC, Bessman MJ, Claverys JP (1994) *Mol Microbiol* **11**: 323-330
37. Kamath AV, Yanofsky C (1993) *Gene* **134**: 99-102
38. Fleischmann RD, Adams MD, White O, Clayton RA, Kirhness EF, Kerkavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick MJ, et al. (1995) *Science* **269**: 496-512
39. Kakuma T, Nishida J, Tsuzuki T, Sekiguchu M (1995) *J Biol Chem* **270**: 25942-25948
40. Cai JP, Kakuma T, Wsuzuki T, Sekiguchu M (1995) *Carcinogenesis* **16**: 2343-2350
41. Wani G, D'Ambrosio SM (1995) *Carcinogenesis* **16**: 277-283

42. Kang D, Nishida J, Iyama A, Nakabeppu Y, Furuichi M, Fujiwara T, Sekiguchi M, Takeshige K (1995) *J Biol Chem* **270**: 14659-14665
43. Wu C, Nagasaki H, Maruyama K, Nakabeppu Y, Sekiguchi M, Yuasa Y (1995) *Biochem Biophys Res Commun* **214**: 1239-1245
44. Okamoto K, Toyokuni S, Kim WJ, Ogawa O, Kakehi Y, Arao S, Hiai H, Yoshida O (1996) *Int J Cancer* **65**: 437-441
45. Porter DW, Nelson VC, Fivash MJ, Kasprzak KS (1996) *Chem Res Toxicol* **9**: 1375-1381
46. Frick DN, Bassman MJ (1995) *J Biol Chem* **270**: 1529--1534
47. Thorne NM, Hankin S, Wilkinson MC, Nunez C, Barraclough R, McLennan AG (1995) *Biochem J* **311**: 717-721
48. Frick DN, Townsend BD, Bassman MJ (1995) *J Biol Chem* **270**: 24086-24091
49. Koonin EV (1993) *Nucleic Acids Res* **21**: 4847
50. *Lin J, Abeygunawardana C, Frick DN, Bessman* MJ, Mildvan AS (1996) *Biochemistry* **35**: 6715-6726
51. Hayakawa H, Taketomi A, Sakumi K, Kuwano M, Sekiguchi M (1995) *Biochemistry* **34**: 89-95
52. Shigenaga MK, Gimeno CJ, Ames BN (1989) *Proc Natl Acad Sci USA* **86**: 9697-9701
53. Shigenaga MK, Aboujaoude EN, Chen Q, Ames BN (1994) *Methods Enzymol* **234**: 16-33
54. Bessho T, Tano K, Kasai H, Ohtsuka E, Nishimura S (1993) *J Biol Chem* **268**: 19416-19421
55. Bessho T, Roy R, Yamamoto K, Kasai H, Nishimura S, Tano K, Mitra S (1993) *Proc Natl Acad Sci USA* **90**: 8901-8904
56. Białkowski K, Cysewski P, Oliński R (1996) *Z Naturforsch* **51c**: 119-122
57. Białkowski K, Oliński R (1995) *Doniesienia Zjazdowej, XXXI Zjazd Polskiego Towarzystwa Biochemicznego* **111**
58. Białkowski K (1996) *Praca doktorska*, Akademia Medyczna w Bydgoszczy
59. Oliński R, Zastawny T, Budzbon J, Skokowski J, Zegarski W, Dizdaroglu M (1992) *FEBS Lett* **309**: 193-198
60. Jaruga P, Zastawny TH, Skokowski J, Dizdaroglu M, Oliński R (1994) *FEBS Lett* **341**: 59-64
61. *Oliński R, Zastawny TH, Foksiński M, Barecki* A, Dizdaroglu M (1995) *Free Radic Biol Med* **18**: 807-813
62. Białkowski K, Kowara R, Windorbska W, Oliński R (1996) *Cancer Lett* **99**: 93-97
63. Oliński R (1993) *Post Hig Med Dośw* **47**: 463-474

II. CELE BADAŃ

Warunkiem *sine qua non* prowadzenia badań dotyczących funkcji, regulacji i biologii enzymu jest posiadanie wiarygodnej metody oznaczania jego aktywności enzymatycznej. W czasie, gdy autor tej rozprawy rozpoczynał swoje zmagania z białkiem MTH1, w literaturze naukowej można było odnaleźć jedynie metody oznaczania aktywności oczyszczonych preparatów tego enzymu. Taki stan rzeczy wymógł na autorze opracowanie własnej (i jak do tej pory jedynej), ilościowej metody oznaczania aktywności 8-oxo-dGTPazowej białka MTH1 w komórkach i tkankach, która daje możliwość śledzenia zmian komórkowego poziomu aktywności enzymu pod wpływem różnych bodźców działających na organizm lub komórki hodowane *in vitro*. Ogólna idea ilościowego oznaczania specyficznej aktywności 8-oxo-dGTPazy w materiale biologicznym została wykreowana przez autora jeszcze w końcowym okresie przygotowywania pracy doktorskiej. Jednak jej usprawnianie, optymalizacja, badanie wpływu różnych czynników na jej skuteczność, modyfikacje w zależności od rodzaju używanego materiału badawczego oraz walidacja każdego etapu procedury, trwały przez kolejne lata. Stąd też wydzielono grupę celów metodycznych, których realizacja warunkowała osiągnięcie zasadniczych celów poznawczych.

Skuteczne oczyszczanie puli wolnych nukleotydów z mutagennych substratów dla syntezy DNA (np. 8-oxo-dGTP) obniża ryzyko powstawania mutacji punktowych w DNA komórki, a tym samym przeciwdziała jej transformacji nowotworowej. Dlatego też główne cele poznawcze dotyczyły poszukiwania i opisu działania efektorów i stanów fizjologicznych komórki, które wpływają na poziom antymutagennej aktywności białka MTH1.

Pod każdym z wymienionych poniżej celów podano w nawiasie numery porządkowe publikacji, w których opisano ich realizację.

Cele metodyczne

1. Opracowanie i optymalizacja procedur syntezy i oczyszczania 5'-mono-, di- oraz trifosforanu 8-oksy-2'-deoksyguanozyny (odpowiednio: 8-oxo-dGMP, 8-oxo-dGDP i 8-oxo-dGTP), jako związków niezbędnych dla wykonywania

oznaczeń aktywności pirofosfohydrolazy 8-oksy-2'-deoksyguanozyno-5'-trifosforanu (8-oxo-dGTPazy) (publikacja nr 2).

2. Opracowanie metody selektywnego monitorowania aktywności 8-oxo-dGTPazowej białka MTH1 w lizatach komórek i tkanek zawierających interferujące aktywności enzymatyczne (publikacja nr 2).
3. Optymalizacja składu i stężeń składników mieszanin reakcyjnych używanych w standardowych oznaczeniach aktywności 8-oxo-dGTPazowej oraz badaniach kinetycznych (publikacja nr 2 i 6).
4. Opracowanie procedur ekstrakcji 8-oxo-dGTPazy z komórek hodowanych *in vitro* oraz tkanek ssaków z uwzględnieniem wpływu typowych składników medium homogenizacyjnego na odzysk aktywności, takich jak detergenty, białka stabilizujące, inhibitory proteaz, związki redukujące (publikacje nr 2, 3, 8).
5. Opracowanie metody ilościowego oznaczania produktu reakcji katalizowanej przez 8-oxo-dGTPazę w obecności różnych inhibitorów enzymu, opartej na wysokosprawnej chromatografii cieczowej z detekcją absorpcji promieniowania ultrafioletowego (publikacje nr 2 i 6).
6. Walidacja opracowanej procedury oznaczania w materiale biologicznym aktywności 8-oxo-dGTPazowej białka MTH1 (publikacje nr 2 i 6).

Cele poznawcze

1. Charakterystyka parametrów kinetycznych reakcji hydrolizy 8-oxo-dGTP katalizowanej przez białka MTH1 ssaków (publikacje nr 2 i 6).
2. Charakterystyka specyficzności substratowej ludzkiego białka MTH1 opracowaną metodą, opartą na pomiarach inhibitorowych właściwości difosforanowych analogów substratów (publikacja nr 6).
3. Eksperymentalna weryfikacja hipotezy o wpływie aktywności 8-oxo-dGTPazowej białka MTH1 na zawartość 8-oksy-2'-deoksyguanozyny w DNA komórek (publikacja 3).
4. Poszukiwanie oraz opis działania inhibitorów aktywności 8-oxo-dGTPazowej białka MTH1 u ssaków, jako związków o potencjale mutagennym:
   a) charakterystyka wpływu karcynogennych jonów metali (Cd^{2+} i Ni^{2+}) na aktywność 8-oxo-dGTPazową oraz poziom 8-oksy-2'-deoksyguanozyny w DNA na podstawie eksperymentów *in vivo* (publikacje 2, 4 i 5),

b) kinetyczna charakterystyka inhibicji aktywności ludzkiej 8-oxo-dGTPazy przez nukleozydo-5'-difosforany oraz stworzenie teoretycznego modelu ich wpływu na efektywność degradacji 8-oxo-dGTP przez białko MTH1 *in vivo* (publikacje 2 i 5).

5. Poszukiwanie wyjaśnienia dla zróżnicowanego poziomu ekspresji genu *MTH1* w różnych typach komórek fizjologicznych oraz nadekspersji tego genu w komórkach nowotworowych. Określenie wpływu różnych, fizjologicznych stanów komórek w populacji na poziom aktywności 8-oxo-dGTPazy:
   a) oznaczenie poziomu aktywności 8-oxo-dGTPazy w różnych fazach cyklu komórkowego (publikacja nr 7),
   b) próba określenia relacji pomiędzy aktywnością 8-oxo-dGTPazy i kontaktem międzykomórkowym (publikacja nr 7),
   c) poszukiwanie potencjalnej zależności pomiędzy aktywnością 8-oxo-dGTPazy i tempem proliferacji komórek (publikacje nr 7 i 8).

III. MATERIAŁY, METODY, WYNIKI, DYSKUSJA I LITERATURA

Zgodnie z obraną formułą rozprawy habilitacyjnej wyniki badań oraz ich dyskusję przedstawiono w postaci opublikowanych prac. Publikacja nr 1, z uwagi na jej przeglądowy charakter oraz rok ukazania się drukiem, została zamieszczona we wstępie jako wprowadzenie do tematyki badawczej oraz zapis stanu wiedzy na temat białka MTH1 z roku 1997. Publikacje nr 2, 3, 4, 6, 7 i 8 są oryginalnymi pracami eksperymentalnymi. Praca nr 5 zawiera podsumowanie eksperymentów dotyczących inhibicji enzymatycznej aktywności ssaczych białek MTH1 przez jony metali oraz dyskusję uzyskanych wyników w szerszym kontekście karcynogennego potencjału jonów metali. Artykuł nr 9, zamykający cykl publikacji przedstawionych w rozprawie, jest aktualnym przeglądem wiedzy na temat metabolizmu oraz roli 8-oksyguaniny w procesie nowotworzenia. Uwzględnia on bieżące odkrycia naukowe z zakresu regulacji ekspresji genu *MTH1*, biologicznych funkcji białka MTH1 i jego znaczenia w procesie karcynogenezy.

2.

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A novel assay of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity

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ABSTRACT

8-Oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) is a product of oxidative modification of dGTP, that can be misincorporated into DNA, causing AT→CG mutations. Cells are protected against 8-oxo-dGTP by 8-oxo-dGTP 5′-pyrophosphohydrolases (8-oxo-dGTP-ases) that convert it to 8-oxo-dGMP. Thus, inhibition of 8-oxo-dGTPases may lead to cancer. To elucidate the involvement of 8-oxo-dGTPases in carcinogenesis, an assay of the 8-oxo-dGTPase activity is required. This paper presents such an assay developed for Chinese hamster ovary (CHO) cells that can be applied to any biological material. It includes: (i) a convenient method for preparing 8-oxo-2′-deoxyguanosine 5′-phosphates; (ii) an HPLC/UV quantification of 8-oxo-dGTP hydrolysis products and (iii) separation of 8-oxo-dGTPase activity from interfering 8-oxo-dGTP phosphatase(s). The 8-oxo-dGTPase activity of CHO cells depends on magnesium, has a pH optimum of 8.5, K_m for 8-oxo-dGTP of 9.3 μM, and is inhibited by 8-oxo-dGDP, the product of interfering 8-oxo-dGTP phosphatases. The latter must be removed from the assayed samples by ultrafiltration through 30 kDa cut-off membranes. The method was used to test the inhibition by cadmium ions of the activity of 8-oxo-dGTPase in CHO cells. The cells cultured with 0.3–3 μM cadmium(II) acetate for up to 24 h had their 8-oxo-dGTPase activity suppressed in a Cd(II) concentration-dependent manner, down to 70% of the control value.

INTRODUCTION

The *mutT*⁻ mutants of *Escherichia coli*, the first discovered mutator strain of these bacteria (1), were characterized by at least a 1000-fold increase in frequency of a specific unidirectional A→C point mutation (2). The expression product of the wild-type *mutT* gene, an enzyme, hydrolyzes the canonical ribonucleoside and 2′-deoxyribonucleoside 5′-triphosphates to the corresponding nucleoside monophosphates and inorganic pyrophosphate, with great preference for dGTP (3). The enzyme hydrolyzes most specifically the products of dGTP and GTP oxidative modification, 8-oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) (4) and 8-oxo-GTP (5,6). Thus, the high spontaneous mutation rate observed in *E.coli mutT*⁻ mutants results from the lack of 8-oxo-dGTP pyrophosphatase (8-oxo-dGTPase) activity.

8-Oxo-dGTP can be generated by endogenous oxidants arising in normal cell metabolism. Its mutagenicity results from mispairing properties of 8-oxoguanine (reviewed in 7,8). If not decomposed by MutT protein, 8-oxo-dGTP can be incorporated into DNA opposite cytosine or adenine (4,9,10). The 8-oxo-G:A mispair, if not repaired (11), may result in AT→CG transversion (12). *Escherichia coli* MutT protein also prevented DNA transcription errors by efficiently hydrolyzing 8-oxo-GTP that could be otherwise misincorporated into nascent RNA (6).

Activity of the mammalian homologues of MutT protein (13), demonstrating very similar kinetic properties (14), imply an antimutagenic function: their expression in *E.coli mutT*⁻ mutants reduces greatly (15) or reverts to normal level (16,17) the high rate of A→C mutations. Thus, one may expect that a knock-out mutation of the gene coding for 8-oxo-dGTPase should form a mutator eukaryotic cell. This, in turn, would lead to a high rate of point mutation and to high frequency of cell transformation. Also, a chronic exposure of cells (animals) to inhibitors of 8-oxo-dGTPase might elevate the point mutation rate. Such inhibitors might be carcinogenic in animals.

To investigate the potential involvement of 8-oxo-dGTPases in the mechanisms of mutagenic and carcinogenic processes, we must measure the level of the enzyme activity in cells exposed to different carcinogenic agents. Since, to our best knowledge, a reliable enzymatic assay of 8-oxo-dGTPase activity in cultured cells and animal tissues has not yet been proposed, we decided to develop such an assay. The assay was worked out on cultured Chinese hamster ovary (CHO) cells, but can be easily adapted to any biological material. The assay was then used to verify *in vivo* our former *in vitro* findings that certain carcinogenic metals, including Cd(II), inhibited the enzymatic activity of isolated bacterial (MutT) and human (MTH1) 8-oxo-dGTPases (18). We also describe synthesis and one-step purification of 8-oxo-2′-deoxyguanosine 5′-phosphates required in the procedure.

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MATERIALS AND METHODS

Chemicals

DEAE-cellulose DE52 was purchased from Whatman (England). Bovine catalase was from Boehringer Mannheim (Germany). Ascorbic acid, bovine serum albumin (BSA), dithiothreitol (DTT), sodium dihydrogen phosphate, disodium hydrogen phosphate, 2′-deoxyguanosine (dG) and its 5′- mono-, di- and triphosphates: dGMP (free acid), dGDP (disodium salt), and dGTP (trisodium salt), as well as disodium ethylenediaminetetraacetate (Na_2EDTA), hydrochloric acid, 30% (w/w) hydrogen peroxide, methanol (HPLC grade), phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, sodium chloride, triethylammonium bicarbonate (TEAB) and Tris-(hydroxymethyl)aminomethane (Tris) were from Sigma Chemical Co. (USA). Ham's F-12 nutrient mixture, fetal bovine albumin, glutamine, penicillin, streptomycin and Trypsin/Versene mixture were purchased from Biofluids Inc. (USA). Deionized, double-distilled water was used in all experiments.

Preparation of 8-oxo-2′-deoxyguanosine 5′-mono-, 5′-di- and 5′-triphosphates (8-oxo-dGMP, 8-oxo-dGDP and 8-oxo-dGTP, respectively)

All the 8-oxo-dG 5′-phosphates were prepared using the same procedure based on oxidation of the corresponding dG 5′-phosphates with H_2O_2 and ascorbic acid. Ascorbic acid (100 mg) and 50 mg of dGTP, dGDP or dGMP were dissolved in 40 ml of 20 mM phosphate buffer, pH 7.0, in a glass flask. Reaction was initiated by adding 0.5 ml 30% H_2O_2; the mixture was left in the dark at room temperature for 3 h. The reaction was terminated by adding 120 µg of catalase, followed by incubation at 37°C for 20 min with vigorous stirring. The reaction mixture was then filtered through a 0.2 µm Nylon membrane vacuum filtration unit (Nalgene, USA) and loaded on a DEAE-cellulose column (2.6 × 19.5 cm) equilibrated with 20 mM TEAB buffer, pH 8.5. The subsequent elution programs of the column at the flow rate of 2 ml/min and pH 8.5 were different depending on the 8-oxo-dG 5′-phosphate being isolated. Thus, for 8-oxo-dGTP, elution with 230 ml of 20 mM TEAB was followed by 2000 ml linear gradient of 20–160 mM TEAB and 1140 ml of 160 mM TEAB. For 8-oxo-dGDP, 190 ml of 20 mM TEAB was followed by 2000 ml linear gradient of 20–140 mM TEAB and 470 ml of 140 mM TEAB. 8-Oxo-dGMP was eluted with 80 ml of 20 mM TEAB, followed by 2000 ml linear gradient of 20–110 mM TEAB and 480 ml of 110 mM TEAB. The effluent was collected in 19 ml fractions whose UV absorbance spectrum was measured in the range of 200–320 nm. The fractions containing individual 8-oxo-dG 5′-phosphates eluted after the corresponding unreacted dG 5′-phosphate peak, exhibiting the characteristic UV spectrum of 8-oxo-dG, were pooled and evaporated to dryness at 45°C under vacuum. TEAB was removed from the preparations by co-evaporation (five times) with water/methanol (1:1). Each final 8-oxo-dG 5′-phosphate sample was dissolved in 2 ml H_2O and its concentration was determined spectrophotometrically using the molar absorbance coefficient for 8-oxo-dG and its phosphate derivatives, $\epsilon_{293} = 10\,300$ (19). The nucleotide solutions were stored at –20°C.

Cell culture and harvesting

The CHO cells, variant K1-BH4, were maintained at 37°C in 750 ml culture flasks, under air containing 5% CO_2, in 25 ml Ham's F-12 nutrient mixture supplemented with 5% fetal bovine serum, 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 µg/ml). Typically, the cells were grown to near confluence and harvested as follows: medium was removed from the flask and cells were washed three times with 15 ml of ice-cold 20 mM Tris-buffered saline, pH 7.4, and scraped to 10 ml of the same saline. The cells were recovered by 10 min centrifugation at 1500 *g* (4°C). Alternatively, the cells were harvested by trypsinization; after removal of the medium, the cells were treated for 1 min with 10 ml of Trypsin/Versene mixture, followed by 10 ml of Ham's medium. Detached cells were collected and washed three times with 15 ml of 20 mM Tris-buffered saline, each time with centrifugation at 1500 *g*, for 10 min, at 4°C.

Cell extracts for determination of 8-oxo-dGTPase activity

Typically, the cells from one culture flask were suspended in 0.25–0.5 ml of 20 mM Tris–HCl hypotonic buffer, pH 7.4, and lysed by three cycles of freezing and thawing in liquid nitrogen and water bath. Alternatively, 20 mM Tris–HCl, pH 7.4, containing 0.5 mM PMSF, 0.5 µg/ml leupeptin and pepstatin A, was used as a lysis buffer. The resulting cell lysates were ultracentrifuged for 3 h at 150 000 *g* (4°C). The supernatants, termed below as 'extracts', were stored at –70°C. To isolate the fraction of low molecular weight proteins, 200 µl of the extract was filtered through a 30 kDa cut-off, low protein-binding ultrafiltration membrane (Ultrafree-MC Filtration Unit, Millipore), with centrifugation at 3000 *g* until complete passage of the sample. The resulting through-fraction ('ultrafiltrate') was used for the 8-oxo-dGTPase activity determination either immediately or after several days storage at –70°C.

Determination of 8-oxo-dGTPase activity

The enzymatic assays were run in 0.5 ml capped polypropylene tubes in an incubation mixture of total volume of 60 µl containing 5–120 µM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl buffer (pH 6–10), other additives as specified in the Results, and 5–20 µl of the cell extract or its ultrafiltrate. In the developmental stage of the assay, 200 µg/ml of BSA and/or 5 mM DTT were also present in certain test solutions. The reaction was initiated by adding the extract or ultrafiltrate, carried out at 37°C for 30–120 min, and terminated by adding 20 µl of 50 mM Na_2EDTA. The reaction mixture was finally analyzed by HPLC. To determine the reaction time course, the volume of reaction mixture was scaled up to 600 µl, and 20 µl aliquots were analyzed by HPLC at 30 min intervals. The analyses were performed using Waters HPLC system consisting of two pumps (model 510), Waters Intelligent Sample Processor (model 710B), UV-VIS Photodiode Array Detector (model 996), Supelcosil LC-18-T column (250 × 4.6 mm, 5 µm grain; Supelco, Switzerland), controlled by a Millennium Chromatography Manager. The reaction mixtures (20 µl) were chromatographed isocratically with 100 mM NaH_2PO_4-NaOH buffer (pH 5.5)/methanol (95:5), at a flow rate of 1 ml/min. Solutions of known concentrations of 8-oxo-dGDP and 8-oxo-dGMP, ranging from 5 to 30 µM, were used for calibration. For quantitative analysis of the formed reaction products, chromatograms acquired at 293 nm were integrated.

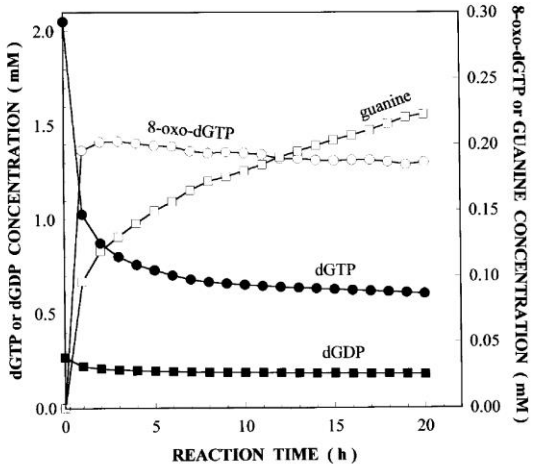

Figure 1. Formation of 8-oxo-dGTP and guanine from 2 mM dGTP reacting with 14.2 mM ascorbic acid and 133 mM H_2O_2 in 20 mM phosphate buffer, pH 7.0, at room temperature. Samples (20 µl) of the reaction mixture were analyzed by HPLC at 60 min intervals as described in Materials and Methods. The main nucleobase derivatives present in the mixture were quantified versus synthetic standards. The dGTP sample used for the reaction contained ~10% of dGDP.

Treatment of CHO cells with cadmium acetate

The CHO cells growing in 36 flasks were treated simultaneously at ~50% confluence with 1 ml of the culture medium (control group) or the same medium containing cadmium acetate to set the final cadmium concentration at 0.3, 1 or 3 µM level. Each group of cells consisted of nine separate cultures. After cadmium was added, the cells were allowed to grow for an additional 2, 6 or 24 h, at which points three cultures of each cadmium concentration were harvested with the cell scraper, lysed in 20 mM Tris–HCl, pH 7.4, and ultracentrifuged. The resulting extracts were ultrafiltered and assayed for 8-oxo-dGTPase activity.

Protein determination

Protein concentration in the cell extracts was determined in triplicate by the method with bicinchoninic acid (20). Crystalline BSA was used as a protein standard.

RESULTS

Synthesis of 8-oxo-dGTP, 8-oxo-dGDP and 8-oxo-dGMP

To establish the optimum reaction conditions for dGTP oxidation to 8-oxo-dGTP with the ascorbic acid/H_2O_2 system (14), the time course of the reaction and the effects of pH, as well as of dGTP and H_2O_2 concentrations on the product yield, were followed by HPLC. The 8-oxo-dGTP yield improved with increasing initial dGTP concentration, but only up to 2 mM dGTP. At 4 mM dGTP, the yield of 8-oxo-dGTP began to decline. H_2O_2 concentration in the reaction mixture of 100–150 mM was the best compromise between the 8-oxo-dGTP yield and reaction selectivity.

The oxidation of dGTP yielded two main UV-absorbing products, 8-oxo-dGTP and guanine (Fig. 1). Since the latter predominated in non-buffered reaction mixtures, preparatory reactions should be carried out in buffered neutral media. Under optimal reaction conditions (2 mM dGTP, 14.2 mM ascorbic acid, 133 mM H_2O_2 in 20 mM phosphate buffer, pH 7.0), the concentration of 8-oxo-dGTP reached its maximum after 3 h of incubation at room temperature (Fig. 1). The same reaction conditions were also used for oxidation of dGDP and dGMP. Dephosphorylation of nucleotides was not observed.

The individual 8-oxo-dG 5′-phosphates, formed in reaction mixtures during oxidation of dGTP, dGDP and dGMP, were purified by DEAE-cellulose column chromatography, using TEAB gradients for elution. Every 8-oxo-derivative being purified, eluted as the last UV-absorbing peak of the chromatographed mixture (Fig. 2A, B and C). This procedure allowed for obtaining high purity preparations of the nucleotides in a single chromatographic step (Figs 2 and 3). The purity of such prepared 8-oxo-dGTP, 8-oxo-dGDP and 8-oxo-dGMP was ascertained by HPLC and spectral analysis (Fig. 3). All three 8-oxo-2′-deoxyguanosine 5′-phosphate preparations had the same UV spectrum characteristic of 8-oxo-dG with the maxima at 245 and 293 nm (Fig. 3B). To further verify the identity of the synthesized 8-oxo-dGTP, it was subjected to hydrolysis by *E.coli* alkaline phosphatase, and the resulting reaction mixture was chromatographed by HPLC. This treatment led to sequential dephosphorylation of this nucleotide to 8-oxo-dGDP, 8-oxo-dGMP and finally to 8-oxo-dG, coeluting with genuine nucleotide and nucleoside standards. The yields were as follows: 8-oxo-dGTP, 6.1%; 8-oxo-dGDP, 10.4%; 8-oxo-dGMP, 4.1%.

Quantitative determination of 8-oxo-dGTP dephosphorylation products

Among the various HPLC techniques, we found reversed-phase chromatography on a Supelcosil LC-18-T column to be the best for fast resolution of 8-oxo-dGTP (retention time, t_R = 5.2 min) and all the products of its dephosphorylation, 8-oxo-dGDP (t_R = 6.3 min), 8-oxo-dGMP (t_R = 8.9 min) and 8-oxo-dG (t_R = 22.4 min), using isocratic elution with 100 mM NaH_2PO_4-NaOH buffer (pH 5.5)/methanol (95:5). The elution order of the compounds on the Supelcosil LC-18-T column depends on the number of phosphate residues in the molecules, 8-oxo-dGTP being the first and 8-oxo-dG the last eluting peak (Fig. 4).The detection limit at 293 nm for 8-oxo-dGMP was 5 pmol per injection, corresponding to 0.25 µM 8-oxo-dGMP when 20 µl of the reaction mixture was analyzed. It means that hydrolysis of 0.6% of the 40 µM 8-oxo-dGTP to 8-oxo-dGMP could be detected. The acquisition of the chromatograms at 293 nm ensured not only high sensitivity but also selectivity of 8-oxo-dG derivatives detection. No UV-absorbing compounds originating from the CHO cells were detected at this wavelength when typical enzymatic reaction mixtures were chromatographed.

Enzymatic hydrolysis of 8-oxo-dGTP by CHO cell extracts

Preliminary experiments revealed that at pH above 7.0, in the presence of 5 mM $MgCl_2$, 8-oxo-dGTP was hydrolyzed by the extract of CHO cells mainly to 8-oxo-dGDP (Fig. 4A). The 8-oxo-dGMP sought was a minor second hydrolysis product. To obtain the time course data for 8-oxo-dGTP hydrolysis, 40 µM 8-oxo-dGTP was reacted in the presence of 5 mM $MgCl_2$, 100 mM Tris–HCl, at pH 8.5, with the extract of CHO cells; total protein concentration in the reaction mixture was 132 µg/ml. As shown in Figure 5A, the predominant product was indeed 8-oxo-dGDP which after 330 min incubation at 37°C constituted 65% of both 8-oxo-dGDP and 8-oxo-dGMP. To determine if that 8-oxo-dGMP

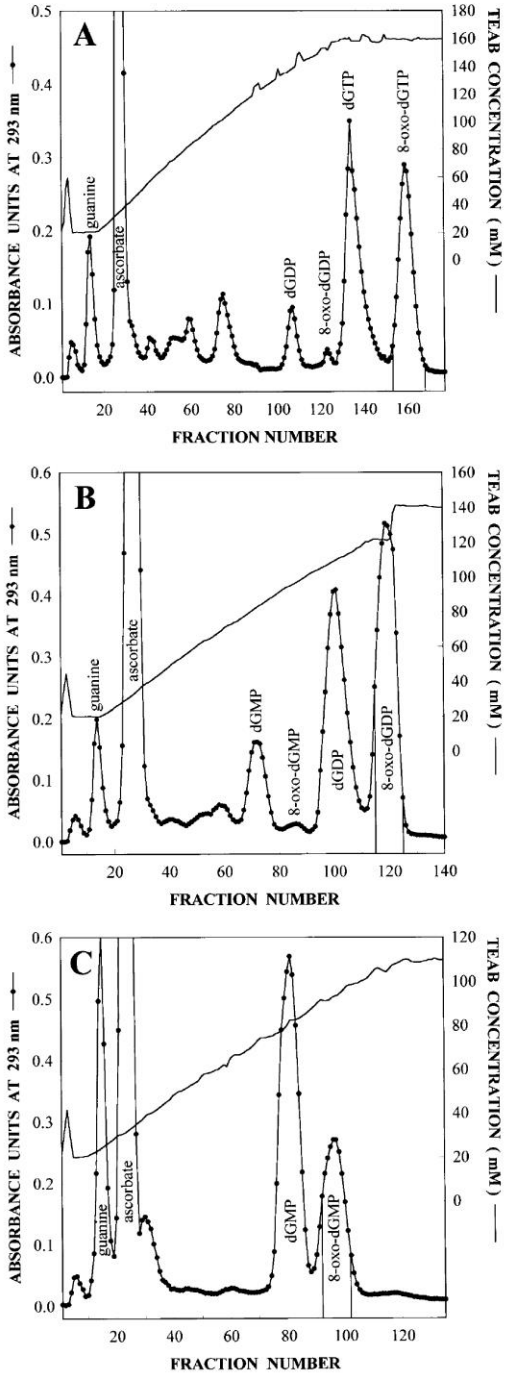

Figure 2. Purification of 8-oxo-dGTP (**A**), 8-oxo-dGDP (**B**) and 8-oxo-dGMP (**C**) by anion exchange chromatography on DEAE-cellulose column with TEAB gradient. Each 8-oxo-dG 5′-phosphate was prepared by oxidation of dGTP, dGDP or dGMP with the H_2O_2/ascorbate system. The reaction mixtures (40 ml) were loaded without preconcentration on the column equilibrated with 20 mM TEAB (pH 8.5), and eluted with gradient of TEAB (pH 8.5), as described in Materials and Methods. 8-Oxo-derivatives of the substrate nucleotides were eluted always as the last peak. Fractions of each 8-oxo-dG 5′-phosphate marked with vertical lines were pooled.

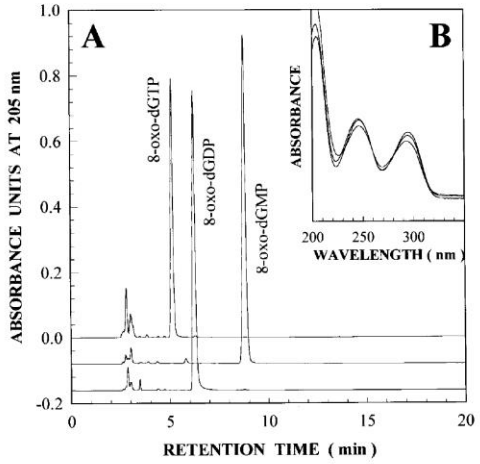

Figure 3. Purity check of the synthesized 8-oxo-dG 5′-phosphates. (**A**) Water solutions of the final preparations of 8-oxo-dGTP, 8-oxo-dGDP and 8-oxo-dGMP were chromatographed by HPLC as described in the Materials and Methods. Presented chromatograms were acquired by absorbance detector at 205 nm that ensured effective detection of UV-absorbing compounds. (**B**) UV spectra of water-diluted final preparations of the nucleotides measured in Beckman DU 68 spectrophotometer.

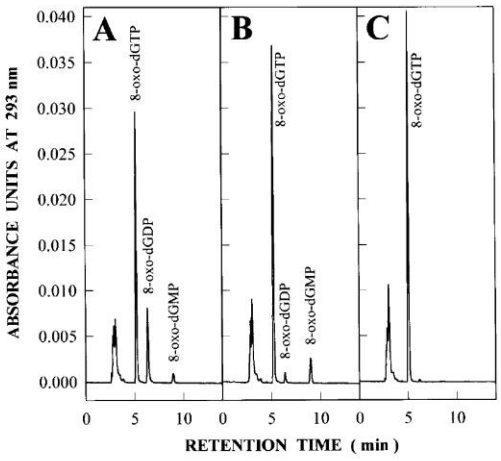

Figure 4. HPLC separation of 8-oxo-dGTP and its hydrolytic dephosphorylation products, 8-oxo-dGDP and 8-oxo-dGMP. (**A**) Chromatogram of 8-oxo-dGTP after incubation with extract of CHO cells. 8-Oxo-dGTP (40 µM), 5 mM $MgCl_2$ and 100 mM Tris–HCl, pH 8.5 were incubated with extract (7.9 µg protein) in total volume of 60 µl for 30 min. The main product of 8-oxo-dGTP hydrolysis in this case is 8-oxo-dGDP. (**B**) Chromatogram of 8-oxo-dGTP after incubation with ultrafiltrate of CHO cell extract used in (A). Reaction was carried out under the same conditions. 8-Oxo-dGMP is the main product. (**C**) Chromatogram of a control reaction mixture prepared as in (B) except that Na_2EDTA was added to the mixture before the ultrafiltrate. Hydrolysis of 8-oxo-dGTP was not detected. The peaks eluting near the column dead volume ($t_R \approx 3$ min) are produced mainly by EDTA.

originated from dephosphorylation of 8-oxo-dGDP or directly from 8-oxo-dGTP, 40 µM 8-oxo-dGDP was incubated at 37°C in the presence of 5 mM $MgCl_2$ with the same amount of CHO extract

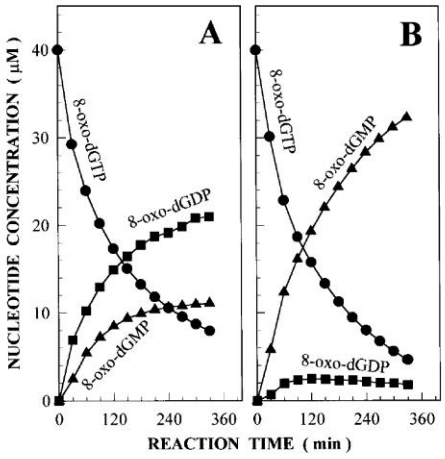

Figure 5. Time course of the hydrolytic dephosphorylation of 8-oxo-dGTP by the CHO cell extract (**A**) and its ultrafiltrate (**B**). Mixtures of 40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl, pH 8.5 containing extract (100 μl, 79 μg protein) or its ultrafiltrate (100 μl) were incubated in a total volume of 600 μl. Aliquots of 20 μl were chromatographed every 30 min, as described in Materials and Methods.

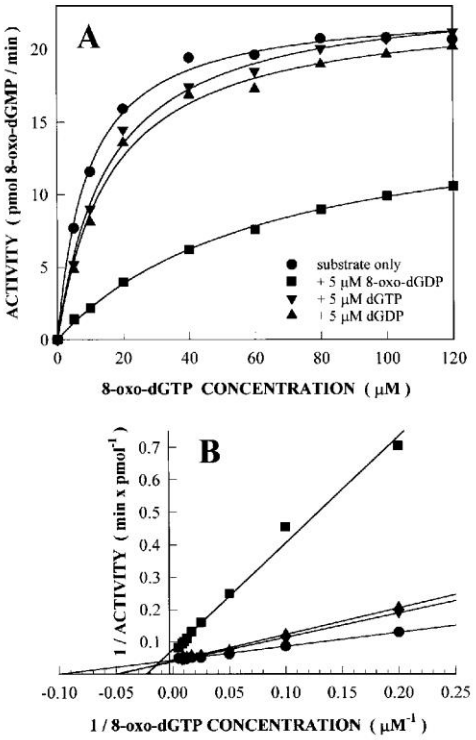

Figure 6. The substrate concentration dependence of the 8-oxo-dGTPase activity of CHO cells as determined in the absence or presence of 5 μM potential enzyme inhibitors: 8-oxo-dGDP, dGTP and dGDP. Ultrafiltrate of CHO cell extract (protein concentration in extract, 3.8 mg/ml) was incubated for 30 min at 37°C with 5 mM $MgCl_2$, 100 mM Tris–HCl, pH 8.5 and 8-oxo-dGTP concentrations ranging from 5 to 120 μM. This set of reactions was repeated in the presence of 5 μM 8-oxo-dGDP, dGTP or dGDP. The reactions were terminated with 20 μl of 50 mM Na_2EDTA, and 20 μl aliquots of the mixtures were chromatographed to determine amount of 8-oxo-dGMP formed. (**B**) Lineweaver–Burk double reciprocal plot of the data.

as above, at pH 5–8.5. No decomposition of 8-oxo-dGDP was detected for several hours, meaning that the observed 8-oxo-dGMP was a product of pyrophosphohydrolase (8-oxo-dGTPase), and not just unspecific phosphatase activity of the CHO extract. Approximately 90% of the phosphatase activity catalyzing 8-oxo-dGDP formation was inhibited by the addition of EDTA [molar ratio EDTA/Mg(II) = 3.3].

Selective determination of the 8-oxo-dGTPase activity and kinetic properties of the enzyme from CHO cells

Upon incubation with the cell extract, 8-oxo-dGTP is rapidly consumed by the interfering phosphatase(s), making it impossible to measure reliably the 8-oxo-dGTPase activity (Figs 4A and 5A). However, 8-oxo-dGTPase activity may be separated from that of the phosphatase(s) by ultrafiltration through low protein-binding regenerated cellulose membranes with 30 kDa molecular cut-off pores, using Ultrafree MC Filtration Units, Millipore, USA (ultrafilters of some other companies gave poor results). As depicted in Figure 4B, an ultrafiltrate obtained from the CHO cell extract hydrolyzed 8-oxo-dGTP almost exclusively to 8-oxo-dGMP. Thus, the 8-oxo-dGTPase activity was associated with a small protein of molecular mass <30 kDa. This activity could be completely inhibited by adding EDTA, signifying its dependence on magnesium. Therefore, EDTA could be used for termination of the enzymatic reaction (Fig. 4C). After the addition of EDTA, neither generation of 8-oxo-dGMP nor loss of 8-oxo-dGTP could be detected in the reaction mixture for up to three days at room temperature. The pH optimum for 8-oxo-dGTPase activity was found to be 8.5.

To investigate the kinetics of the hydrolysis by CHO cell ultrafiltrate at optimum pH, 8-oxo-dGTP was incubated with ultrafiltrate prepared from the same extract used previously in the time course experiment, under the same conditions (compare Fig. 5A). As shown in Figure 5B, this time 8-oxo-dGTP was hydrolyzed predominantly to 8-oxo-dGMP, with only minor (5% in 330 min) formation of 8-oxo-dGDP. The formation of 8-oxo-dGMP was linear until ~40% of 8-oxo-dGTP was consumed. The reaction followed Michaelis–Menten kinetics (Figure 6), and the K_m value for 8-oxo-dGTP hydrolysis at pH 8.5 was 9.3 μM.

Standard reaction conditions for 8-oxo-dGTPase activity determination

The optimum reaction mixture for 8-oxo-dGTPase activity assay in CHO cells included: 40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl, pH 8.5, and cell extract ultrafiltrate in total volume of 60 μl. The reaction was initiated by the addition of the ultrafiltrate (usually 10 μl of ultrafiltrate obtained from cell extract containing 0.5–3 mg protein/ml), carried out at 37°C for 30–60 min, and terminated by addition of 20 μl of 50 mM Na_2EDTA. Blank samples were prepared by adding Na_2EDTA before ultrafiltrate, followed by incubation at 37°C. Under these 'standard reaction conditions', 8-oxo-dGTPase was almost completely saturated with the substrate (Fig. 6A), and the substrate hydrolysis did not

exceed 25%. The activity unit (U) was defined as the amount of enzyme converting 1 pmol of 8-oxo-dGTP to 8-oxo-dGMP per min under standard reaction conditions. The enzyme activity was linearly proportional to the amount of ultrafiltrate added to the reaction mixture. To compare different cell extracts, their activities determined in ultrafiltrates were expressed in relation to protein concentrations in the corresponding extracts (protein concentrations in the ultrafiltrates were too low for reliable determination). It means that activity of 10 µl of an ultrafiltrate was divided by the amount of protein (in mg) present in 10 µl of the extract from which the ultrafiltrate was prepared. The 8-oxo-dGTPase activity in stored ultrafiltrates was relatively stable in time. Not more than 20% loss of the enzyme activity was observed when the ultrafiltrate was left at room temperature for 64 h without addition of any protease inhibitors or protein stabilizers (data not shown).

The effects of dGTP, dGDP and 8-oxo-dGDP on the activity of 8-oxo-dGTPase

The influence of 5 µM dGTP, dGDP or 8-oxo-dGDP on the K_m and V_{max} parameters of 8-oxo-dGTP hydrolysis by the CHO cell ultrafiltrate was investigated. As found, both dGTP and dGDP were weak competitive inhibitors of 8-oxo-dGTPase activity, increasing the K_m value from 9.3 to 15.5 and 16.4 µM, and causing negligible changes of V_{max} from 22.9 to 23.9 and 23.0 pmol/min, respectively (Fig. 6). In contrast, 8-oxo-dGDP quite considerably affected both K_m and V_{max} of the 8-oxo-dGTPase activity. In the presence of 5 µM 8-oxo-dGDP, K_m increased to 61.7 µM and V_{max} decreased to 15.9 pmol/min (Fig. 6). The influence of different 8-oxo-dGDP concentrations on the activity is shown in Figure 7: 2.5 µM 8-oxo-dGDP caused a 50% and 40 µM 8-oxo-dGDP caused a 93% drop in the 8-oxo-dGTPase activity towards 40 µM 8-oxo-dGTP. Under the same conditions, 40 µM dGTP, an alternative substrate for 8-oxo-dGTPase (3), produced only a 4% inhibition. Hydrolysis of dGTP in this reaction mixture was undetectable.

Influence of cell harvesting methods, protease inhibitors and protein stabilizers on the recovery of 8-oxo-dGTPase activity

CHO cell cultures, grown in 10 flasks (started at 10^6 cells/flask), were terminated after 4 days and processed separately. The cells of five flasks were harvested manually with cell scrapers, and the remaining cells were harvested by trypsinization. The cells were further lysed in 20 mM Tris–HCl, pH 7.4, ultracentrifuged, ultrafiltered and analyzed for 8-oxo-dGTPase using standard reaction conditions. The mean enzyme activity in cells harvested by scraping was 1677 ± 185 SE ($n = 5$) and in cells harvested by trypsinization the activity was 865 ± 68 SE ($n = 5$) U/mg protein. Thus, the trypsinization procedure yielded only 50% of the 8-oxo-dGTPase activity that could be recovered from the same number of CHO cells by the scraping method ($P = 0.015$).

To compare the influence of protease inhibitors on the recovery of 8-oxo-dGTPase activity, 16 cultures of CHO cells were harvested by scraping. Eight of the collected cell samples were lysed by freezing and thawing in 20 mM Tris–HCl, pH 7.4, and the remaining samples were lysed in 20 mM Tris–HCl, pH 7.4, containing 0.5 mM PMSF and 0.5 µg/ml pepstatin A and leupeptin. The ultrafiltrates were analyzed for 8-oxo-dGTPase activity under standard reaction conditions. The mean activity in the cells lysed without protease inhibitors was 1206 ± 45 SE ($n = 8$) U/mg protein, while that in the cells lysed with PMSF, leupeptin and pepstatin A was significantly lower, 790 ± 49 SE ($n = 8$) U/mg protein ($P = 0.0002$). Hence, in our standard assays the inhibitors were not used.

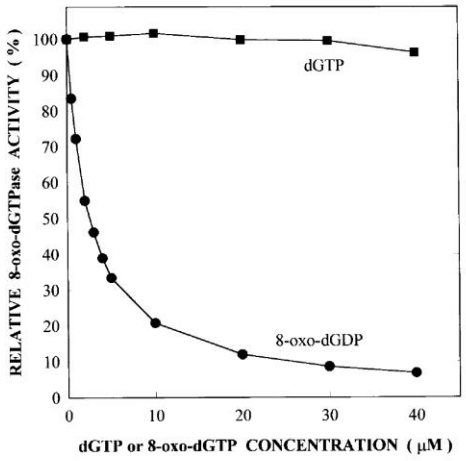

Figure 7. Inhibitory effect of 8-oxo-dGDP and dGTP on the activity of 8-oxo-dGTPase from CHO cells. The enzyme activity in the ultrafiltrate was determined under standard reaction conditions except that the reaction mixtures contained increasing concentrations of 8-oxo-dGDP or dGTP ranging from 0 to 40 µM. Neither 8-oxo-dGDP nor dGTP decomposed under these conditions.

Addition of 200 µg/ml of BSA and/or 5 mM DTT to reaction mixture had no significant effect on the 8-oxo-dGTPase activity.

Validation of the 8-oxo-dGTPase activity assay

The following conditions were found to be essential for the optimal analytical procedure of 8-oxo-dGTPase activity determination in cultured cells: (i) manual harvesting of the cells with cell scrapers; (ii) lysis of the cells in three cycles of freezing and thawing in the presence of hypotonic buffer, 20 mM Tris–HCl, pH 7.4; (iii) ultracentrifugation of the lysed cells (3 h at 150 000 *g*; 4°C) and determination of protein concentration in the resulting extract; (iv) ultrafiltration of the extract through 30 kDa molecular weight cut-off, low protein binding cellulose filters and (v) determination of the 8-oxo-dGTPase activity under standard conditions and quantitation of 8-oxo-dGMP formed by HPLC with UV detection.

The reproducibility of 8-oxo-dGTPase activity assay was investigated at different stages of the analytical procedure. Several independent experiments, based on seven repetitions ($n = 7$), were completed in order to estimate the contribution of a particular analytical step to the final standard error value of the assay. The results showed that the relatively least reproducible step of the entire procedure, determining the precision of the assay, was the ultrafiltration step (3.8% SE of the mean value), the HPLC analysis being the most precise step (0.18% SE). Overall, the standard error ($n = 3$) of the assay used to evaluate cadmium effects in CHO cells (below) varied from 1.6 to 15% of the mean values.

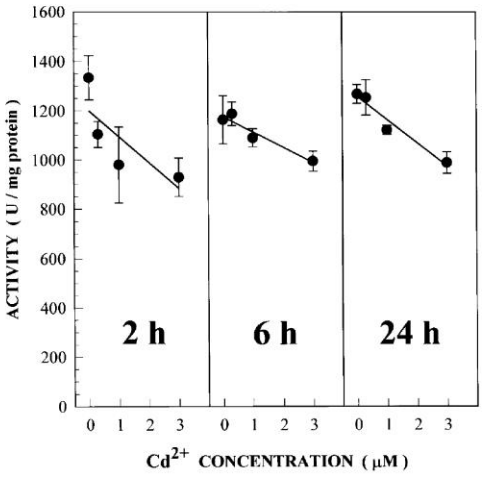

Figure 8. Activity of 8-oxo-dGTPase in CHO cells grown with 0, 0.3, 1 or 3 μM cadmium acetate for 2, 6 and 24 h. The error bars represent standard error values for three independent cultures treated in the same way. Linear regression lines were drawn to express the trend of activity to decrease with increasing cadmium concentration.

An example of the assay application: effect of cadmium on the activity of 8-oxo-dGTPase in CHO cells

Cadmium chloride has been shown previously to strongly inhibit isolated bacterial and human 8-oxo-dGTPases (18). To test this effect *in vivo*, the present assay was used to investigate the influence of cadmium ions on 8-oxo-dGTPase activity in cultured CHO cells. The cells were grown in the presence of 0.0, 0.3, 1.0 or 3.0 μM $CdCl_2$ in the culture medium for 2, 6 and 24 h, and then washed, harvested, and assayed for 8-oxo-dGTPase activity as described previously. As shown in Figure 8, an observed sequential decrease of 8-oxo-dGTPase activity correlated well with the increasing concentration of Cd(II). After the 2, 6 and 24 h incubation with 3 μM Cd(II), the enzyme activity decreased to 66, 89 and 78% of the control value (cadmium-free cultures), respectively. Analysis of variance (ANOVA) revealed that the observed cadmium concentration-dependent decrease of 8-oxo-dGTP pyrophosphatase activity was statistically highly significant for the 6 and 24 h treatments ($P = 0.013$ and 0.0006, respectively), and only weakly so for the 2 h exposure ($P = 0.076$).

DISCUSSION

8-Oxo-dGTPase is an enzyme hydrolyzing 8-oxo-dGTP, the strongly mutagenic (12) substrate for DNA synthesis. Thus, any factor causing a decrease of the 8-oxo-dGTPase activity could be potentially mutagenic and carcinogenic. To investigate the role of 8-oxo-dGTPase in carcinogenesis, a reliable assay of this enzyme's activity *in vivo* is essential. The analytical procedure for selective 8-oxo-dGTPase determination in CHO cells, developed in the present study, constitutes such an assay.

Pure preparations of the enzyme substrate and product are indispensable for any successful enzymatic activity determination. Two general approaches were reported for the 8-oxo-dGTP and 8-oxo-dGMP synthesis: a multistep chemical synthesis starting from 2′-deoxyguanosine (21) or a direct oxidation of dGTP and dGMP at the C8 position by oxygen radical-generating systems, such as ascorbic acid/EDTA/Fe^{2+} (12), ascorbic acid/O_2/Fe^{2+} (22), ascorbic acid/EDTA/Fe^{2+}/O_2 (23) or ascorbic acid/H_2O_2 (14). After trying the other approaches, we decided to adopt and optimize the method of dGTP and dGMP oxidation described by Mo *et al.* (14). In all methods based on oxidation of dG nucleotides, the main problem is the separation of their 8-oxo-derivatives from unreacted substrates and many other unidentified products. Previously described methods of 8-oxo-dGTP purification applied several steps of chromatographic separation, usually consisting of a combination of low-pressure anion exchange chromatography on DEAE-Sephadex followed by subsequent two steps of reversed-phase HPLC separation (12,22,23) or anion-exchange HPLC on Spherisorb SAX column with subsequent low pressure separation on DEAE-MemSep anion-exchange chromatographic cartridge (14). In contrast, our procedure allows purification of several milligrams of 8-oxo-dGTP, 8-oxo-dGDP or 8-oxo-dGMP in a single chromatographic run on DEAE-cellulose column (Fig. 2).

Another critical requirement of an enzymatic assay is precise quantification of the reaction product. The oldest (4,14) and most frequently used method of 8-oxo-dGTPase activity determination applies the radiolabeled [α-^{32}P]8-oxo-dGTP. The enzymatic reaction product ^{32}P-8-oxo-dGMP is separated from the substrate by thin layer chromatography and quantified autoradiographically (24). The inconveniences of this method include the synthesis of the short-lived radiolabeled and unlabeled 8-oxo-dGTP and low precision of autoradiographic quantitation. In a more recent HPLC method, 8-oxo-dGMP was separated on a Beckman Ultrasphere C_{18} column and measured electrochemically (21). However, the instability of the electrochemical detector made this method inconvenient for use with our reaction mixtures, containing many electrochemically-active constituents. In the present method we used the Supelcosil LC-18-T column, which ensures excellent separation of all the products of 8-oxo-dGTP hydrolysis. For detection and quantification of 8-oxo-dG phosphates, we used a photodiode array UV-VIS detector that offered very low background noise level of ~1×10^{-5} absorbance units, allowing very sensitive detection of 8-oxo-dG and its phosphates (minimum 5 pmol/injection).

The 8-oxo-dGTPase activity assays described to date can be successfully used only with purified enzymes and should not be applied to mammalian cell or tissue extracts. Upon incubation with such extracts, 8-oxo-dGTP is hydrolyzed to 8-oxo-dGDP. The presence of 8-oxo-dGTP phosphatase activity, hydrolyzing 8-oxo-dGTP to 8-oxo-dGDP in the presence of magnesium ions in alkaline solutions, was previously demonstrated in human (14,25) and mouse cells (17). This activity drastically lowers the substrate concentration for 8-oxo-dGTPase in reaction mixture. Moreover, we showed that 8-oxo-dGDP is a strong inhibitor of the 8-oxo-dGTPase activity. This observation also has another practical implication: the 8-oxo-dGTP preparation for 8-oxo-dGTPase assay should be virtually free of 8-oxo-dGDP. Because the latter is also produced by non-enzymatic decomposition of 8-oxo-dGTP in solution, long term storage of 8-oxo-dGTP preparation should be avoided. As little as 5% 8-oxo-dGDP in 8-oxo-dGTP preparations decreases the observed activity of 8-oxo-dGTPase by ~50%.

Considering the above, it is obvious that in order to be accurately determined, the 8-oxo-dGTP pyrophosphatase activity must be separated from 8-oxo-dGTP phosphatase activities. To achieve this, we propose the use of 30 kDa cut-off ultrafiltration

membranes for fast separation of both activities. Apparently, the molecular mass of the interfering phosphatase(s) is >30 kDa, in contrast with that of 8-oxo-dGTPase(s) having molecular mass of 18 kDa, as found in humans (14), mice (17) and rats (16). The following characteristic features confirm the identity of the CHO cell 8-oxo-dGTPase with other mammalian MutT homologues: (i) molecular mass below 30 kDa; (ii) total Mg(II)-dependence of the enzyme activity (Fig. 4C); (iii) maximum activity at pH 8.5 and (iv) K_m = 9.3 μM for 8-oxo-dGTP hydrolysis at pH 8.5 (Figure 6). In comparison, the purified human enzyme has been characterized as 18 kDa protein, demonstrating Mg(II)-dependent activity towards 8-oxo-dGTP with pH optimum of 8.0 and K_m = 12.5 μM (14). We also characterized the human enzyme from cultured fibroblasts: the pH optimum was 8.5 and K_m for 8-oxo-dGTP hydrolysis was 8.5 μM (unpublished results).

The present method of 8-oxo-dGTPase separation by ultrafiltration enables kinetic studies of the enzyme and estimation of the total enzyme activity in cells. It should be noted, however, that this method is not perfect since some of the interfering 8-oxo-dGTP phosphatase may get through the membrane (Figs 4B and 5B) and, possibly, the membrane may retain some of the 8-oxo-dGTPase present in cell extract. Both will result in underestimation of 8-oxo-dGTPase activity. In effect, the ultrafiltration step is a critical factor for reproducibility of the whole analytical procedure. Individual ultrafiltration membranes present some differences in their ultrafiltration capacity. Therefore, at least three separate ultrafiltrates for each enzyme activity determination should be prepared from an individual cell extract. Also, the extracts in which 8-oxo-dGTPase activity is to be compared should not differ substantially in total protein concentration because the ultrafiltration efficiency can be affected by total amount of macromolecules applied onto the ultrafiltration membrane.

In the formerly proposed assays, BSA and DTT were used in the reaction media as 8-oxo-dGTPase stabilizers (14,16,17). In our investigations, neither of these additives increased the observed activity of the CHO enzyme under standard reaction conditions. Also, the activity of 8-oxo-dGTPase in ultrafiltrates kept for days at room temperature without these stabilizers was stable. Therefore, there was no reason to include BSA and/or DTT in the cell lysis buffer or the reaction medium. We compared two methods of cell harvesting, scraping and trypsinization, for the recovery of 8-oxo-dGTPase from CHO cells. The results indicate that trypsinization should be avoided since it lowers by 50% the recovered enzyme activity in comparison with that obtained from cells harvested by scraping. PMSF, leupeptin and pepstatin A are protease inhibitors widely used as additives in cell lysis and homogenization buffers to preserve the activity of extracted enzymes. However, since a mixture of these three protease inhibitors decreased the recovery of 8-oxo-dGTPase activity by 35%, we did not use these compounds in our standard procedure of cell processing.

To demonstrate the usefulness of our 8-oxo-dGTPase activity assay, the assay was used to investigate the influence of *in vivo* treatment with cadmium ions on the enzyme activity in CHO cells. We observed a decrease in enzyme activity with increasing concentration of Cd(II) ions ranging from 0 to 3 μM after 2, 6 and 24 h of treatment. These results accord with previously published data (18) demonstrating the inhibition of purified *E.coli* and human 8-oxo-dGTPases by Cd(II) *in vitro*. The inhibitory effect of cadmium ions on the activity of 8-oxo-dGTPase provides one possible mechanism involved in the introduction of the 8-oxo-dG lesion into DNA, observed by others in cadmium treated cells (26) and thereby could explain the mutagenic and carcinogenic potential of this metal (27). This conclusion might be supported by the presence of the characteristic AT→CG transversions among the most frequent point mutations in CHO cells exposed to cadmium (28).

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REFERENCES

1 Treffers,H.P., Spinelli,V. and Belser,N.O. (1954) *Proc. Natl. Acad. Sci. USA*, **40**, 1064–1071.
2 Yanofsky,C., Cox,E.C. and Horn,V. (1966) *Proc. Natl. Acad. Sci. USA*, **55**, 274–281.
3 Bhatnagar,S.K. and Bessman,M.J. (1988) *J. Biol. Chem.*, **263**, 8953–8957.
4 Maki,H. and Sekiguchi,M. (1992) *Nature*, **355**, 273–275.
5 Hayakawa,H., Taketomi,A., Sakumi,K., Kuwano,M. and Sekiguchi,M. (1995) *Biochemistry*, **34**, 89–95.
6 Taddei,F., Hayakawa,H., Bouton,M., Cirinesi,A., Matic,I., Sekiguchi,M. and Radman,M. (1997) *Science*, **278**, 128–130.
7 Grollman,A.P. and Moriya,M. (1993) *Trends Genet.*, **9**, 246–249.
8 Sekiguchi,M. (1996) *Genes Cells*, **1**, 139–145.
9 Pavlov,Y.I., Minnick,D.T., Izuta,S. and Kunkel,T.A. (1994) *Biochemistry*, **33**, 4695–4701.
10 Kamath-Loeb,A.S., Hizi,A., Kasai,H. and Loeb,L.A. (1997) *J. Biol. Chem.*, **272**, 5892–5898.
11 Tajiri,T., Maki,H. and Sekiguchi,M. (1995) *Mutat. Res.*, **336**, 257–267.
12 Cheng,K.C., Cahill,D.S., Kasai,H., Nishimura,S. and Loeb,L.A. (1992) *J. Biol. Chem.*, **267**, 166–172.
13 Bessman,M.J., Frick,D.N. and O'Handley,S.F. (1996) *J. Biol. Chem.*, **271**, 25059–25062.
14 Mo,J.Y., Maki,H. and Sekiguchi,M. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 11021–11025.
15 Sakumi,K., Furuichi,M., Tsuzuki,T., Kakuma,T., Kawabata,S., Maki,H. and Sekiguchi,M. (1993) *J. Biol. Chem.*, **268**, 23524–23530.
16 Cai,J.P., Kakuma,T., Tsuzuki,T. and Sekiguchi,M. (1995) *Carcinogenesis*, **16**, 2343–2350.
17 Kakuma,T., Nishida,J., Tsuzuki,T. and Sekiguchi,M. (1995) *J. Biol. Chem.*, **270**, 25942–25948.
18 Porter,D.W., Yakushiji,H., Nakabeppu,Y., Sekiguchi,M., Fivash,M.J.,Jr and Kasprzak,K.S. (1997) *Carcinogenesis*, **18**, 1785–1791.
19 Kasai,H. and Nishimura,S. (1984) *Nucleic Acids Res.*, **12**, 2137–2145.
20 Smith,P.K., Krohn,R.I., Hermanson,G.T., Mallia,A.K., Gartner,F.H., Provenzano,M.D., Fujimoto,E.K., Goeke,N.M., Olson,B.J. and Klenk,D.C. (1985) *Anal. Biochem.*, **150**, 76–85.
21 Porter,D.W., Nelson,V.C., Fivash,M.J.,Jr and Kasprzak,K.S. (1996) *Chem. Res. Toxicol.*, **9**, 1375–1381.
22 Hatahet,Z., Purmal,A.A. and Wallace,S.S. (1993) *Nucleic Acids Res.*, **21**, 1563–1568.
23 Purmal,A.A., Kow,Y.W. and Wallace,S.S. (1994) *Nucleic Acids Res.*, **22**, 3930–3935.
24 Akiyama,M., Maki,H., Sekiguchi,M. and Horiuchi,T. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3949–3952.
25 Kang,D., Nishida,J., Iyama,A., Nakabeppu,Y., Furuichi,M., Fujiwara,T., Sekiguchi,M. and Takeshige,K. (1995) *J. Biol. Chem.*, **270**, 14659–14665.
26 Mikhailova,M.V., Littlefield,N.A., Hass,B.S., Poirier,L.A. and Chou,M.W. (1997) *Cancer Lett.*, **115**, 141–148.
27 IARC (1993) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Beryllium, Cadmium, Mercury and Exposures in the Glass Manufacturing Industry*. IARC, Lyon, France, Vol. 58, pp. 119–237.
28 Yang,J.L., Chao,J.I. and Lin,J.G. (1996) *Chem. Res. Toxicol.* **9**, 1360–1367.

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Original Contribution

HIGHER ACTIVITY OF 8-OXO-2′-DEOXYGUANOSINE 5′-TRIPHOSPHATE PYROPHOSPHOHYDROLASE (8-OXO-dGTPase) COINCIDES WITH LOWER BACKGROUND LEVELS OF 8-OXO-2′-DEOXYGUANOSINE IN DNA OF FETAL COMPARED WITH MATERNAL MOUSE ORGANS

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Abstract—Mammalian homologues of *Escherichia coli* MutT, a protein having 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity, are thought to play the same role in preventing the incorporation of promutagenic 8-oxo-2′-deoxyguanosine (8-oxo-dG) into DNA. One could thus expect that higher activity of 8-oxo-dGTPase should correlate with a lower background level of 8-oxo-dG in nuclear DNA. During transplacental carcinogenesis experiments, in control healthy Swiss mice on day 18 of gestation we found consistently lower levels of 8-oxo-dG in DNA in fetal livers and lungs (1.74 ± 0.04 SE and 1.49 ± 0.08 SE 8-oxo-dG/10^5 dG, respectively; pooled organs of fetuses of 8 dams) as compared with maternal organs (3.05 ± 0.20 SE and 3.08 ± 0.17 SE 8-oxo-dG/10^5 dG, respectively; $n = 8$). The 8-oxo-dGTPase activity determination in the same organs revealed that the lower levels of 8-oxo-dG in fetal DNA did, indeed, coincide with higher 8-oxo-dGTPase activity (48.8 ± 2.6 SE and 52.5 ± 2.5 SE U/mg protein in livers and lungs, respectively); and vice versa, higher 8-oxo-dG levels in DNA of maternal organs were associated with lower levels of 8-oxo-dGTPase activity (24.3 ± 1.3 SE and 4.7 ± 0.6 SE U/mg protein, as above). Without excluding other reasons for the relatively low 8-oxo-dG background in DNA of fetal tissues (e.g., higher level of antioxidants and antioxidative enzymes; more efficient DNA repair), this inverse relationship may support or at least does not contradict the concept of a guardian role of 8-oxo-dGTPase against 8-oxo-dGTP mutagenicity in mammalian cells. © 1999 Elsevier Science Inc.

Keywords—8-oxo-dGTPase, MTH1, MutT, 8-Oxo-2′-deoxyguanosine, Oxidative DNA damage, Mutagenesis, Free radicals

INTRODUCTION

The *mutT*$^-$ mutants of *Escherichia coli* were the first mutator phenotype organisms ever reported [1]. They were characterized by at least a 1000-fold increase in frequency of a specific A → C point mutation [2]. The expression product of the wild-type *mutT* gene has been initially identified as a pyrophosphatase that hydrolyzes the canonical ribonucleoside- and 2′-deoxyribonucleoside 5′-triphosphates to the corresponding nucleoside monophosphates and inorganic pyrophosphate, with strong preference for dGTP [3, 4]. The enzyme decomposes even more effectively the products of dGTP and GTP oxidative modification, 8-oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) [5] and 8-oxoguanosine 5′-triphosphate (8-oxo-GTP) [6,7], that are likely to be generated by endogenous oxidants arising in normal cell metabolism and under oxidative stress conditions. Thus, the high spontaneous mutation rate observed in *E. coli mutT*$^-$ mutants results from the lack of functional 8-oxo-dGTP pyrophosphatase (8-oxo-dGTPase) that prevents incorporation of 8-oxo-dGTP into nascent DNA. Mutagenicity of 8-oxo-dGTP results from mispairing properties of 8-oxoguanine [8,9]. 8-Oxo-dGTP can be incorporated into DNA opposite cytosine or adenine [5,10,11]. The 8-oxo-G : A mispair, if not repaired [12], may result in AT → CG transversion [13]. *Escherichia coli* MutT protein also prevents DNA transcription errors by

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hydrolyzing 8-oxo-GTP that could be otherwise misincorporated into nascent RNA [7].

The mammalian 8-oxo-dGTPases are homologous with bacterial enzymes in terms of both kinetic properties [14,15] and amino acid sequences [16–18]. They have been thought to play the same antimutagenic role in eukaryotic cells as MutT plays in prokaryotes. If this is really the case, then it should be possible to find an inverse correlation between 8-oxo-2′-deoxyguanosine content in DNA and 8-oxo-dGTPase activity in the cells. Searching for such a correlation was the main goal of the present experiment in which the levels of 8-oxo-dG and activity of 8-oxo-dGTPase were determined in livers and lungs of healthy pregnant mice and their fetuses on day 18 of gestation. These mice comprised a part of the control group in a bigger transplacental carcinogenesis bioassay that will be published elsewhere). To achieve this goal, we adapted the 8-oxo-dGTPase assay, that we originally developed for cultured cells [15], to investigating mammalian tissues. The adaptation, reported here in full detail, confirms a more general practicality of this assay.

MATERIALS AND METHODS

Chemicals

8-Oxo-2′-deoxyguanosine 5′-triphosphate and 5′-monophosphate were synthesized as previously described [15]. 8-Oxo-2′-deoxyguanosine was synthesized by Dr. Victor Nelson from SAIC Frederick (Frederick, MD, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Six-week-old Swiss outbred (NIH/NCr) mice were bred with two females to one male. The animals were kept under pathogen-free conditions, on hardwood bedding, with 12-h fluorescent light/12-h dark cycle, at 24 ± 2°C and 50 ± 5% relative humidity. They were fed NIH-31 diet and had free access to tap water. The presence of a vaginal plug denoted the first day of gestation. The pregnant mice were randomly divided into two groups, eight animals each, and euthanized with CO_2 on the 18^{th} day of gestation. Maternal and fetal livers and lungs were immediately collected and frozen in liquid nitrogen. 8-Oxo-dGTPase activity was determined in livers and lungs of one group and 8-oxo-dG was determined in the other group of mice and their fetuses. Because of the small size of the fetuses, the lungs and livers of fetuses from each dam were pooled for analyses, respectively.

Determination of 8-oxo-dG level in nuclear DNA

The level of 8-oxo-dG in nuclear DNA was determined using the enzymatic hydrolysis procedure according to Adachi et al. [19]. Separate dilutions of genuine 8-oxo-dG and dG were used as standards. DNA hydrolysates were analyzed by a high-performance liquid chromatography (HPLC) system consisting of a Hewlett Packard (Palo Alto, CA, USA) 1050 pump, a Waters Intelligent Sample Processor model 710B, a Waters 490E Programmable Multiwavelength Detector (Waters Co., Milford, MA, USA), an ESA (Chelmsford, MA, USA) Coulochem II 5200A electrochemical detector (guard cell: 700 mV, standard analytical cell model 5010: working electrode E1 at 300 mV) and a Supelcosil LC-18-S (250 × 4.6 mm, 5 μm grain) (Supelco, Switzerland) column equipped with a 2-cm guard column. Twenty microliter aliquots of the DNA hydrolysates were chromatographed at 1 ml/min flow rate, using 100 mM sodium acetate-orthophosphoric acid, pH 5.2/methanol (92:8) as an eluent. Chromatograms were acquired and integrated by an ESA 500 Chromatography Data System.

Determination of 8-oxo-dGTPase activity

8-Oxo-dGTPase activity was determined using a modified method developed by us previously for cultured cells [15]. The tissues (100–250 mg) were homogenized on ice with 1.5 ml of cold 20 mM Tris-HCl, pH 7.4, in a motor-driven, 7-ml Potter-Elvehjem homogenizer. The homogenates were centrifuged for 15 min at 2500 × *g* (4°C). Supernatants were further ultracentrifuged for 1 h at 100,000 × *g* (4°C) in a fixed-angle Ti 50 rotor (Beckman Instruments, Fullerton, CA, USA). The resulting supernatants (0.5 ml), termed below as *extracts*, were collected for further processing. Three 100-μl portions of each extract were ultrafiltered through 30 kDa cut-off, low protein-binding regenerated cellulose membranes (Ultrafree-MC Filtration Units, Millipore, Bedford, MA, USA). This step separated the 8-oxo-dGTP pyrophosphatase activity from 8-oxo-dGTP phosphatase and 8-oxo-dGMP phosphatase activities. The extracts and ultrafiltrates were stored at −70°C for subsequent determination of the protein concentration and 8-oxo-dGTPase activity. The determination 8-oxo-dGTPase activity in the ultrafiltrates was carried out in 0.5-ml capped polypropylene tubes, as follows. A 60-μl volume of the reaction solution, containing 40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.5), and ultrafiltrate, was incubated at 37°C for 120 min. The reaction was initiated by the addition of the ultrafiltrate (5 μl) and terminated by adding 20 μl of 50 mM Na_2EDTA. Blank samples were prepared with Na_2EDTA added before the ultrafil-

trate, followed by incubation at 37°C. The reaction solutions were analyzed by HPLC for the amount of 8-oxo-dGMP formed. Our Waters HPLC system consisted of two pumps (model 510), autosampler (model 717 plus), UV-VIS Photodiode Array Detector (model 996), Supelcosil LC-18-T column (250 × 4.6 mm, 5 μm grain). It was controlled by a Millennium 2000 Chromatography Manager. Twenty microliters aliquots of the reaction mixtures were chromatographed isocratically with 100 mM NaH_2PO_4-NaOH buffer (pH 5.5)/methanol (95:5), at a flow rate of 1 ml/min. Solutions of known concentrations of 8-oxo-dGMP, ranging from 5 to 30 μM, were used for calibration. For quantification of the reaction product, chromatograms acquired at 295 nm were integrated. The activity unit (U) was defined as the amount of enzyme converting 1 pmol of 8-oxo-dGTP to 8-oxo-dGMP per min under the above reaction conditions. The mean 8-oxo-dGTPase activity in the tissue extract was calculated from determinations of three separate ultrafiltrates of a given extract. To compare different organ extracts, their activities determined in ultrafiltrates were expressed in relation to protein concentration in the corresponding extracts. Protein concentration in the extracts was determined by the biuret method [20], using crystalline bovine serum albumin as a standard.

Statistical analysis

The significance of differences between means was tested with the use of the unpaired Student's *t*-test.

RESULTS

8-Oxo-dG levels

The 8-oxo-dG level in liver DNA of 8 pregnant mice ranged from 2.45 to 3.98 per 10^5 dG molecules (mean value 3.05 ± 0.20 SE). It was significantly higher (p = .00002) than in DNA of the fetal livers, where the level ranged from 1.46 to 1.92 per 10^5 dG (mean value 1.74 ± 0.04 SE). The maternal lungs also contained significantly higher (p = .000001) levels of 8-oxo-dG in DNA (range: 2.40 to 3.82 8-oxo-dG/10^5 dG; mean value 3.08 ± 0.17 SE) in comparison to fetal lungs (range: 1.33 to 1.89 8-oxo-dG /10^5 dG; mean value 1.49 ± 0.08 SE).

8-oxo-dGTPase activity

The mean activity of 8-oxo-dGTPase in maternal livers was 24.3 ± 1.3 SE U/mg protein and ranged from 17.9 to 29.8 U/mg protein. Fetal livers had a twofold higher mean 8-oxo-dGTPase activity ($p = 10^{-9}$), 48.8 ± 2.6 SE U/mg protein, and the values for individual samples ranged from 35.4 to 70.4 U/mg protein. The mean activity of 8-oxo-dGTPase in maternal lungs was 4.7 ± 0.6 SE U/mg protein, ranging from 2.5 to 7.3 U/mg protein, whereas that in the fetal lungs was by an order of magnitude higher, with the mean of 52.5 ± 2.5 SE U/mg protein and range from 43.2 to 67.8 U/mg protein. Thus, for pregnant mice and their fetuses, and inverse relationship was observed between the cellular activity of 8-oxo-dGTPase and the level of nuclear 8-oxo-dG in both organs studied. The results are summarized in Fig. 1.

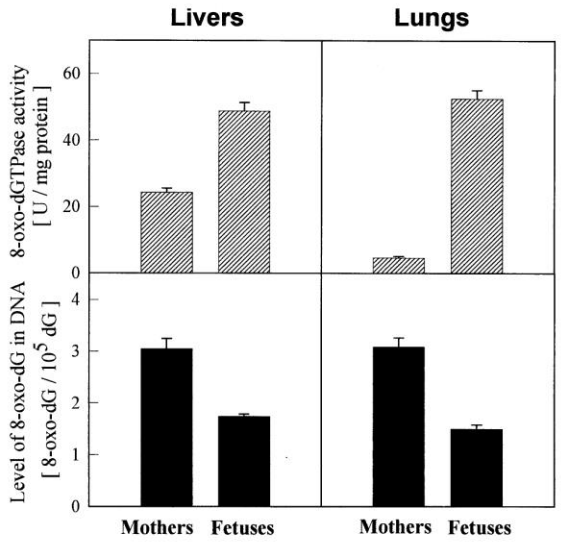

Fig. 1. Activity of 8-oxo-dGTPase and 8-oxo-dG levels in nuclear DNA of maternal and fetal mouse organs. Error bars depict SE of mean values of eight determinations.

Notes on the 8-oxo-dGTPase activity assay

Our method for selective determination of 8-oxo-dGTP pyrophosphatase activity, developed originally for analysis of cultured cells [15], was applied here to determine 8-oxo-dGTPase activity in mouse tissue extracts. The extracts of the mammalian cells contain high activities of interfering enzymes that decompose 8-oxo-dGTP to 8-oxo-dGDP and 8-oxo-dGMP to 8-oxo-dG. In addition, 8-oxo-dGDP is a very strong inhibitor of 8-oxo-dGTPase activity [15]. For these reasons it is necessary to separate the 8-oxo-dGTPase from interfering enzymes by ultrafiltration. This allows for its selective and reproducible determination. Fig. 2 presents a chromatographic separation of the substrate and product of the reaction with typical ultrafiltrate of mouse liver extract that contains 8-oxo-dGTPase activity without interfering phosphatase activities. The presence of the latter would be signified by the presence of 8-oxo-dGDP and 8-oxo-dG peaks with retention times of 6.3 min and 22.4 min,

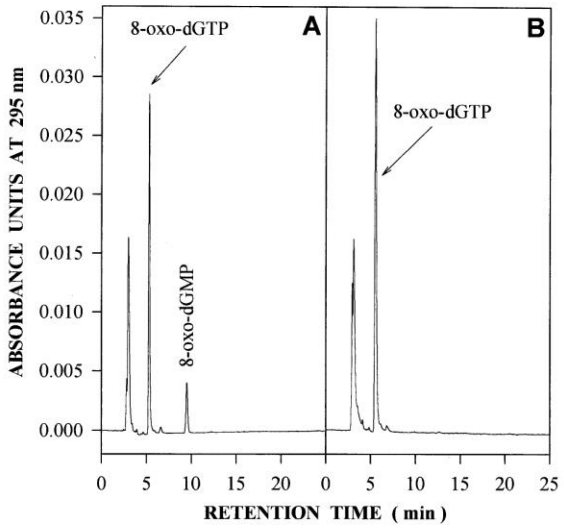

Fig. 2. High-performance liquid chromatography analysis of a typical 8-oxo-dGTPase assay reaction mixture prepared and chromatographed as specified in the text. (A) Shows 8-Oxo-dGMP as the sole product of 8-oxo-dGTP hydrolysis, thus demonstrating the effectiveness of separation of 8-oxo-dGTPase from interfering phosphatase activities by ultrafiltration (see text). (B) Blank sample prepared by adding Na_2EDTA to substrate solution before introduction of the ultrafiltrate.

respectively, as determined in control HPLC analyses (not shown).

DISCUSSION

Investigations of transplacental carcinogenesis carried out in our laboratory [21] resulted in an intriguing observation that the levels of 8-oxo-dG in DNA of fetal organs were always lower than those in the corresponding maternal organs. This consistent difference seems to reflect the age of the animals. It confirms previous findings of 8-oxo-dG accumulation in DNA of aging tissues and cells [22,23]. We found this phenomenon to be a good model for investigations of possible 8-oxo-dGTPase involvement in controlling the content of 8-oxo-dG in nuclear DNA of mammalian cells.

Although the antimutagenic function of bacterial 8-oxo-dGTPase (MutT) is well established [2,8], this function has not been demonstrated for this class of enzymes in eukaryotic cells. Transfection of the human 8-oxo-dGTPase gene into *mutT*$^-$-*E. coli* resulted in partial reversal [24], and transfection of mouse and rat genes resulted in a complete reversal [17,18] of the high A → C point mutation rate, typical for incorporational mutagenicity of 8-oxo-dGTP observed in these mutants. There is no experimental evidence that 8-oxo-dGTPase activity may be responsible for limiting the amount of 8-oxo-dGTP being incorporated into nuclear DNA of mammalian cells. There are basic differences in the localization of this enzyme in pro- and eukaryotic cells. Whereas bacterial 8-oxo-dGTPase is localized in the same compartment that the genomic DNA is replicated, the mammalian enzyme is not. The latter was found in cytosol and mitochondria, but not in the nucleus [25]. It is thus likely that mitochondrial 8-oxo-dGTPase sanitizes the free deoxynucleotide pool surrounding replicating mitochondrial DNA. It is not known, however, if and how the pool of free deoxyribonucleotides is sanitized in cell nucleus. This makes the hypothetical participation of mammalian 8-oxo-dGTPase in decreasing the content of 8-oxoguanine in nuclear DNA uncertain.

A eukaryotic cell line or organism carrying the knock-out mutation of the gene coding for 8-oxo-dGTPase would be the best model to measure the influence of 8-oxo-dGTPase on the 8-oxo-dG content in nuclear DNA and the A → C transversion rate. Unfortunately, such a cell line or organism has not been developed yet. A correlation between cellular 8-oxo-dGTPase activity and 8-oxo-dGTP levels could also provide a reliable answer to this problem. We are lacking a method for determination of 8-oxo-dGTP levels in the deoxynucleotide cellular pools. In this situation, a search for correlation between 8-oxo-dGTPase activity and the nuclear 8-oxo-dG contents seems to be a reasonable compromise in testing a possible causative association between these two factors. As found in our experiments, higher activity of 8-oxo-dGTPase in the fetal organs, as compared with maternal organs, did, indeed, coincide with lower levels of 8-oxo-dG in the fetal organs than in maternal organs. A similar inverse relationship between the level of expression of 8-oxo-dGTPase mRNA and 8-oxo-dG content in DNA was also reported recently by Kennedy et al. [26]. Hence, without excluding other reasons for the relatively low 8-oxo-dG background in DNA of fetal tissues (e.g., higher level of antioxidants and antioxidative enzymes; more efficient DNA repair), this inverse correlation may support or at least does not contradict the concept of a guardian role of 8-oxo-dGTPase against 8-oxo-dGTP in mammalian cells. Such a role seems to be especially significant for the rapidly growing fetal organs with high vulnerability to genotoxic damage. It may also help to ameliorate the DNA-damaging effects of oxidative stress observed at birth, likely to result from the sudden increase of tissue oxygen tension [27].

Further research is needed to strengthen the emerging correlation between endogenous 8-oxo-dG level in nuclear DNA and cytosolic 8-oxo-dGTPase activity, or disprove it. We believe that our novel in vivo 8-oxo-dGTPase activity assay will help in this research.

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REFERENCES

[1] Treffers, H. P.; Spinelli, V.; Belser, N. O. A factor (or mutator gene) influencing mutation rates in Escherichia coli. *Proc. Natl. Acad. Sci. USA* **40:**1064–1071; 1954.

[2] Yanofsky, C.; Cox, E. C.; Horn, V. The unusual mutagenic specificity of an *E. coli* mutator gene. *Proc. Natl. Acad. Sci. USA* **55:**274–281; 1966.

[3] Bhatnagar, S. K.; Bessman, M. J. Studies on the mutator gene, *mutT* of *Escherichia coli*. Molecular cloning of the gene, purification of the gene product, and identification of a novel nucleoside triphosphatase. *J. Biol. Chem.* **263:**8953–8957; 1988.

[4] Bhatnagar, S. K.; Bullions, L. C.; Bessman, M. J. Characterization of the *mutT* nucleoside triphosphatase of *Escherichia coli*. *J. Biol. Chem.* **266:**9050–9054; 1991.

[5] Maki, H.; Sekiguchi, M. *MutT* protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature* **355:**273–275; 1992.

[6] Hayakawa, H.; Taketomi, A.; Sakumi, K.; Kuwano, M.; Sekiguchi, M. Generation and elimination of 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate, a mutagenic substrate for DNA synthesis, in human cells. *Biochemistry* **34:**89–95; 1995.

[7] Taddei, F.; Hayakawa, H.; Bouton, M.; Cirinesi, A.; Matic, I.; Sekiguchi, M.; Radman, M. Counteraction by *MutT* protein of transcriptional errors caused by oxidative damage. *Science* **278:**128–130; 1997.

[8] Grollman, A. P.; Moriya, M. Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet.* **9:**246–249; 1993.

[9] Sekiguchi, M. MutT-related error avoidance mechanism for DNA synthesis. *Genes Cells* **1:**139–145; 1996.

[10] Pavlov, Y. I.; Minnick, D. T.; Izuta, S.; Kunkel, T. A. DNA replication fidelity with 8-oxodeoxyguanosine triphosphate. *Biochemistry* **33:**4695–4701; 1994.

[11] Kamath-Loeb, A. S.; IIizi, A.; Kasai, II.; Loeb, L. A. Incorporation of the guanosine triphosphate analogs 8-oxo-dGTP and 8-NH2-dGTP by reverse transcriptases and mammalian DNA polymerases. *J. Biol. Chem.* **272:**5892–5898; 1997.

[12] Tajiri, T.; Maki, H.; Sekiguchi, M. Functional cooperation of *MutT*, *MutM* and *MutY* proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat. Res.* **336:**257–267; 1995.

[13] Cheng, K. C.; Cahill, D. S.; Kasai, H.; Nishimura, S.; Loeb, L. A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G—T and A—C substitutions. *J. Biol. Chem.* **267:**166–72; 1992.

[14] Mo, J. Y.; Maki, H.; Sekiguchi, M. Hydrolytic elimination of a mutagenic nucleotide, 8-oxo-dGTP, by human 18-kDa protein: sanitization of nucleotide pool. *Proc. Natl. Acad. Sci. USA* **89:**11021–1025; 1992.

[15] Bialkowski, K.; Kasprzak, K. S. A novel assay of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity. *Nucleic Acids Res.* **26:**3194–201; 1998.

[16] Furuichi, M.; Yoshida, M. C.; Oda, H.; Tajiri, T.; Nakabeppu, Y.; Tsuzuki, T.; Sekiguchi, M. Genomic structure and chromosome location of the human *mutT* homologue gene MTH1 encoding 8-oxo-dGTPase for prevention of A:T to C:G transversion. *Genomics* **24:**485–490; 1994.

[17] Kakuma, T.; Nishida, J.; Tsuzuki, T.; Sekiguchi, M. Mouse MTH1 protein with 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphatase activity that prevents transversion mutation. cDNA cloning and tissue distribution. *J. Biol. Chem.* **270:**25942–25948; 1995.

[18] Cai, J. P.; Kakuma, T.; Tsuzuki, T.; Sekiguchi, M. cDNA and genomic sequences for rat 8-oxo-dGTPase that prevents occurrence of spontaneous mutations due to oxidation of guanine nucleotides. *Carcinogenesis* **16:**2343–2350; 1995.

[19] Adachi, S.; Zeisig, M.; Moller, L. Improvements in the analytical method for 8-hydroxydeoxyguanosine in nuclear DNA. *Carcinogenesis* **16:**253–258; 1995.

[20] Gornall, A. G.; Bardawill, C. J.; David, M. M. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:**751–766; 1949.

[21] Sipowicz, M. A.; Amin, S.; Desai, D.; Kasprzak, K. S.; Anderson, L. M. Oxidative DNA damage in tissues of pregnant mice and fetuses caused by the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pirydyl)-1-butanone (NNK). *Cancer Lett.* **117:**87–91; 1997.

[22] Fraga, C. G.; Shigenaga, M. K.; Park, J. W.; Degan, P.; Ames, B. N. Oxidative damage to DNA during aging: 8-hydroxy-2′-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. USA* **87:**4533–4537; 1990.

[23] Homma, Y.; Tsunoda, M.; Kasai, H. Evidence for the accumulation of oxidative stress during cellular ageing of human diploid fibroblasts. *Biochem. Biophys. Res. Commun.* **203:**1063–1068; 1994.

[24] Sakumi, K.; Furuichi, M.; Tsuzuki, T.; Kakuma, T.; Kawabata, S.; Maki, H.; Sekiguchi, M. Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. *J. Biol. Chem.* **268:**23524–23530; 1993.

[25] Kang, D.; Nishida, J.; Iyama, A.; Nakabeppu, Y.; Furuichi, M.; Fujiwara, T.; Sekiguchi, M.; Takeshige, K. Intracellular localization of 8-oxo-dGTPase in human cells, with special reference to the role of the enzyme in mitochondria. *J. Biol. Chem.* **270:**14659–14665; 1995.

[26] Kennedy, C. H.; Cueto, R.; Belinsky, S. A.; Lechner, J. F.; Pryor, W. A. Overexpression of hMTH1 mRNA: a molecular marker of oxidative stress in lung cancer cells. *FEBS Lett.* **429:**17–20; 1998.

[27] Randerath, K.; Zhou, G.-D.; Monk, S. A.; Randerath, E. Enhanced levels in neonatal rat liver of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-hydroxydeoxyguanosine), a major mutagenic oxidative DNA lesion. *Carcinogenesis,* **18:**1419–1421; 1997.

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Cadmium(II), unlike nickel(II), inhibits 8-oxo-dGTPase activity and increases 8-oxo-dG level in DNA of the rat testis, a target organ for cadmium(II) carcinogenesis

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8-Oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) is an enzyme which prevents incorporation into DNA of promutagenic 8-oxo-2′-deoxyguanosine (8-oxo-dG) from a deoxynucleotide pool damaged by endogenous oxidants. Its inhibition may thus be carcinogenic. We previously found that Cd(II) inhibited 8-oxo-dGTPase in both cell free systems and cultured cells. To verify this finding in a relevant animal model, we investigated the effects of Cd(II) on cellular 8-oxo-dGTPase activity and nuclear DNA 8-oxo-dG levels in the rat testis, a target organ for Cd(II) carcinogenesis. Ni(II), which does not induce testicular tumors in rats and is a weaker *in vitro* inhibitor of 8-oxo-dGTPase than Cd(II), was investigated as a comparison. Male F344/NCr rats were given a single s.c. dose of 20 μmol Cd(II) acetate, 90 μmol Ni(II) acetate or 180 μmol sodium acetate (controls) per kg body wt and killed 2, 8, 24 or 48 h later (three rats/time point). Cd(II) caused a gradual decrease in testicular 8-oxo-dGTPase activity with time. It became significant only after 8 h post-injection ($P < 0.05$) and resulted in a final 50% loss of the enzyme activity at 48 h ($P < 0.01$). Although the results for Ni(II) at 8 h and later were apparently lower than the controls, the decrease did not reach statistical significance. Treatment of rats with Cd(II) led to an early and progressive increase (from 130% at 2 h to 200% at 48 h versus the controls) of the 8-oxo-dG level in testicular DNA ($P < 0.05$ or better). Ni(II) acetate also tended to raise the testicular 8-oxo-dG level, but the increase was transient, with an apparent maximum at 8 h, and did not approach statistical significance ($P < 0.2$). Thus, Cd(II), unlike Ni(II), is able to inhibit 8-oxo-dGTPase activity and to raise 8-oxo-dG levels in rat testicular DNA. However, the time course of both effects indicates that 8-oxo-dGTPase inhibition is most likely not the sole cause of the increase in 8-oxo-dG.

Introduction

Cadmium is toxic and carcinogenic to humans and animals (1,2). One possible pathogenic pathway of its action includes mediation of promutagenic DNA damage, as demonstrated by various assays, including DNA strand break and 8-oxo-2′-deoxyguanosine (8-oxo-dG) determination (3–7). Since Cd(II) is not redox active under physiological conditions, this type of damage can be inflicted by Cd(II) only indirectly, e.g. through inflammatory cells and/or inhibition of cellular antioxidant and DNA repair systems (5,6,8–10).

Besides being generated directly in the DNA, an 8-oxo-dG lesion may be incorporated into DNA from 2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) produced in the deoxynucleotide pool through oxidation of dGTP by endogenous metabolic oxidants (11,12). Such a potentially mutagenic incorporation is countered in cells by a specific 8-oxo-dGTP pyrophosphohydrolase (8-oxo-dGTPase) (11,12). Hence, inhibition of this protective enzyme should enhance mutagenesis and cancer. Our previous *in vitro* experiments revealed that 8-oxo-dGTPase was sensitive to inhibition by several carcinogenic metals, including Cd(II) (13). Cd(II) was also inhibitory toward 8-oxo-dGTPase activity in cultured Chinese hamster ovary (CHO) cells (14). The goal of the present study was to test this Cd(II) effect *in vivo*, in an appropriate animal model. To achieve this goal, 8-oxo-dGTPase activity and 8-oxo-dG levels were determined in rat testes, which is the main target organ for Cd(II)-induced carcinogenesis (1,2,15), at time intervals up to 48 h following a single s.c. injection of Cd(II) acetate. Similar effects of nickel(II), which was shown to slightly inhibit 8-oxo-dGTPase activity *in vitro* (13) and exert limited toxic effects (carcinogenicity was not sought) in the rat testis (16), were investigated for comparison.

Abbreviations: CHO, Chinese hamster ovary; 8-oxo-dG, 8-oxo-2′-deoxyguanosine (8-hydroxy-2′-deoxyguanosine); 8-oxo-dGMP, 8-oxo-2′-deoxyguanosine 5′-monophosphate; 8-oxo-dGDP, 8-oxo-2′-deoxyguanosine 5′-diphosphate; 8-oxo-dGTP, 2′-deoxyguanosine 5′-triphosphate; 8-oxo-dGTPase, 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase.

Materials and methods

Chemicals

Ni(II) acetate tetrahydrate, Cd(II) acetate dihydrate and anhydrous sodium acetate were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). 8-Oxo-dGTP and 8-oxo-2′-deoxyguanosine 5′-monophosphate (8-oxo-dGMP) were synthesized as described in our previous paper (14). 8-Oxo-dG was synthesized by Dr Victor Nelson of SAIC Frederick (Frederick, MD). All other chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Animals and treatment

Male F344/NCr rats, 6–7 weeks old, weighing 120–200 g, were randomly divided into three groups of 12 animals. The animals were housed on hardwood bedding in a room with a 12 h fluorescent light/12 h dark cycle, at 24 ± 2°C and 50 ± 5% relative humidity. They were fed NIH-31 Open Formula 6% Modified Diet (Zeigler Brothers, Gardners, PA) and had free access to drinking water. The rats were given a single dose of one of the following salts: Cd(II) acetate, 20 μmol/kg body wt; Ni(II) acetate, 90 μmol/kg body wt; sodium acetate, 180 μmol/kg body wt (control group). These doses of metal acetates, except that of sodium acetate, are known to initiate carcinogenesis in rats (cadmium in testes, nickel in kidneys) (reviewed in refs 1,2,10). The injections were s.c. at the nape of the neck, in 2 ml of water/kg body wt. Three rats of each group were killed with CO_2 2, 8, 24 and 48 h post-injection. Their testes were collected immediately and frozen in liquid nitrogen.

Determination of 8-oxo-dGTPase activity

8-Oxo-dGTPase activity was determined in one testis of each rat according to the following protocol based on the general method developed previously by us for cultured cells and mouse tissues (14,17). The whole testis was homogenized for 10 s in 3.5 ml of ice-cold 20 mM Tris–HCl, pH 7.4, with a

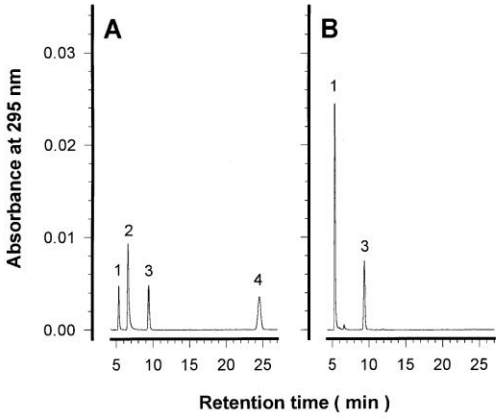

Fig. 1. HPLC separation of the substrate (1) and products (2–4) of 8-oxo-dGTP hydrolysis catalyzed by (**A**) a total protein extract of normal rat testis and (**B**) a ⩽30 kDa ultrafiltrate of that extract. Mixtures (60 µl) containing 40 µM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl (pH 8.5) and 5 µl of testicular protein extract or its ultrafiltrate were incubated at 37°C for 60 min. The hydrolysis was stopped by addition of 20 µl 50 mM Na_2EDTA. Aliquots of 20 µl of the mixtures were chromatographed as described in Materials and methods. 1, 8-oxo-dGTP; 2, 8-oxo-dGDP; 3, 8-oxo-dGMP; 4, 8-oxo-dG.

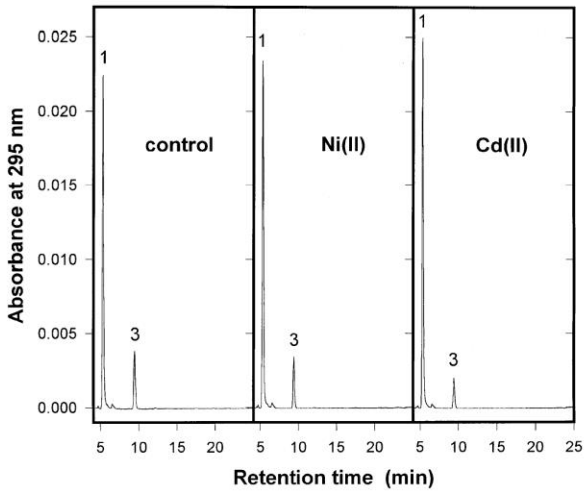

Fig. 2. The treatment of rats with metal salts does not change the specificity of 8-oxo-dGTP decomposition by ultrafiltrates of testicular protein extracts. Exemplary HPLC chromatograms demonstrating the presence of pure 8-oxo-dGTP pyrophosphatase activity in the ultrafiltrates of rats 24 h after a single s.c injection of 180 µmol/kg body wt Na(I) acetate (control), 90 µmol/kg body wt Ni(II) acetate or 20 µmol/kg body wt Cd(II) acetate. Numbers indicate the same compounds as in Figure 1.

Brinkmann Polytron homogenizer PT 10/35 equipped with a 1 cm (diameter) foam reducing generator with saw teeth. The homogenate was centrifuged for 15 min at 2800 *g* (4°C). The resulting supernatant was ultracentrifuged for 1 h at 100 000 *g* (4°C). Three 150 µl portions of each supernatant, termed below 'extract', were ultrafiltered through 30 kDa cut-off, low protein-binding regenerated cellulose membrane (Ultrafree-MC Filtration Units; Millipore, Bedford, MA). This step separates the assayed 8-oxo-dGTP pyrophosphatase activity from the interfering 8-oxo-dGTP phosphatase and 8-oxo-dGMP phosphatase activities (Figure 1). The extracts and ultrafiltrates were stored at –70°C for subsequent determinations of protein concentration and 8-oxo-dGTPase activity. The former was done by the biuret method (18), while the latter was accomplished as follows. A 60 µl volume of the reaction solution containing 40 µM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl (pH 8.5) and ultrafiltrate was incubated at 37°C for 30 min. The reaction was initiated by addition of the ultrafiltrate (5 µl) and terminated by addition of 20 µl 50 mM Na_2EDTA. Blank samples were prepared with Na_2EDTA introduced before the ultrafiltrate, followed by incubation at 37°C.

The reaction solutions were analyzed by HPLC for the amount of 8-oxo-dGMP formed. Our Waters HPLC system consisted of two pumps (model 510), an autosampler (model 717 plus), a UV-VIS photodiode array detector (model 996), a Supelcosil LC-18-T column (250×4.6 mm, 5 µm grain; Supelco, Bellefonte, PA) and was controlled by a Millennium^{32} Chromatography Manager. Aliquots (20 µl) of the reaction mixtures were chromatographed isocratically with 100 mM NaH_2PO_4–NaOH buffer, pH 5.5, methanol (95:5), at a flow rate of 1 ml/min. Solutions of known concentrations of 8-oxo-dGMP, ranging from 1.25 to 15 µM, were used for calibration. For quantification of the reaction product, chromatograms acquired at 295 nm were integrated.

The enzymatic activity unit (U) was defined as the amount of enzyme converting 1 pmol 8-oxo-dGTP to 8-oxo-dGMP per min under the above reaction conditions. The mean 8-oxo-dGTPase activity in the tissue extract was calculated from determinations in three separate ultrafiltrates of the same extract and finally expressed in relation to total protein concentration in the extract.

Determination of 8-oxo-dG level in nuclear DNA

The level of 8-oxo-dG in nuclear DNA was determined in the second testis of each rat, using the enzymatic hydrolysis procedure according to Adachi *et al.* (19). Separate dilutions of genuine 8-oxo-dG and deoxyguanosine were used as standards. DNA hydrolysates were analyzed by a HPLC system consisting of a Hewlett Packard 1050 pump, a Waters Intelligent Sample Processor model 710B, a Waters 490E Programmable Multiwavelength Detector, an ESA Coulochem II 5200A electrochemical detector (guard cell, 700 mV, standard analytical cell model 5010; working electrode E1 at 300 mV) and a Supelcosil LC-18-S (250×4.6 mm, 5 µm grain) column equipped with a 2 cm guard column. Aliquots (20 µl) of the DNA hydrolysates were chromatographed at 1 ml/min flow rate, with 100 mM sodium acetate–orthophosphoric acid, pH 5.2, methanol (92:8) as eluent. Chromatograms were acquired and integrated by an ESA 500 Chromatography Data System.

Statistical analysis

The significance of differences between means was tested using Student's *t*-test.

Results

8-Oxo-dGTPase activity

As illustrated in Figure 1, 8-oxo-dGTPase activity in the rat testis could be measured using our assay developed originally for cultured cells (14). Unlike in the whole testis extract (Figure 1A), no degradation of 8-oxo-dGTP to products other than 8-oxo-dGMP was observed in the ultrafiltrate (Figure 1B). Thus, like the mouse liver and lungs (17), the rat testis does not contain proteins with phosphatase activity which could pass the 30 kDa cut-off ultrafilters. It is also important to notice that the metal treatments did not change the specificity of the enzymatic activity in the ultrafiltrates, as signified by the presence of 8-oxo-dGMP and absence of other possible products, e.g. 8-oxo-2′-deoxyguanosine 5′-diphosphate (8-oxo-dGDP) and/or 8-oxo-dG, of 8-oxo-dGTP hydrolysis (Figure 2).

Treatment of rats with Cd(II) acetate resulted in a significant gradual decrease in testicular 8-oxo-dGTPase activity with time after injection (Figure 3). This decrease first became apparent as early as 8 h post-injection ($P < 0.05$) and finally resulted in a 50% loss of enzyme activity at 48 h ($P < 0.01$). Although the results for Ni(II) at 8 h and later after the injection were lower than the controls, the differences did not reach statistical significance. Likewise, the slight variations in 8-oxo-dGTPase activity observed with time in the control testes were not significant (Figure 3).

8-oxo-dG levels in nuclear DNA

As shown in Figure 4, treatment of rats with Cd(II) acetate led to a marked increase in the 8-oxo-dG level in testicular DNA that remained statistically significant over the entire period of the experiment, with $P < 0.05$ or better. It reached

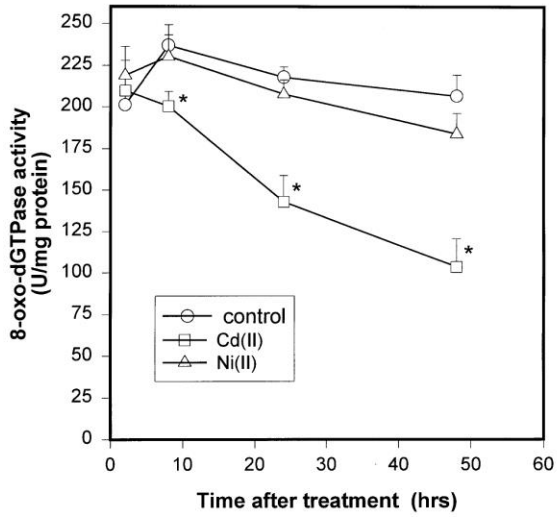

Fig. 3. 8-Oxo-dGTPase activity in testes of rats 2, 8, 24 and 48 h after a single s.c. injection of 180 μmol/kg body wt Na(I) acetate (control), 90 μmol/kg body wt Ni(II) acetate or 20 μmol/kg body wt Cd(II) acetate. Error bars denote SE. *$P < 0.05$ or better versus the corresponding control value.

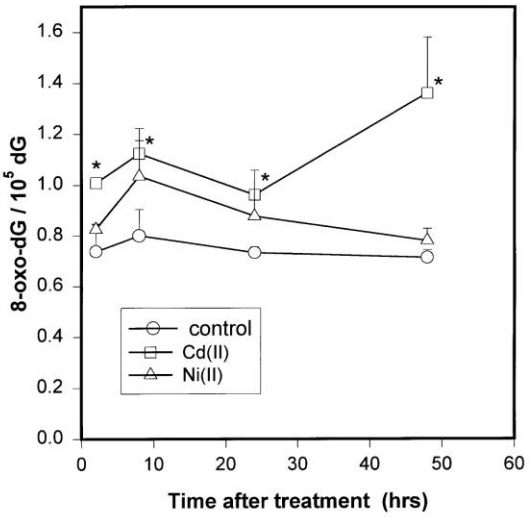

Fig. 4. The levels of 8-oxo-dG in DNA of the rat testes 2, 8, 24 and 48 h after a single s.c. injection of 180 μmol/kg body wt Na(I) acetate (control), 90 μmol/kg body wt Ni(II) acetate or 20 μmol/kg body wt Cd(II) acetate. Error bars denote SE. *$P < 0.05$ or better versus the corresponding control value.

nearly 200% of the control value at 48 h. Ni(II) acetate also tended to increase the testicular 8-oxo-dG level, but that increase was transient with an apparent maximum at 8 h and its difference from the control did not approach statistical significance ($P < 0.2$ versus the corresponding control for 8 h).

Discussion

8-Oxo-dGTPase is an enzyme hydrolyzing 8-oxo-dGTP, one of the mutagenic products of active oxygen attack on the nucleotide pool (11,12), to 8-oxo-dGMP and pyrophosphate. Unlike 8-oxo-dGDP, which may be derived from 8-oxo-dGTP by phosphatase hydrolysis, 8-oxo-dGMP cannot be rephosphorylated back to the triphosphate (12). Therefore, 8-oxo-dGTPase is regarded as an antimutagenic enzyme 'sanitizing' the cellular nucleotide pool (20). Its inhibition is expected to increase incorporational mutagenicity of 8-oxo-dGTP, signified by AT→CG transversions (11,12,21).

The major problem in determination of 8-oxo-dGTPase activity in tissue extracts has been interference from other cellular phosphatases which degrade the substrate triphosphate through intermediate phosphates, 8-oxo-dGDP and 8-oxo-dGMP, to 8-oxo-dG (Figure 1A). Thus, the substrate and product of the 8-oxo-dGTPase-catalyzed reaction are hydrolyzed by other enzymes. Furthermore, 8-oxo-dGDP is a very strong inhibitor of 8-oxo-dGTPase (14). Fortunately, the 18 kDa rat 8-oxo-dGTPase (22) can be easily separated from the larger interfering enzymes by simple ultrafiltration through 30 kDa cut-off, low protein-binding, regenerated cellulose membranes (Figure 1B). In our previous experiment, this procedure proved successful for intact animal tissues (17). The present study confirms the effectiveness of ultrafiltration and also the entire novel assay in analysis of a tissue damaged by toxic metals (Figure 2).

The 8-oxo-dGTP signature AT→CG transversion, mentioned above, is frequent among point mutations identified in the hypoxanthine (guanine) phosphoribosyl-transferase gene of cadmium-exposed CHO cells (7). Cd(II) was also found to inhibit bacterial and human 8-oxo-dGTPases *in vitro* (13) and the hamster enzyme in cultured CHO cells (14), as well as to increase 8-oxo-dG levels in DNA of cultured cells (4) and in testes, but not lung, of glutathione-depleted rats (3). In the latter paper, oxidative DNA damage has been related to the inhibition by Cd(II) of DNA repair when glutathione biosynthesis is also inhibited. Since Cd(II), being non-redox active under physiological conditions, cannot oxidize DNA bases directly, these observations, as well as our present results, are consistent with, or at least do not contradict, the general notion that the observed oxidative DNA damage (by metabolic oxidants) is assisted by this metal through inhibition of cellular antioxidant and DNA protection/repair systems, of which 8-oxo-dGTPase is a member (23). It is, however, very likely that Cd(II) can also enhance oxidative DNA damage by boosting endogenous oxidation, e.g. by triggering inflammation (10,24,25). Our results, which differ from those reported by Hirano *et al.* (3) in the sense that we observed elevation of testicular 8-oxo-dG by Cd(II) in rats even without prior depletion of glutathione, may support that notion. The difference might be due to a different strain of rat and/or higher Cd(II) dose used in the present experiment. Species-, strain- and dose-related variations in response to Cd(II), including the severity of the inflammatory/necrotizing reactions in testes, have been reported (26 and references therein).

The decrease in 8-oxo-dGTPase activity with time after injection was concurrent in our experiment with increasing level of 8-oxo-dG. It would be tempting, therefore, to speculate about a causative relationship between these two effects. However, the dynamics of these effects appear to be dissimilar enough to cast doubt upon the existence of such a relationship: 2 h after Cd(II) injection, the 8-oxo-dGTPase activity remained unchanged while the 8-oxo-dG level was already significantly elevated above the control. Thus, at least at this time point, the increase in oxidative DNA damage could not be a result of inhibition of the enzyme. Nonetheless, a contribution of the

latter to the elevation of 8-oxo-dG level at later times, especially past 24 h, cannot be excluded.

Ni(II) tended to suppress 8-oxo-dGTPase activity versus that of the control at ≥8 h after treatment. The very limited extent of this effect seems to be consistent with the much lower inhibitory potential of Ni(II) toward 8-oxo-dGTPase observed *in vitro* (13), as well as a generally weaker toxicity of Ni(II) in testes (16,27) as compared with that for Cd(II) (1,2,16,26). The testicular 8-oxo-dG levels in Ni(II)-treated rats, consistently higher than in the controls [with an apparent maximum 8 h after Ni(II), with $P < 0.2$ versus the control for this time point but $P < 0.05$ versus the mean of all control values], might indicate some oxidative damage to the DNA. However, under the present experimental conditions, this effect was too weak to be significant. It should be verified on larger groups of rats. A rationale for a closer look at possible generation of oxidative DNA damage by Ni(II) in the testis comes from the strong oxidation-mediating properties of the Ni(II) complex with protamine P2, a DNA-binding protein abundantly present in the testes and sperm heads (28,29).

In conclusion, Cd(II) acetate treatment results in marked inhibition of cellular 8-oxo-dGTPase activity and increases in the nuclear 8-oxo-dG level in the rat testis, the target organ of Cd(II)-induced carcinogenesis. The increase in oxidative DNA damage precedes the decrease in 8-oxo-dGTPase activity, indicating that the first appearance of 8-oxo-dG in DNA is not due to its incorporation from the nucleotide pool. However, the results do not exclude the possibility that a further decrease in 8-oxo-dGTPase activity with time after Cd(II) treatment may contribute to such incorporation. The very limited effects of Ni(II) observed in the present study, although suggestive of some damaging trends toward testicular 8-oxo-dGTPase and DNA, did not reach statistical significance and await confirmation on larger animal groups.

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References

1. IARC (1993) *Beryllium, Cadmium, Mercury and Exposures in the Glass Manufacturing Industry*, Monographs on the Evaluation of Carcinogenic Risks to Humans No. 58. IARC, Lyon, pp. 119–237.
2. Magos,L. (1991) Epidemiological and experimental aspects of metal carcinogenesis: physicochemical properties, kinetics, and the active species. *Environ. Health Perspect.*, **95**, 157–189.
3. Hirano,T., Yamaguchi,Y. and Kasai,H. (1997) Inhibition of 8-hydroxyguanine repair in testes after administration of cadmium chloride to GSH-depleted rats. *Toxicol. Appl. Pharmacol.*, **147**, 9–14.
4. Mikhailova,M.V, Littlefield,N.A., Hass,B.S., Poirier,L.A. and Chou,M.W. (1997) Cadmium-induced 8-hydroxydeoxyguanosine formation, DNA strand breaks, and antioxidant enzyme activities in lymphoblastoid cells. *Cancer Lett.*, **115**, 141–148.
5. Calevro,F., Beyersmann,D. and Hartwig,A. (1998) Effect of cadmium(II) on the extent of oxidative DNA damage in primary brain cell cultures from *Pleurodeles* larvae. *Toxicol. Lett.*, **94**, 217–225.
6. Hartwig,A. (1995) Current aspects in metal genotoxicity. *BioMetals*, **8**, 3–11.
7. Yang,J.-L., Chao,J.-I. and Lin,J.-G. (1996) Reactive oxygen species may participate in the mutagenicity and mutational spectrum of cadmium in Chinese hamster ovary-K-1 cells. *Chem. Res. Toxicol.*, **9**, 1360–1367.
8. Yang,C.-F., Shen,H.-M., Shen,Y., Zhuang,Z.-X. and Ong,C.-N. (1997) Cadmium-induced oxidative cellular damage in human fetal lung fibroblasts (MRC-5 cells). *Environ. Health Perspect.*, **105**, 712–716.
9. Hartwig,A. (1994) Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: a review. *Environ. Health Perspect.*, **102** (suppl. 3), 45–50.
10. Kasprzak,K.S. (1995) Possible role of oxidative damage in metal-induced carcinogenesis. *Cancer Invest.*, **13**, 411–430.
11. Maki,H. and Sekiguchi,M. (1992) MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature*, **355**, 273–275.
12. Hayakawa,H., Taketomi,A., Sakumi,K., Kuwano,M. and Sekiguchi,M. (1995) Generation and elimination of 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate, a mutagenic substrate for DNA synthesis in human cells. *Biochemistry*, **34**, 89–95.
13. Porter,D.W., Yakushiji,H., Nakabeppu,Y., Sekiguchi,M., Fivash,M.J.Jr and Kasprzak,K.S. (1997) Sensitivity of *Escherichia coli* (MutT) and human (MTH1) 8-oxo-dGTPases to *in vitro* inhibition by the carcinogenic metals, nickel(II), copper(II), cobalt(II) and cadmium(II). *Carcinogenesis*, **18**, 1785–1791.
14. Bialkowski,K. and Kasprzak,K.S. (1998) A novel assay of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity. *Nucleic Acids Res.*, **26**, 3194–3201.
15. Waalkes,M.P., Coogan,T.P. and Barter,R.A. (1992) Toxicological principles of metal carcinogenesis with special emphasis on cadmium. *Crit. Rev. Toxicol.*, **22**, 175–201.
16. Hoey,M.J. (1966) The effects of metallic salts on the histology and functioning of the rat testis. *J. Reprod. Fertil.*, **12**, 461–471.
17. Bialkowski,K., Bialkowska,A., Anderson,L.M. and Kasprzak,K.S. (1999) Higher activity of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) coincides with lower background levels of 8-oxo-2′-deoxyguanosine in DNA of fetal compared with maternal mouse organs. *Free Radic. Biol. Med.*, in press.
18. Gornall,A.G., Bardawill,C.J. and David,M.M. (1949) Determination of serum proteins by means of the biuret method. *J. Biol. Chem.*, **177**, 751–766.
19. Adachi,S., Zeisig,M. and Moller,L. (1995) Improvements in the analytical method for 8-hydroxydeoxyguanosine in nuclear DNA. *Carcinogenesis*, **16**, 253–258.
20. Mo,J.-Y., Maki,H. and Sekiguchi,M. (1992) Hydrolytic elimination of a mutagenic nucleotide, 8-oxo-dGTP, by human 18 kilodalton protein: sanitization of nucleotide pool. *Proc. Natl Acad. Sci. USA*, **89**, 11021–11025.
21. Cheng,K.C., Cahill,D.S., Kasai,H., Nishimura,S. and Loeb,L.A. (1992) 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. *J. Biol. Chem.*, **267**, 166–172.
22. Cai,J.-P., Kakuma,T., Tsuzuki,T. and Sekiguchi,M. (1995) cDNA and genomic sequences for rat 8-oxo-dGTPase that prevents occurrence of spontaneous mutation due to oxidation of guanine nucleotides. *Carcinogenesis*, **16**, 2343–2350.
23. Grollman,A.P. and Moriya,M. (1993) Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet.*, **9**, 246–249.
24. Kasprzak,K.S. (1996) Oxidative DNA damage in metal-induced carcinogenesis. In Chang,L.W., Magos,L. and Suzuki,T. (eds) *Toxicology of Metals*. CRC Press, Boca Raton, FL, pp. 299–320.
25. Beyersmann,D. and Hechtenberg,S. (1997) Cadmium, gene regulation, and cellular signalling in mammalian cells. *Toxicol. Appl. Pharmacol.*, **144**, 247–261.
26. Waalkes,M.P. and Rehm,S. (1994) Chronic toxic and carcinogenic effects of cadmium chloride in male DBA/2NCr and NFS/NCr mice: strain-dependent association with tumors of the hematopoietic system, injection site, liver, and lung. *Fundam. Appl. Toxicol.*, **23**, 21–31.
27. Xie,J., Funakoshi,T., Shimada,H. and Kojima,S. (1995) Effects of chelating agents on testicular toxicity in mice caused by acute exposure to nickel. *Toxicology*, **103**, 147–155.
28. Bal,W., Lukszo,J. and Kasprzak,K.S. (1997) Mediation of oxidative DNA damage by nickel(II) and copper(II) complexes with the N-terminal sequence of human protamine HP2. *Chem. Res. Toxicol.*, **10**, 915–921.
29. Liang,R., Senturker,S., Shi,X., Bal,W., Dizdaroglu,M. and Kasprzak,K.S. (1999) Effects of Ni(II) and Cu(II) on DNA interaction with the N-terminal sequence of human protamine P2: enhancement of binding and mediation of oxidative DNA strand scission and base damage. *Carcinogenesis*, **20**, 893–898.

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Inhibition of antimutagenic enzymes, 8-oxo-dGTPases, by carcinogenic metals. Recent developments

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Abstract

Nickel, cadmium, cobalt, and copper are carcinogenic to humans and/or animals, but the underlying mechanisms are poorly understood. Our studies have been focused on one such mechanism involving mediation by the metals of promutagenic oxidative damage to DNA bases. The damage may be inflicted directly in DNA or in the deoxynucleotide pool, from which the damaged bases are incorporated into DNA. Such incorporation is prevented in cells by 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphatases (8-oxo-dGTPases). Thus, inhibition of these enzymes should enhance carcinogenesis. We have studied effects of Cd(II), Cu(II), Co(II), and Ni(II) on the activity of isolated bacterial and human 8-oxo-dGTPases. Cd(II) and Cu(II) were strongly inhibitory, while Ni(II) and Co(II) were much less suppressive. After developing an assay for 8-oxo-dGTPase activity, we confirmed the inhibition by Cd(II) in cultured cells and in the rat testis, the target organ for cadmium carcinogenesis. 8-Oxo-dGTPase inhibition was accompanied by an increase in the 8-oxo-dG level in testicular DNA.

Keywords: Cadmium; Cobalt; Copper; Nickel; 8-Oxo-dGTPase

1. Introduction

The emergence of 8-oxoguanine, the major product of reactive oxygen species (ROS) attack, in genomic DNA has become the focus of intensive studies because of its promutagenic properties [1–4]. Elevated levels of this damaged base have been observed in organs of rats exposed to Ni(II) and Co(II) [5–8], or accumulating excessive amounts of Cu(II) [9]. Certain biocomplexes of these metals are known to activate oxygen species under physiological conditions and enhance oxidative damage to DNA and other molecules [10–12]. In addition, oxidative damage, inflicted by endogenous ROS, may be augmented through inhibition by metals of antioxidant and DNA repair mechanisms [11–17]. These 'inhibitory pathways' might explain, at least in part, why Cd(II), which is a potent carcinogen [18] but not a redox active metal under physiological conditions, mediates oxidative damage [19], including emergence of 8-oxoguanine in genomic DNA [20,21].

It is presumed that 8-oxoguanine can arise in DNA due to a direct attack of ROS on chromatin, or through incorporation from an oxidatively damaged nucleotide pool, namely, from 8-oxo-2′-deoxyguanosine-5′-triphosphate (8-oxo-dGTP), during DNA synthesis or repair [2–4,22,23]. A class of enzymes, designated MutT in *Escherichia coli* [24] and MTH1 in humans [25], was found to prevent such incorporation and thus act as an antimutagenic guard [1,22,23,26]. Mutagenicity of 8-oxo-dGTP is a consequence of mispairing properties of 8-oxoguanine (reviewed in Refs. [1,26]) which can be incorporated into DNA opposite cytosine or adenine [4,22,27]. The 8-oxo-G:A mispair may result in AT→CG transversion mutations [3,28,29].

The *mutT*$^-$ mutants of *Escherichia coli* have been the first mutator phenotype organisms ever discovered [24] that were characterized by a 1000- to 10 000-fold increase in frequency of a specific A→C point mutation [30]. The expression product of the *mutT* gene, an enzyme, has been identified as a pyrophosphatase that preferentially hydrolyzes 8-oxo-dGTP, the product of oxidative modification of dGTP, to 8-oxo-dGMP [22,31]. 8-Oxo-dGTP is likely to be generated by endogenous ROS arising in normal cell metabolism and, to a greater extent, under oxidative stress. Thus, the high spontaneous mutation rate in *mutT*$^-$ *Escherichia coli* is thought to result from the lack of functional 8-oxo-dGTP

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pyrophosphatase (8-oxo-dGTPase) (reviewed in Ref. [1]). Mammalian homologs of MutT, such as MTH1, share a homology with the bacterial enzyme in terms of kinetic properties [23,32] and, to a lesser degree, amino acid sequences [25,33,34]. They may play the same antimutagenic role in eukaryotes as MutT plays in bacteria [35].

MutT and MTH1 are activated by Mg(II) that can make them sensitive to inhibition by other metals. If so, we hypothesized that inhibition of mammalian 8-oxo-dGTPases might, at least in part, account for the increased 8-oxoguanine levels found in DNA exposed in vivo to Ni(II), Co(II), Cd(II), or Cu(II) [5–9,20,21]. In order to test this hypothesis, we first determined effects of the above metals on the enzymatic activity of isolated bacterial MutT and human MTH1 proteins, and found that Cd(II) and Cu(II) were more inhibitory than Ni(II) and Co(II) [36]. In the next step, the experiments were focused on corroborating the effects of Cd(II) in cultured cells [32] and those of Cd(II) and Ni(II) in the rat testes, the target organ for Cd(II)-, but not Ni(II)-induced carcinogenesis [37]. The present paper summarizes and discusses the results of the above investigations [32,36,37].

2. Materials and methods

The methodology of our in vitro investigations, based on HPLC, has been described elsewhere [36]. The effects of Ni(II), Co(II), Cu(II), and Cd(II), introduced as chlorides, on the enzymatic activity of MTH1 and MutT were tested in the presence of 8 mM Mg(II), 100 mM ammonium acetate, pH 7.4, and variable concentrations of the other metals. The metal concentrations that suppressed enzymatic activity of either enzyme to 50% of its original value (IC_{50}) were determined using the fractional inhibition method.

The K1-BH4 Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum. At approximately 50% confluence, they were treated for 2, 6, and 24 h with the same medium containing 0, 0.3, 1 or 3 μM Cd(II) [32].

Male Fischer F344/NCr rats, 6–7 weeks old, were given a single subcutaneous dose of 20 μmol Cd(II) acetate, 90 μmol Ni(II) acetate, or 180 μmol sodium acetate (controls) per kg body weight. The rats were killed 2, 8, 24, and 48 h after injection. Their testes were collected and stored in liquid nitrogen [37].

The assay of the enzymatic activity of 8-oxo-dGTPase and the syntheses of reference compounds have been explained elsewhere [32]. Briefly, cell or tissue homogenates were ultracentrifuged and the resulting supernatants ('extracts') were collected. They were next ultrafiltered through a 30 kDa cut-off low protein-binding ultrafiltration membrane with centrifugation at 3000 *g*. This step allows for separation of the pyrophosphatase activity from interfering phosphatase activities in the extracts (explained in Fig. 1). The substrate and products were separated and quantified by HPLC. The mean 8-oxo-dGTPase activity in the cell extract was expressed in relation to total protein concentration in the extract. An activity unit was defined as the amount of enzyme converting 1 pmol of 8-oxo-dGTP to 8-oxo-dGMP per min under standard reaction conditions [32].

To test possible correlation between the activity of 8-oxo-dGTPase in the testis with the occurrence of 8-oxo-dG in nuclear DNA of the same organ in rats treated with Cd(II) and Ni(II), 8-oxo-dG was assayed by HPLC with electrochemical detection, according to Adachi et al. [38].

3. Results

3.1. Inhibition of isolated 8-oxo-dGTPases

All the investigated metals, Cd(II), Cu(II), Ni(II), and Co(II), inhibited the activity of MutT and MTH1 in a concentration-dependent manner, though to different extents. The IC_{50} concentrations for these metals are given in Table 1 [36]. They indicate that, for either enzyme, Cu(II) and Cd(II) were stronger inhibitors than Co(II) and Ni(II), with the corresponding IC_{50} values differing by at least one order of magnitude. Also, the human MTH1 enzyme appeared to be more sensitive to inhibition by the metals than the bacterial enzyme. Possible reasons for the latter difference, based on the non-competitive nature of the inhibition and some dissimilarities in amino acid makeup of these two enzymes, are given elsewhere [36].

3.2. Inhibition of 8-oxo-dGTPase in CHO cells

The strong inhibitory potential of Cd(II) versus 8-oxo-dGTPases in a cell-free system was next verified in living cells. Cd(II) inhibited 8-oxo-dGTPase activity in cultured CHO cells in a concentration-dependent manner (Fig. 2). The effects were measured up to only 3 μM Cd(II) since higher concentrations of Cd(II) were toxic to cells. The enzyme activity decreased to 66, 89, and 78% of the respective control values, for the 2, 6, and 24 h incubations, respectively ($P = 0.076$, 0.013, and 0.0006, respectively, by ANOVA). Surprisingly, for each given Cd(II) concentration, longer exposures did not significantly affect the level of inhibition observed after 2 h of incubation [32].

3.3. Inhibition of 8-oxo-dGTPase activity and increase of 8-oxo-dG level in the rat testis

The assay of 8-oxo-dGTPase activity, developed originally by us for cultured cells [32], proved to be useful for analysis of the rat testis as well [37]. As illustrated in Fig. 3, unlike the whole extract of the testis (Fig. 3(A)), the ultrafiltrate of this extract (Fig. 3(B)) was free from enzymes degrading 8-oxo-dGTP to products other than 8-oxo-dGMP. Thus, the rat testis does not contain proteins with phosphatase activity that could pass through the 30 kDa cut-off ultrafilters, the crucial operation of our assay. The metal treatments did not

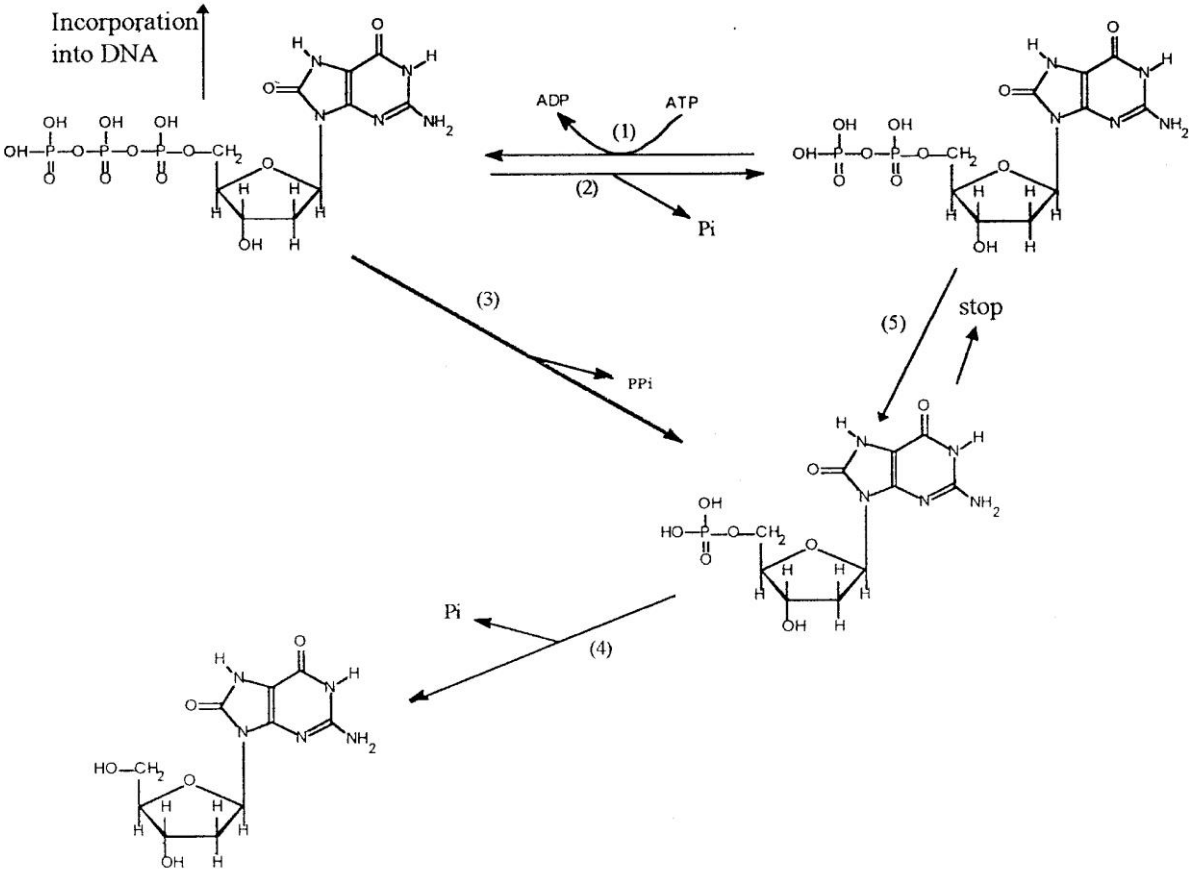

Fig. 1. Metabolic fate of 8-oxo-dGTP. The numbers at arrows indicate the following cellular enzymatic activities: (1) diphosphonucleoside kinase; (2) 8-oxo-2′-deoxyguanosine 5′-triphosphate phosphatase; (3) 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphatase (8-oxo-dGTPase; e.g., MutT, MTH1 and their homologs); (4) 8-oxo-2′-deoxyguanosine 5′-monophosphate phosphatase; (5) 8-oxo-2′-deoxyguanosine 5′-diphosphate phosphatase. Pi, orthophosphate. PPi, pyrophosphate. ATP, adenosine 5′-triphosphate. ADP, adenosine 5′-diphosphate.

Table 1
IC_{50} values for MTH1 and MutT [a,b]

Metal	IC_{50} (μM)	
	MTH1	MutT
Ni(II)	801±97	1459±96
Co(II)	376±71	8788±1003
Cu(II)	17±2	107±7
Cd(II)	30±8	60±6

[a] From [36].
[b] IC_{50}, metal concentration suppressing the enzyme activity to 50% of its original value. The numbers represent means±SE determined from three experiments.

change the specificity of the enzymatic activity in the ultrafiltrates, as indicated by the presence of 8-oxo-dGMP and the absence of other possible products of 8-oxo-dGTP hydrolysis, such as 8-oxo-dGDP and/or 8-oxo-dG (Fig. 4).

Treatment of rats with Cd(II) acetate resulted in a significant gradual decrease of testicular 8-oxo-dGTPase activity with time after the injection (Fig. 5). This decrease first became apparent as early as 8 h post-injection ($P<0.05$) and finally resulted in a 50% loss of the enzyme activity in 48 h ($P<0.01$). Although the results for Ni(II) at 8 h and later after the injection were lower than the controls, the differ-

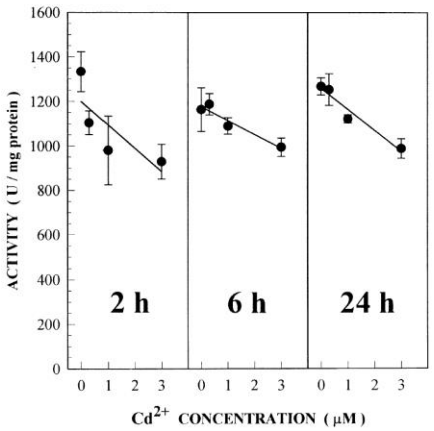

Fig. 2. Activity of 8-oxo-dGTPase in CHO cells cultured in the presence of 0–3 μM Cd(II) for 2, 6, or 24 h. The bars represent the standard error for three independent cultures treated in the same way [32].

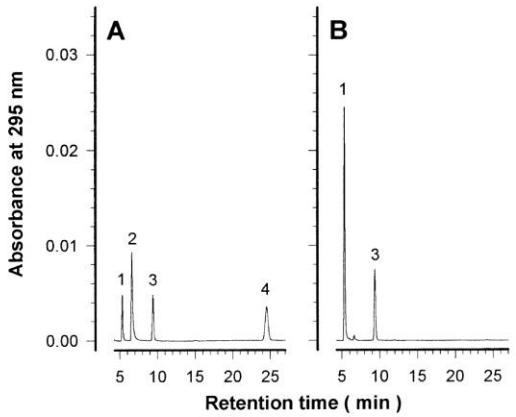

Fig. 3. HPLC separation of the substrate (1) and products (2–4) of 8-oxo-dGTP hydrolysis catalyzed by (A) a total protein extract of normal rat testis, and (B) a ≤30-kDa ultrafiltrate of that extract: (1) 8-oxo-dGTP; (2) 8-oxo-dGDP; (3) 8-oxo-dGMP; (4) 8-oxo-dG [37].

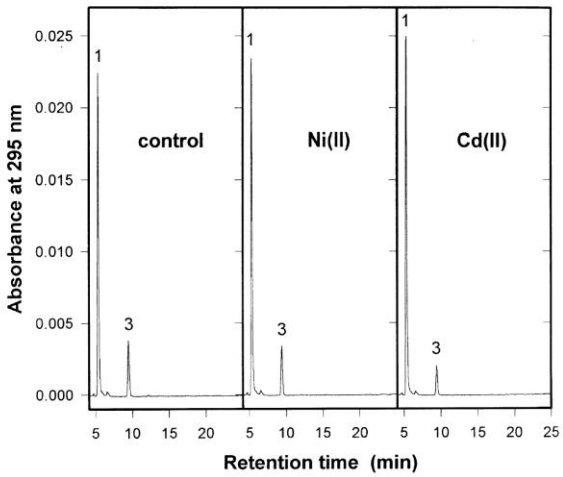

Fig. 4. Exemplary HPLC profiles demonstrating that the treatment of rats with metal salts did not change the specificity of enzymatic hydrolysis of 8-oxo-dGTP by ultrafiltrates of testicular protein extracts 24 h after treatment. Numbers indicate the same compounds as in Fig. 3 [37].

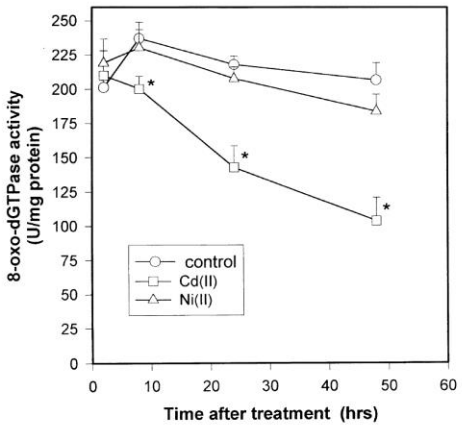

Fig. 5. 8-Oxo-dGTPase activity in testes of rats 2–48 h after a single subcutaneous injection of a metal salt. The bars denote the standard error ($N=3$). Asterisks denote $P<0.05$ or better vs. the corresponding control value [37].

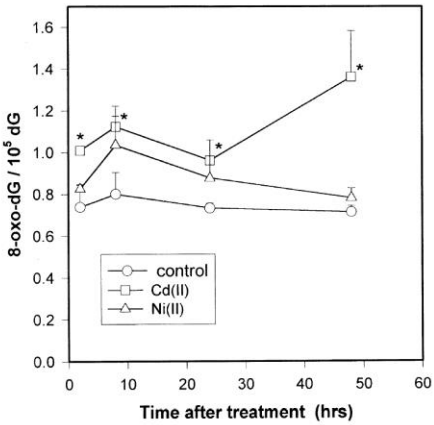

Fig. 6. The levels of 8-oxo-dG in testicular DNA 2–48 h after a single subcutaneous injection of a metal salt. The bars denote the standard error ($N=3$). Asterisks denote $P<0.05$ or better vs. the corresponding control value [37].

ences did not reach statistical significance. Likewise, the slight variations in 8-oxo-dGTPase activity observed with time in the control testes were not significant (Fig. 5) [37].

As shown in Fig. 6, treatment of rats with Cd(II) acetate led to a marked increase of the 8-oxo-dG level in testicular DNA that remained statistically significant over the entire period of the experiment, with $P<0.05$ or better. It reached nearly 200% of the control value in 48 h. Ni(II) acetate also tended to increase the testicular 8-oxo-dG level, but that increase was transient with an apparent maximum at 8 h, and its difference from the control did not approach statistical significance ($P<0.2$ versus the corresponding control for 8 h) [37].

4. Discussion

4.1. Selection of Cd(II) to study the inhibition of cellular 8-oxo-dGTPase activity

The metals tested in our studies had been found in various in vivo experiments to increase the levels of 8-oxoguanine in DNA [5–9,20,21], but the mechanisms involved in this effect remained unknown. One might assume that three of these metals, Cu(II), Co(II), and Ni(II), being redox active at physiological pH, should be capable of generating the 8-oxoguanine (or 8-oxo-dG) lesion in DNA by a direct interaction with chromatin and endogenous oxidants. They should enhance oxidation of dGTP to 8-oxo-dGTP as well. Because, at the same time, the metals can also inhibit 8-oxo-dGTPase activity, this may increase the 8-oxoguanine level in DNA even further. However, for these particular metals, distinguishing between the direct generation of 8-oxoguanine

within genomic DNA and incorporation of this lesion there from 8-oxo-dGTP, and thus assessing possible contribution of the incorporational pathway to the final effect, is not an easy task. Cd(II), which is not redox active under physiological conditions, but strongly inhibits 8-oxo-dGTPase activity, seemed to be a better metal to use. We presumed that endogenous (metabolic) oxidants generate enough 8-oxo-dGTP to assure 8-oxoguanine incorporation into DNA, especially when 8-oxo-dGTPase is inhibited. Therefore, our investigations in cells and animals were focused on Cd(II).

CHO cells were selected for our experiments because the AT→CG transversions, typical for 8-oxo-dGTP mutagenicity, first seen in *mutT*$^-$ bacteria [30], have also been frequent among point mutations identified in the *hprt* gene of Cd(II)-exposed CHO cells [39]. The rat testis, in turn, is a sensitive target for Cd(II)-induced carcinogenesis [18]. Cadmium(II) was also found to increase 8-oxo-dG levels in DNA of cultured lymphoblastoid cells [20] and in testes of glutathione-depleted rats [21]. In the latter study, oxidative DNA damage has been related to the inhibition by Cd(II) of DNA repair when glutathione biosynthesis is also inhibited. Since Cd(II) cannot oxidize DNA bases directly, these observations, as well as our present results, are consistent with, or at least do not contradict, the general notion that the observed oxidative DNA damage (by metabolic oxidants) is assisted by this metal through inhibition of cellular antioxidant and DNA protection/repair systems, of which 8-oxo-dGTPase is a member [1].

4.2. 8-Oxo-dGTPase activity versus 8-oxo-dG level in the rat testis

Decrease of 8-oxo-dGTPase activity with time after injection of Cd(II) concurred in our experiments with an increasing level of 8-oxo-dG. We might anticipate, therefore, that a causative relationship exists between these two effects. However, 2 h after the injection, 8-oxo-dGTPase activity remained unchanged while the 8-oxo-dG level was already significantly elevated above the control. Thus, at least at this time point, the increase in oxidative DNA damage cannot be associated with inhibition of the enzyme. It is very likely that Cd(II) can promptly enhance oxidative damage directly in DNA, e.g., by boosting endogenous oxidation. Nonetheless, a contribution of 8-oxo-dGTPase inhibition to the elevation of 8-oxo-dG level at later times, especially after 24 h, cannot be excluded.

Nickel(II) tended to suppress 8-oxo-dGTPase activity versus that of the control at ≥ 8 h after treatment. The very limited magnitude of this effect seems to be consistent with the much lower inhibitory potential of Ni(II) toward 8-oxo-dGTPase, observed in vitro [36], as well as generally lower susceptibility of the testis to Ni(II) [40] than to Cd(II) toxicity [18,41]. The testicular 8-oxo-dG levels in Ni(II)-treated rats, invariably higher than in the controls, with an apparent maximum 8 h after Ni(II), might indicate some oxidative damage to the DNA. However, under the experimental conditions applied, this effect was too weak (about 19% increase of the mean value over the mean of all control values, $P < 0.05$) to have biological significance.

4.3. Biological relevance of the observed effects

The in vitro IC_{50} values found in our studies for Ni(II) and Co(II) [36] appear to be too high to have an importance in vivo. The corresponding much lower values for Cd(II) and Cu(II) may be more biologically relevant, as confirmed for Cd(II) in CHO cells and rat testis. We must remember, however, that both epidemiological and experimental studies point at particulate, water-insoluble, metal compounds as the most potent carcinogens [18,42,43]. Phagocytosis would be the major way of their uptake by target cells [44]. Once phagocytized, such particles are fragmented and slowly solubilized [45], producing gradients of released metal ions that may reach local concentrations high enough to damage cytosolic proteins, including 8-oxo-dGTPase. Further, inhibition of the enzyme could be favored by accumulation of metals, also those administered systemically as promptly soluble salts, in specific cell organelles, e.g., in mitochondria [46], having their own 8-oxo-dGTPase activity [47]. Therefore, possible inhibitory effects of various carcinogenic metal species, both soluble and 'insoluble', on cellular 8-oxo-dGTPase, warrant further studies.

5. Conclusions

The carcinogenic transition metal ions Ni(II), Cu(II), Co(II), and Cd(II) are able to inhibit both bacterial and human 8-oxo-dGTPases. Cd(II) and Cu(II) are stronger inhibitors than Ni(II) and Co(II), and the human MTH1 enzyme is relatively more sensitive to inhibition than the bacterial MutT enzyme. Cd(II) has been proven to markedly inhibit 8-oxo-dGTPase activity in cultured cells and rat testes. In the latter, increase of oxidative DNA damage preceded the decrease in 8-oxo-dGTPase activity, indicating that the first appearance of 8-oxo-dG in DNA was not due to its incorporation from the nucleotide pool. However, the results do not exclude the possibility that further decrease in 8-oxo-dGTPase activity in time after Cd(II) treatment may contribute to such incorporation. The very limited effects of Ni(II) observed, although suggestive of some damaging trends toward testicular 8-oxo-dGTPase and DNA, await confirmation in larger animal groups and dose/response experiments.

6. Abbreviations

CHO cells	line K1-BH4 of Chinese hamster ovary cells
HPLC	high pressure liquid chromatography

IC_{50}	metal concentration that suppresses enzymatic activity to 50% of its original value
MTH1	human 8-oxo-dGTPase
MutT	8-oxo-dGTPase from *Escherichia coli*
ROS	reactive oxygen species
8-oxo-dG	8-oxo-2′-deoxyguanosine
8-oxo-dGTP	8-oxo-2′-deoxyguanosine 5′-triphosphate
8-oxo-dGDP	8-oxo-2′-deoxyguanosine 5′-diphosphate
8-oxo-dGMP	8-oxo-2′-deoxyguanosine 5′-monophosphate
8-oxo-dGTPase	8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphatase

References

[1] A.P. Grollman, M. Moriya, Trends Genet. 9 (1993) 246.
[2] S. Shibutani, M. Takeshita, A.P. Grollman, Nature 349 (1991) 431.
[3] K.C. Cheng, D.S. Cahill, H. Kasai, S. Nishimura, L.A. Loeb, J. Biol. Chem. 267 (1992) 166.
[4] Y.I. Pavlov, D.T. Minnick, S. Izuta, T.A. Kunkel, Biochemistry 33 (1994) 4695.
[5] K.S. Kasprzak, B.A. Diwan, N. Konishi, M. Misra, J.M. Rice, Carcinogenesis 11 (1990) 647.
[6] K.S. Kasprzak, B.A. Diwan, J.M. Rice, M. Misra, C.W. Riggs, R. Olinski, M. Dizdaroglu, Chem. Res. Toxicol. 5 (1992) 809.
[7] K.S. Kasprzak, T.H. Zastawny, S.L. North, C.W. Riggs, B.A. Diwan, J.M. Rice, M. Dizdaroglu, Chem. Res. Toxicol. 7 (1994) 329.
[8] K.S. Kasprzak, P. Jaruga, T.H. Zastawny, S.L. North, C.W. Riggs, R. Olinski, M. Dizdaroglu, Carcinogenesis 18 (1997) 271.
[9] F. Yamamoto, H. Kasai, Y. Togashi, N. Takeichi, T. Hori, S. Nishimura, Jpn. J. Cancer 84 (1993) 508.
[10] N. Spear, S.D. Aust, Arch. Biochem. Biophys. 317 (1995) 142.
[11] K.S. Kasprzak, Cancer Invest. 13 (1995) 411.
[12] K.S. Kasprzak, in: L. Chang (Ed.), Toxicology of Metals, Lewis Publishers, Boca Raton, FL, 1996, pp. 299–320.
[13] K.S. Kasprzak, in: N.D. Hadjiliadis (Ed.), Cytotoxic, Mutagenic and Carcinogenic Potential of Heavy Metals Related to Human Environment, Kluwer, Dordrecht, 1997, pp. 73–92.
[14] S.J. Stohs, D. Bagchi, Free Radical Biol. Med. 18 (1995) 321.
[15] R.E. Rodriguez, M. Misra, B.A. Diwan, C.W. Riggs, K.S. Kasprzak, Toxicology 107 (1996) 131.
[16] M. Misra, R.E. Rodriguez, K.S. Kasprzak, Toxicol. Lett. 58 (1991) 121.
[17] A. Hartwig, Environ. Health Perspect. 102 (Suppl. 3) (1994) 45.
[18] IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, vol. 58, Cadmium, Mercury, Beryllium and the Glass Industry, International Agency for Research on Cancer, Lyon, 1993.
[19] L.-J. Yang, J.I. Chao, J.-G. Lin, Chem. Res. Toxicol. 9 (1996) 1360.
[20] M.V. Mikhailova, N.A. Littlefield, B.S. Hass, L.A. Poirier, M.W. Chou, Cancer Lett. 115 (1997) 141.
[21] T. Hirano, Y. Yamaguchi, H. Kasai, Toxicol. Appl. Pharmacol. 4 (1997) 9.
[22] H. Maki, M. Sekiguchi, Nature 355 (1992) 273.
[23] J.-Y. Mo, H. Maki, M. Sekiguchi, Proc. Natl. Acad. Sci. USA 89 (1992) 11021.
[24] H.P. Treffers, V. Spinelli, N.O. Belser, Proc. Natl. Acad. Sci. USA 40 (1954) 1064.
[25] M. Furuichi, M.C. Yoshida, H. Oda, T. Tajiri, Y. Nakabeppu, T. Tsuzuki, M. Sekiguchi, Genomics 24 (1994) 485.
[26] M. Sekiguchi, Genes Cells 1 (1996) 139.
[27] A.S. Kamath-Loeb, A. Hizi, H. Kasai, L.A. Loeb, J. Biol. Chem. 272 (1997) 5892.
[28] T. Tajiri, H. Maki, M. Sekiguchi, Mutat. Res. 336 (1995) 257.
[29] M. Inoue, H. Kamiya, K. Fujikawa, Y. Ootsuyama, N. Murata-Kamiya, T. Osaki, K. Yasumoto, H. Kasai, J. Biol. Chem. 273 (1998) 11069.
[30] C. Yanofsky, E.C. Cox, V. Horn, Proc. Natl. Acad. Sci. USA 55 (1966) 274.
[31] H. Hayakawa, A. Taketomi, K. Sakumi, M. Kuwano, M. Sekiguchi, Biochemistry 34 (1995) 89.
[32] K. Bialkowski, K.S. Kasprzak, Nucleic Acids Res. 26 (1998) 3194.
[33] T. Kakuma, J. Nishida, T. Tsuzuki, M. Sekiguchi, J. Biol. Chem. 270 (1995) 25942.
[34] J.P. Cai, T. Kakuma, T. Tsuzuki, M. Sekiguchi, Carcinogenesis 16 (1995) 2343.
[35] R.G. Fowler, R.M. Schaaper, FEMS Microbiol. Rev. 21 (1997) 43.
[36] D.W. Porter, H. Yakushiji, Y. Nakabeppu, M. Sekiguchi, M.J. Fivash Jr., K.S. Kasprzak, Carcinogenesis 18 (1997) 1785.
[37] K. Bialkowski, A. Bialkowska, K.S. Kasprzak, Carcinogenesis 20 (1999) 1621.
[38] S. Adachi, M. Zeisig, L. Moller, Carcinogenesis 16 (1995) 253.
[39] J.-L. Yang, J.-I. Chao, J.-G. Lin, Chem. Res. Toxicol. 9 (1996) 1360.
[40] J. Xie, T. Funakoshi, H. Shimada, S. Kojima, Toxicology 103 (1995) 147.
[41] M.P. Waalkes, S. Rehm, Fundam. Appl. Toxicol. 23 (1994) 21.
[42] K. Hughes, M.E. Meek, R. Newhook, P.K.L. Chan, Regul. Toxicol. Pharmacol. 22 (1995) 213.
[43] A.R. Oller, M. Costa, G. Oberdorster, Toxicol. Appl. Pharmacol. 143 (1997) 152.
[44] X. Huang, C.B. Klein, M. Costa, Carcinogenesis 15 (1994) 545.
[45] H.F. Hildebrand, M. Collyn D'Hooghe, P. Shirali, C. Bailly, J.P. Kerckaert, Carcinogenesis 11 (1990) 1943.
[46] K.S. Kasprzak, L.A. Poirier, Carcinogenesis 6 (1985) 1819.
[47] D. Kang, J. Nishida, A. Iyama, Y. Nakabeppu, M. Furuichi, T. Fujiwara, M. Sekiguchi, K. Takeshige, J. Biol. Chem. 270 (1995) 14659.

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Original Contribution

INHIBITION OF 8-OXO-2′-DEOXYGUANOSINE 5′-TRIPHOSPHATE PYROPHOSPHOHYDROLASE (8-OXO-dGTPase) ACTIVITY OF THE ANTIMUTAGENIC HUMAN MTH1 PROTEIN BY NUCLEOSIDE 5′-DIPHOSPHATES

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Abstract—The hMTH1 protein, a human homologue of *E. coli* MutT protein, is an enzyme converting 8-oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) to 8-oxo-2′-deoxyguanosine 5′-monophosphate (8-oxo-dGMP) and inorganic pyrophosphate. It is thought to play an antimutagenic role by preventing the incorporation of promutagenic 8-oxo-dGTP into DNA. As found in our previous investigations, 8-oxo-2′-deoxyguanosine 5'-diphosphate (8-oxo-dGDP) strongly inhibited 8-oxo-dGTPase activity of MTH1. Following this finding, in the present study we have tested the canonical ribo- and deoxyribonucleoside 5′-diphosphates (NDPs and dNDPs) for possible inhibition of 8-oxo-dGTP hydrolysis by hMTH1 extracted from CCRF-CEM cells (a human leukemia cell line). Among them, the strongest inhibitors appeared to be dGDP (Ki = 74 μM), dADP (Ki = 147 μM), and GDP (Ki = 502 μM). Other dNDPs and NDPs, such as dCDP, dTDP, ADP, CDP, and UDP were much weaker inhibitors, with Ki in the millimolar range. Based on the present results and published data, we estimate that the strongest inhibitors, dGDP and dADP, at physiological concentrations not exceeding 5 μM and GDP at mean concentration of 30 μM, taken together, can decrease the cellular hMTH1 enzymatic activity vs. 8-oxo-dGTP (expected to remain below 500 pM) by up to 15%. The other five NDPs and dNDPs tested cannot markedly affect this activity. © 2003 Elsevier

Keywords—MTH1, 8-Oxo-dGTPase, 8-Oxo-dGTP pyrophosphohydrolase, Antimutagenic enzyme, Oxidative damage, Activity inhibition, Free radicals

INTRODUCTION

The human MTH1 protein (hMTH1), homologue of *E. coli* MutT, is an enzyme decomposing 8-oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) to 8-oxo-2′-deoxyguanosine 5′-monophosphate (8-oxo-dGMP) and inorganic pyrophosphate [1]. 8-Oxo-dGTP, a promutagenic product of oxidative damage to dGTP, may be formed in cells upon normal aerobic metabolism and oxidative stress. By removing this product from the deoxyribonucleotide pool of substrates for DNA synthesis, hMTH1 is thought to play an antimutagenic role (for review see [2]).

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The *hMTH1* gene is located on the chromosome 7p22 [3]. It consists of 5 exons and can produce up to seven types (1, 2A, 2B, 3A, 3B, 4A, and 4B) of mRNAs as a result of the existence of multiple sites of transcription initiation and alternative RNA splicing processes [4]. Type 1 mRNA and the18 kDa hMTH1 variant are predominant products of *hMTH1* [5]. The enzymatic activity of purified 18 kDa human 8-oxo-dGTPase is Mg^{2+}-dependent [1,6]. The enzyme shows its maximum activity under slightly alkaline conditions [1] and demonstrates a broad specificity towards nucleoside 5′-triphosphates. Although hMTH1 decomposes most effectively 2-hydroxy-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP) [7,8], 2-hydroxyadenosine 5′-triphosphate (2-OH-ATP) [9], 8-oxo-dGTP [1], and 8-oxo-2′-deoxyadenosine 5′-triphosphate (8-oxo-dATP) [7], it is also capable of hydrolyzing less effectively 8-oxoguanosine 5′-triphosphate (8-oxo-GTP) [10],

8-chloro-2′-deoxyguanosine 5′-triphosphate (8-Cl-dGTP) [11], and canonical deoxyribonucleoside and ribonucleoside 5′-triphosphates such as dGTP [1].

Four isolated forms of hMTH1 protein (p18, p21, p22, and p26) demonstrate equal activity towards 8-oxo-dGTP [5]. Substrate specificity of hMTH1 for different nucleoside 5′-triphosphates does not depend on evolutionarily conservative phosphohydrolase module of the protein, corresponding to amino acids Gly-36 to Gly-58 [8]. Tryptophan 117 of hMTH1 is responsible for binding 8-oxoguanine and 2-OH-adenine-containing nucleotides [8,12], whereas aspartic acid 119 is essential only for 2-OH-adenine moiety recognition [8]. It explains the different substrate specificities of bacterial MutT protein and its human homologue hMTH1, since both amino acids are not conserved in *E. coli* MutT protein.

A better insight into the role of mammalian MTH1 proteins has been acquired by the creation of *MTH1* nullizygous cell lines and mice [13]. These transgenic mice, defective in *MTH1* gene and devoid of 8-oxo-dGTPase activity, demonstrated higher incidence of lung, liver, and stomach cancers accompanied by 2-fold increase in spontaneous mutation frequency in *Hprt* gene, as compared to wild-type mice [14]. Nevertheless, this slightly higher mutation rate has not been confirmed in a more recent study that showed the same level of mutation frequency in *rpsL* reporter gene of *E. coli*, introduced into both $MTH1^{+/+}$ and $MTH1^{-/-}$ backgrounds [15].

As we have found in our previous study, admixture of 8-oxo-2′-deoxyguanosine 5'-diphosphate (8-oxo-dGDP) in the 8-oxo-dGTP substrate strongly inhibited Chinese hamster 8-oxo-dGTPase activity [16]. The diphosphate is structurally similar to the substrate triphosphate but is not hydrolyzed by the enzyme. Therefore, considering the fact that physiological concentrations of 2'-deoxyribonucleoside 5′-diphosphates (dNDPs) and ribonucleoside 5′-diphosphates (NDPs) in the cell exceed a potential concentration of the substrate (8-oxo-dGTP) by many orders of magnitude, we have hypothesized that the canonical dNDPs and NDPs may significantly inhibit 8-oxo-dGTPase activity in vivo. The present study was designed to test this hypothesis.

MATERIALS AND METHODS

Chemicals

8-Oxo-2′-deoxyguanosine 5′-triphosphate and 8-oxo-2′-deoxyguanosine 5′-monophosphate were synthesized as described previously [16]. All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Preparation of human 8-oxo-dGTPase

Human leukemia CCRF-CEM cells, demonstrating very high 8-oxo-dGTPase activity (own unpublished data) were used as a source of the enzyme. The cells were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine, as described elsewhere [17]. They were harvested by scraping and washed three times with phosphate-buffered saline. A pellet of $\sim 2 \times 10^8$ cells was brought to 10 ml with a hypotonic 20 mM Tris-HCl buffer, pH 7.4, and homogenized by ultrasonication for 10 s on ice. The homogenate was centrifuged for 15 min at 2800 $\times$ *g* (4°C) and tissue debris was discarded. The supernatant was then ultracentrifuged for 1 h at 100,000 $\times$ *g* (4°C). The resulting secondary supernatant located between the pellet and fatty upper layer was gently collected and ultrafiltered at 4°C through 30 kDa cut-off, low protein-binding regenerated cellulose membranes (Ultrafree-CL Filter Units, Millipore, Bedford, MA, USA). This step separates the MTH1 protein from interfering phosphatases [16]. Concentrated solution of bovine serum albumin was added to the ultrafiltrate to a final concentration of 50 μg/ml and the sample was dialyzed at 4°C for 6 h against 4 l of 20 mM Tris-HCl, pH 7.4, to remove UV-absorbing, low molecular weight molecules, including dNDPs and NDPs. The dialyzed sample containing hMTH1 was finally concentrated on 5 kDa cut-off, low protein-binding regenerated cellulose membranes (Ultrafree-CL Filter Units, Millipore) by centrifugation at 4500 $\times$ *g* at 4°C.

Kinetic studies of hMTH1 8-oxo-dGTPase activity in the presence of inhibitors

The 8-oxo-dGTPase activity of hMTH1 was tested in the presence of the following prospective inhibitors: dGDP, dADP, dCDP, dTDP, GDP, ADP, CDP, UDP, and uric acid. The latter, abundantly present in cells, was used to account for possible contribution of the purine moiety to the inhibitory effect of nucleotides. The 8-oxo-dGTPase activity was determined in 60 μl samples of the reaction solution, containing 5–40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris-HCl (pH 7.4), 0–200 μM inhibitor, and 5 μl of the enzyme preparation (as above), incubated at 37°C for 20 min. The reaction was initiated by the addition of the enzyme and terminated by adding 20 μl 50 mM Na_2EDTA. Blank samples were prepared with Na_2EDTA introduced before the enzyme preparation, followed by incubation at 37°C. All reactions were performed in triplicate.

The separation and quantification of 8-oxo-dGMP in the reaction solutions was achieved using an HPLC method developed by us previously [16]. Briefly, the Waters HPLC system (Waters Corp., Milford, MA,

USA) used consisted of two pumps (model 510), autosampler (model 717 plus), UV-VIS photodiode array detector (model 996), Luna C18(2) column (250 × 4.6 mm, 5 μm grain; Phenomenex, Torrance, CA, USA), and was controlled by a Millennium^{32} Chromatography Manager (Waters Corp.). Unless otherwise indicated, 30 μl aliquots of the reaction mixtures were chromatographed isocratically with 100 mM phosphate buffer, pH 5.5/methanol (95:5), at a flow rate of 1 ml/min. Solutions of known concentrations of 8-oxo-dGMP, ranging from 1.25 to 15 μM, were used for calibration. For quantification of the reaction product (8-oxo-dGMP), chromatograms acquired at 295 nm were integrated. The activity of 8-oxo-dGTPase was expressed as a rate of 8-oxo-dGMP formation (pmol 8-oxo-dGMP/min) in the reaction solution. Inhibition constants (*Ki*) were determined using the Michaelis-Menten model for competitive inhibition.

RESULTS AND DISCUSSION

Technical pitfalls accompanying kinetic measurements of 8-oxo-dGTPase activity

As demonstrated in our previous papers, total protein extracts of mammalian cells derived from Chinese hamster [16], mouse [18], rat [19], or human [1,20,21] contain many phosphohydrolytic activities toward 8-oxo-dGTP, 8-oxo-dGDP, and 8-oxo-dGMP, making a selective determination of the 8-oxo-dGTPase activity a challenging task. Fortunately, the major 8-oxo-dGTPase is a much smaller protein (18 kDa) than the other enzymes. This feature of 8-oxo-dGTPase allows for a simple separation of this enzyme from the other unspecific enzymes hydrolyzing 8-oxo-dG phosphates by ultrafiltration through 30 kDa cut-off membranes, without applying the time-consuming chromatographic purification [16,18,19,22].

Although the apparent pH optimum for human 8-oxo-dGTPase is 8.5 (Fig. 1), we decided to measure all the kinetic and inhibition parameters at pH 7.4 to better mimic physiological conditions in the cytosol where the human enzyme is predominant [23]. A precise determination of the Michaelis-Menten constant (*Km*) for 8-oxo-dGTP hydrolysis to 8-oxo-dGMP is difficult. As shown in Fig. 2, the activity vs. substrate concentration curve follows the Michaelis-Menten equation only at lower concentrations. At 8-oxo-dGTP concentrations exceeding 40 μM, the measured enzymatic activity of hMTH1 becomes lower than expected, and above 80 μM an obvious inhibition is observed. This phenomenon is most likely produced by low amounts of 8-oxo-dGDP contaminating our 8-oxo-dGTP preparation due to inevitable spontaneous hydrolysis of the latter.

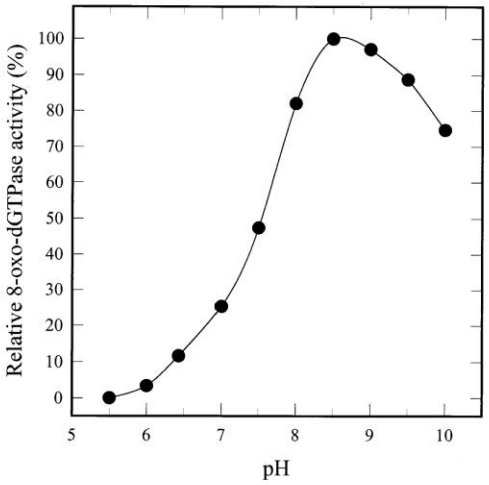

Fig. 1. The effect of pH on 8-oxo-dGTPase activity of the human MTH1 protein. Samples (60 μl of 40 μM 8-oxo-dGTP plus 5 mM $MgCl_2$ and 100 mM Tris-HCl, pH 5.5–10) were reacted for 20 min at 37°C with hMTH1-containing cytosolic ultrafiltrate from CCRF-CEM leukemia cells. The reaction was terminated with 20 μl of 50 mM Na_2EDTA and 30 μl aliquots of the reaction mixtures were analyzed by HPLC to determine 8-oxo-dGMP formation, as described in Materials and Methods. The enzymatic activity is expressed in percentages of the highest observed activity at pH 8.5.

As we have found previously [16], 8-oxo-dGDP is a strong inhibitor of 8-oxo-dGTPases. Its inhibition constant (*Ki*) of 0.5 μM has been calculated for human enzyme by Fujikawa and coworkers [7] according to a competitive inhibition model. This low *Ki* means that an increase of the substrate concentration greatly amplifies the inhibitory effect posed by the concurrently increasing 8-oxo-dGDP contamination of the reaction mixture. Therefore, the most reliable *Km* values can be obtained only for 8-oxo-dGTP of very high purity. *Km* calculated from data obtained for the 0–40 μM concentration range of our substrate preparation was 12.1 μM (Fig. 2). This is essentially the same value as reported by Mo and coworkers for purified human 8-oxo-dGTPase [1]. A purity of our 8-oxo-dGTP and selectivity of 8-oxo-dGTPase reaction catalyzed by our hMTH1 protein preparation are shown in Fig. 3 (chromatograms A and B, respectively).

Inhibition of hMTH1 by canonical nucleoside 5′-diphosphates

Unlike nucleoside 5′-triphosphates, the nucleoside 5′-diphosphates are not hydrolyzed by mammalian 8-oxo-dGTPases. As competitive inhibitors, they bind to the active site of the enzyme and thereby block the binding of the triphosphates. Moreover, nucleoside 5'-diphospates have been recently demonstrated to have higher

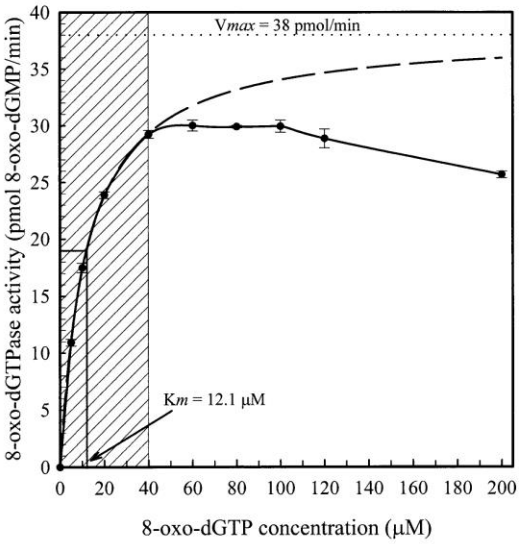

Fig. 2. Determination of the Michaelis-Menten constant (*Km*) for 8-oxo-dGTP hydrolysis catalyzed by hMTH1 at pH 7.4. The experimental activity-substrate concentration curve does not exactly follow the Michaelis-Menten equation due to the presence of low amounts of 8-oxo-dGDP, contaminating 8-oxo-dGTP preparations. The higher the substrate (and contaminant) concentration the stronger the inhibition that can be seen (bulleted line). This mimics inhibition of hMTH1 by the substrate at concentrations above 80 μM 8-oxo-dGTP. Thus, *Km* value should be calculated from the activity measurements performed for the 0–40 μM range of 8-oxo-dGTP concentration. Within this range (diagonally lined area of the graph), experimental points follow exactly the theoretical curve, rectangular hyperbola (dashed line). Error bars denote standard deviation values of three separate activity determinations.

affinity for the active site of human MTH1 than corresponding nucleoside 5′-triphosphates [12]. The strength of the inhibition brought about by a nucleoside 5'-diphosphate seems to depend mainly on the chemical structure of the nucleoside moiety and its affinity for the active site of hMTH1 protein. We have tested inhibitory effects of increasing concentrations (0–200 μM) of eight canonical dNDPs and NDPs, including dGDP, dADP, dCDP, dTDP, GDP, ADP, CDP, and UDP, on the hydrolysis of 5 μM 8-oxo-dGTP to 8-oxo-dGMP catalyzed by hMTH1. Our hMTH1 preparation did not contain any enzymatic activities decomposing nucleoside 5′-diphosphates. An exemplary HPLC chromatogram demonstrating the stability of dGDP incubated with hMTH1 preparation and 8-oxo-dGTP is presented in Fig. 3C.

As shown in Fig. 4, the strongest inhibitors were dGDP, dADP, and GDP. The remaining nucleoside 5′-diphosphates appeared to be much weaker inhibitors (*Ki* values estimated by the approximation of the data shown in Fig. 4 range from 1 to 2 mM). Therefore, only the inhibitory properties of dGDP, dADP, and GDP were investigated in further detail. The effects of 50 μM dGDP, 75 μM dADP, and 200 μM GDP on hMTH1 activity towards 0–40 μM 8-oxo-dGTP are presented in the form of Linewaever-Burk plots (Fig. 5). All three compounds affected mainly the Michaelis-Menten constant but not the maximum velocity of the enzymatic reaction that suggests a competitive mechanism of the inhibition. The individual competitive inhibition constants (*Ki*), defined as the concentration of an inhibitor bringing about a 50% increase in the measured *Km* value, were determined by fitting the experimental data (using the Curve Fit function of Sigma Plot program, SPSS Inc., Chicago, IL, USA) to the equation describing the rate of enzymatic reaction in the presence of a competitive inhibitor. The calculated *Ki* values for dGDP, dADP, and GDP were 74, 147, and 502 μM, respectively. The inhibition of human 8-oxo-dGTPase by dGDP and a very similar *Ki* value of 58 μM have been recently reported elsewhere [12].

To test possible contribution of the base moiety of the diphosphonucleosides to the strength of the inhibition, we investigated the influence of uric acid on the activity of human 8-oxo-dGTPase. Uric acid is a physiological compound produced by human cells as a final product of degradation of purine-containing biomolecules (nucleic acids, nucleotides) and seems to have the most preferable chemical structure with regard to the purine rings of the hMTH1 substrates. As recently reported, the best substrates for the human enzyme besides 8-oxo-dGTP are 2-OH-dATP, 2-OH-ATP, and 8-oxo-dATP [7,9]. This suggests that the presence of oxygens at the C-2 and C-8 positions of the purine ring is favorable in terms of substrate binding to the active site of hMTH1 protein. Although uric acid (2,6,8-trioxypurine) has both such oxygen atoms, we have not observed any inhibition of human 8-oxo-dGTPase activity by uric acid up to its 40 μM concentration (limited by solubility). It means that the sugar phosphate moiety is critical for the high-affinity interaction of a competitive inhibitor with the active site of hMTH1.

Application of 8-oxo-dGTPase inhibition to investigations of substrate specificity of MTH1 proteins

A key to a full understanding of the physiological significance of hMTH1 in vivo is complete information regarding the substrate specificity of this enzyme. A list of substrates for hMTH1 protein still lengthens, thus increasing the number of its prospective roles in the cell [1,7,9–11]. Moreover, the homologous bacterial MutT and mammalian MTH1 proteins isolated from different species differ substantially in their substrate specificity that makes the question about physiological significance of this group of enzymes even more difficult to answer. The results of the present inhibition study may help to

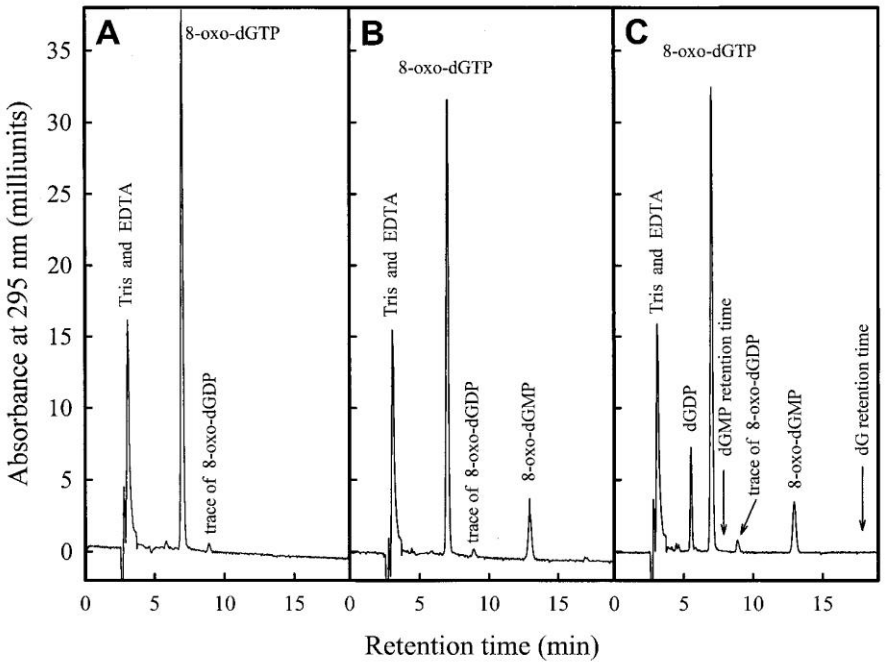

Fig. 3. HPLC analyses of enzymatic reaction solutions. (A) A chromatogram of blank sample showing a purity of synthesized 8-oxo-dGTP. A 60 μl solution containing 40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.4, and hMTH1 preparation was incubated for 20 min at 37°C with 20 μl of 50 mM Na_2EDTA to prevent enzymatic hydrolysis of 8-oxo-dGTP. Of the sample, a 30 μl aliquot was chromatographed as described in Materials and Methods. (B) A chromatogram demonstrating a selective hydrolysis of 8-oxo-dGTP to 8-oxo-dGMP by hMTH1 preparation. A 60 μl solution containing 40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.4, and hMTH1 preparation was incubated at 37°C. After 20 min, the reaction was stopped with 20 μl of 50 mM Na_2EDTA and a 30 μl aliquot was analyzed by HPLC as above. (C) Nucleoside 5′-diphosphates were not hydrolyzed by hMTH1 preparation. An exemplary chromatogram shows 8-oxo-dGTPase reaction solution (as above) containing 50 μM dGDP. Arrows indicate the retention times of potential dGDP degradation products, 2′-deoxyguanosine 5′-monophosphate (dGMP) and 2′-deoxyguanosine (dG).

design future investigations on substrate specificity of MTH1 homologues. The strength of 8-oxo-dGTPase activity inhibition caused by a particular nucleoside 5'-diphosphate reflects the affinity of MTH1 protein for the corresponding nucleoside 5′-triphosphate. The inhibition data presented in Fig. 4 suggest that among the canonical (d)NTPs the best substrates for hMTH1 are dGTP > dATP >> GTP >> dCTP ~ CTP ~ ATP ~ dTTP ~ UTP. This conclusion remains in accord with previous determinations of the substrate specificity of the human MTH1 that were performed with the purified protein [1]. Thus, 8-oxo-dGTPase inhibition studies may be used as a relatively simple and rapid approach for determining substrate specificity of different MTH1 homologues, without the need of purifying the enzyme to homogeneity.

Is human 8-oxo-dGTPase activity inhibited in vivo?

Considering many possible different activities of hMTH1, its main physiological function seems still unclear. To our best knowledge, a concentration of 8-oxo-dGTP or even its presence in the cell has never been established. Since neither 8-oxo-dGMP can be phosphorylated to 8-oxo-dGDP by the human guanylate kinase nor 8-oxo-GDP can be reduced to 8-oxo-dGDP by the human nucleoside 5'-diphosphate reductase [10], the only likely origin of cellular 8-oxo-dGTP seems to be (i) a direct oxidation of dGTP or (ii) an enzymatic phosphorylation of 8-oxo-dGDP (from dGDP oxidation) by nucleoside 5'-diphosphate kinase.

The reported concentrations of dGTP in human cells differ substantially, but its average concentration measured in all investigated mammalian cells was around 5 μM (for review see [24]). If the rate of guanine oxidative damage and repair is similar for DNA and free nucleotide pool, we might predict an 8-oxo-dGTP concentration in the cell to be at least 10^5-fold lower than the concentration of dGTP, namely, around 50 pM. On the other hand, cellular concentrations of dNDPs and NDPs are in the micromolar or even millimolar range [24]; thus, they

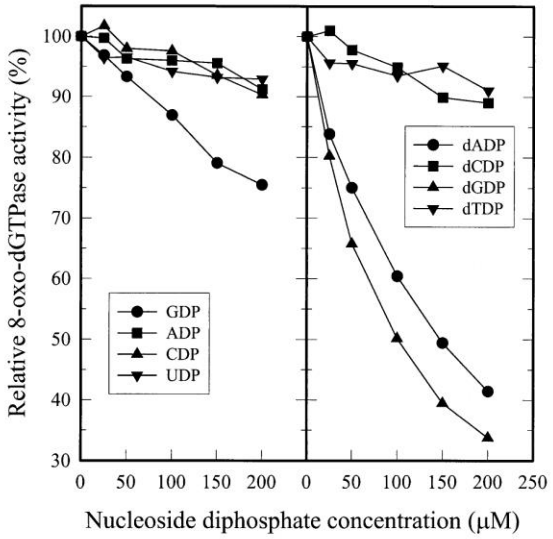

Fig. 4. The inhibition of the 8-oxo-dGTPase activity of hMTH1 by canonical nucleoside 5′-diphosphates. Samples of 5 μM 8-oxo-dGTP plus 5 mM $MgCl_2$ and 100 mM Tris-HCl, pH 7.4, were reacted for 20 min at 37°C with hMTH1-containing cytosolic ultrafiltrate from CCRF-CEM leukemia cells in the presence of increasing concentrations (0–200 μM) of nucleoside 5′-diphosphates. The reaction was stopped with EDTA and 8-oxo-dGTPase activity was determined chromatographically, as described in Materials and Methods, by analyzing 50 μl aliquots of reaction solutions. Relative activity was expressed in percentages of the activity measured without inhibitors.

exceed the hypothetical concentration of 8-oxo-dGTP by at least five to seven orders of magnitude. Moreover, one must consider that potential inhibitory effects of different nucleoside diphosphates on 8-oxo-dGTPase activity are additive.

Assuming the cellular concentrations of 8-oxo-dGTP, dGDP, dADP, and GDP and knowing inhibition constants for the three inhibitors, we can calculate potential in vivo inhibitory effects on 8-oxo-dGTPase activity by comparing the velocities of 8-oxo-dGTP hydrolysis with and without a particular inhibitor. A velocity of the reaction without inhibitor (V_1) is described with the Michaelis-Menten equation (Eqn. 1).

$$V_1 = \frac{Vmax[S]}{[S] + Km} \quad (1)$$

In the presence of a competitive inhibitor, this equation becomes Eqn. 2.

$$V_2 = \frac{Vmax[S]}{[S] + Km(1 + [I]/Ki)} \quad (2)$$

A percentage of the original 8-oxo-dGTPase activity estimated in the presence of the inhibitor may be expressed as $(V_2/V_1) \times 100\%$, as in Eqn. 3.

$$(V_2/V_1) \times 100\% = \frac{[S] + Km}{[S] + Km(1 + [I]/Ki)} \quad (3)$$

where V = velocity of the enzymatic reaction; *Vmax* = maximum velocity of the reaction; *Km* = Michaelis-Menten constant; [S] = substrate concentration; [I] = competitive inhibitor concentration; and *Ki* = inhibition constant.

Since the presumed cellular concentration of the substrate (8-oxo-dGTP) is certainly lower than a *Km* value (12.1 μM) by at least five orders of magnitude, potential changes of 8-oxo-dGTP concentration below 500 pM do not practically change the percentile inhibition caused by diphosphonucleoside inhibitors. In this case, the only factor that influences an extent of the inhibition is a ratio of the cellular concentration of inhibitor to its inhibition constant ([I]/K*i*).

Hence, intracellular concentrations of (d)NDPs are of primary importance to understanding the real effects of (d)NDPs on the 8-oxo-dGTPase sanitizing activity of hMTH1. Unfortunately, reliable data on the cellular concentrations of dGDP and dADP are not available, apparently due to technical problems with their analysis in tissue or cell homogenates. As reviewed by Traut ([24] and references therein), dGTP and dATP concentrations determined in human cells are in the 0.4–6.4 μM and 1.3–3.6 μM ranges, respectively. By analogy to the intracellular concentrations of the other (d)NTP/(d)NDP pools, we consider dGDP and dADP concentrations not to exceed the 5 μM level. Using Eqn. 3 with the presumed cellular concentrations of 8-oxo-dGTP (50 pM) and dGDP (5 μM), we can calculate that, under defined conditions (at the Ki_{dGDP} = 74 μM), hMTH1 protein retains 93.7% of its original 8-oxo-dGTPase activity. Five micromolar dATP, which is a weaker inhibitor (*Ki* = 147 μM), would only decrease that sanitizing activity down to 96.7% of the original value.

The reported concentrations of GDP in different nucleated human cells range from 1.7 to 72 μM [24], with a mean concentration of 30 μM. The latter concentration of GDP would bring the 8-oxo-dGTPase activity down to 94.4%. Summarizing, all three inhibitors acting intracellularly in a concerted manner may inhibit only around 15% of the hMTH1 8-oxo-dGTPase activity. Therefore, we conclude that hMTH1 can effectively perform its nucleotide pool-sanitizing function against 8-oxo-dGTP. Other possible functions of hMTH1, e.g., control of homeostasis of the nucleotide pools and their regulation by phosphonucleoside levels, remain to be explored.

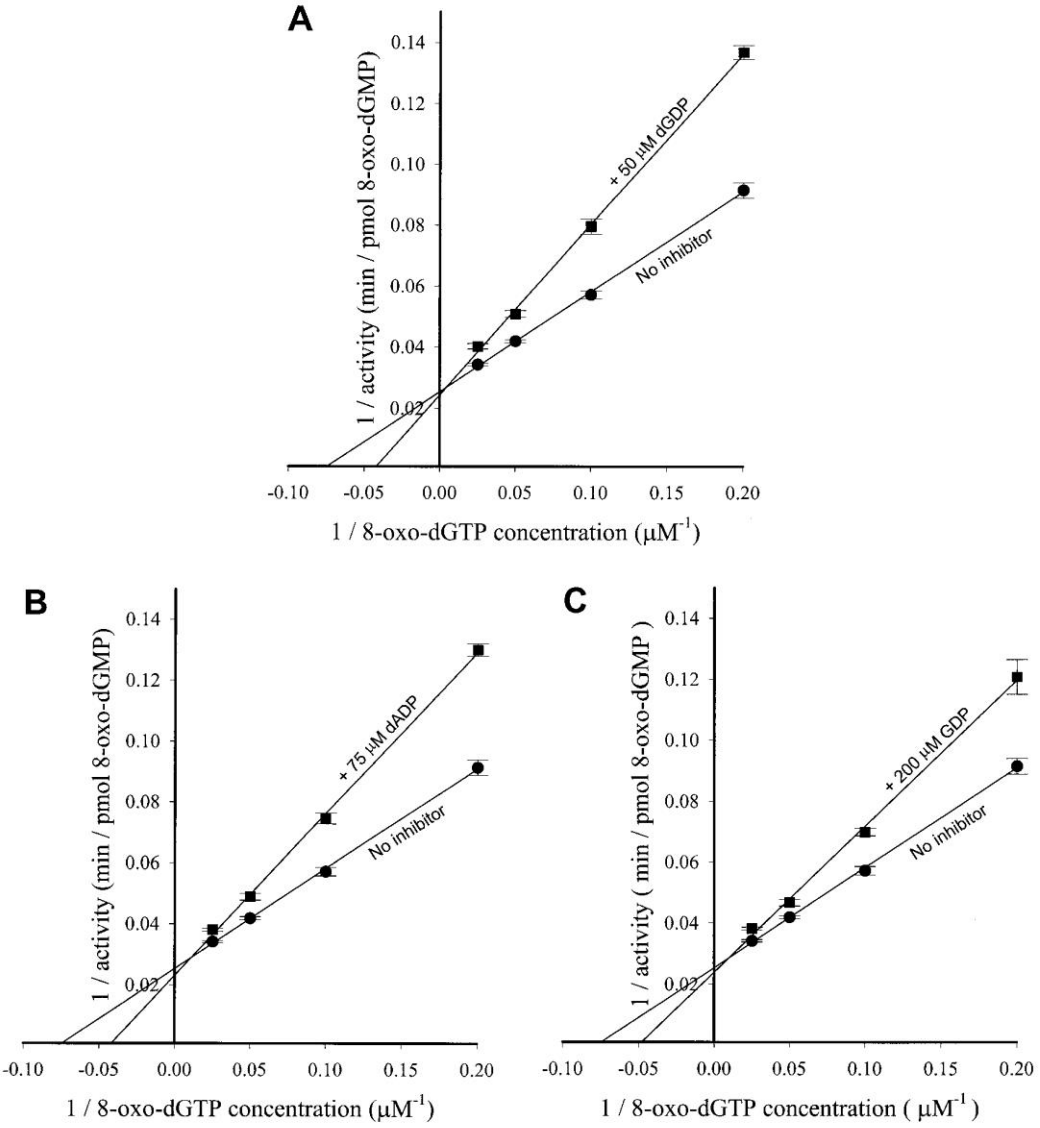

Fig. 5. Determination of inhibition constants (*Ki*) of dGDP, dADP, and GDP towards 8-oxo-dGTPase activity of hMTH1 protein. Samples of 5, 10, 20, or 40 μM 8-oxo-dGTP plus 5 mM $MgCl_2$ and 100 mM Tris-HCl, pH 7.4, were reacted with hMTH1-containing cytosolic ultrafiltrate from CCRF-CEM leukemia cells without inhibitors or with (A) 50 μM dGDP, (B) 75 μM dADP, or (C) 200 μM GDP. The 8-oxo-dGTPase activity was determined as described in Materials and Methods. The activity-substrate concentration curves are presented as double reciprocal plots. Error bars denote standard deviation values.

REFERENCES

[1] Mo, J. Y.; Maki, H.; Sekiguchi, M. Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. *Proc. Natl. Acad. Sci. USA* **89:**11021–11025; 1992.

[2] Nakabeppu, Y. Molecular genetics and structural biology of human MutT homolog, MTH1. *Mutat. Res.* **477:**59–70; 2001.

[3] Furuichi, M.; Yoshida, M. C.; Oda, H.; Tajiri, T.; Nakabeppu, Y.; Tsuzuki, T.; Sekiguchi, M. Genomic structure and chromosome location of the human mutT homologue gene MTH1 encoding 8-oxo-dGTPase for prevention of A: T to C:G transversion. *Genomics* **24:**485–490; 1994.

[4] Oda, H.; Nakabeppu, Y.; Furuichi, M.; Sekiguchi, M. Regulation of expression of the human MTH1 gene encoding 8-oxo-dGTPase. Alternative splicing of transcription products. *J. Biol. Chem.* **272:**17843–17850; 1997.

[5] Oda, H.; Taketomi, A.; Maruyama, R.; Itoh, R.; Nishioka, K.; Yakushiji, H.; Suzuki, T.; Sekiguchi, M.; Nakabeppu, Y. Multiforms of human MTH1 polypeptides produced by alternative translation initiation and single nucleotide polymorphism. *Nucleic Acids Res.* **27:**4335–4343; 1999.

[6] Porter, D. W.; Yakushiji, H.; Nakabeppu, Y.; Sekiguchi, M.; Fivash, M. J. Jr.; Kasprzak, K. S. Sensitivity of Escherichia coli (MutT) and human (MTH1) 8-oxo-dGTPases to in vitro inhibition

by the carcinogenic metals, nickel(II), copper(II), cobalt(II), and cadmium(II). *Carcinogenesis* **18:**1785–1791; 1997.

[7] Fujikawa, K.; Kamiya, H.; Yakushiji, H.; Fujii, Y.; Nakabeppu, Y.; Kasai, H. The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein. *J. Biol. Chem.* **274:**18201–18205; 1999.

[8] Sakai, Y.; Furuichi, M.; Takahashi, M.; Mishima, M.; Iwai, S.; Shirakawa, M.; Nakabeppu, Y. A molecular basis for the selective recognition of 2-hydroxy-dATP and 8-oxo-dGTP by human MTH1. *J. Biol. Chem.* **277:**8579–8587; 2002.

[9] Fujikawa, K.; Kamiya, H.; Yakushiji, H.; Nakabeppu, Y.; Kasai, H. Human MTH1 protein hydrolyzes the oxidized ribonucleotide, 2-hydroxy-ATP. *Nucleic Acids Res.* **29:**449–454; 2001.

[10] Hayakawa, H.; Hofer, A.; Thelander, L.; Kitajima, S.; Cai, Y.; Oshiro, S.; Yakushiji, H.; Nakabeppu, Y.; Kuwano, M.; Sekiguchi, M. Metabolic fate of oxidized guanine ribonucleotides in mammalian cells. *Biochemistry* **38:**3610–3614; 1999.

[11] Fujikawa, K.; Yakushiji, H.; Nakabeppu, Y.; Suzuki, T.; Masuda, M.; Ohshima, H.; Kasai, H. 8-Chloro-dGTP, a hypochlorous acid-modified nucleotide, is hydrolyzed by hMTH1, the human MutT homolog. *FEBS Lett.* **512:**149–151; 2002.

[12] Takahashi, M.; Maraboeuf, F.; Sakai, Y.; Yakushiji, H.; Mishima, M.; Shirakawa, M.; Iwai, S.; Hayakawa, H.; Sekiguchi, M.; Nakabeppu, Y. Role of tryptophan residues in the recognition of mutagenic oxidized nucleotides by human antimutator MTH1 protein. *J. Mol. Biol.* **319:**129–139; 2002.

[13] Tsuzuki, T.; Egashira, A.; Kura, S. Analysis of MTH1 gene function in mice with targeted mutagenesis. *Mutat. Res.* **477:**71–78; 2001.

[14] Tsuzuki, T.; Egashira, A.; Igarashi, H.; Iwakuma, T.; Nakatsuru, Y.; Tominaga, Y.; Kawate, H.; Nakao, K.; Nakamura, K.; Ide, F.; Kura, S.; Nakabeppu, Y.; Katsuki, M.; Ishikawa, T.; Sekiguchi, M. Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase. *Proc. Natl. Acad. Sci. USA* **98:** 11456–11461; 2001.

[15] Egashira, A.; Yamauchi, K.; Yoshiyama, K.; Kawate, H.; Katsuki, M.; Sekiguchi, M.; Sugimachi, K.; Maki, H.; Tsuzuki, T. Mutational specificity of mice defective in the MTH1 and/or the MSH2 genes. *DNA Repair (Amst)* **1:**881–893; 2002.

[16] Bialkowski, K.; Kasprzak, K. S. A novel assay of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity. *Nucleic Acids Res.* **26:** 3194–3201; 1998.

[17] Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Natl. Cancer Inst.* **83:**757–766; 1991.

[18] Bialkowski, K.; Bialkowska, A.; Anderson, L. M.; Kasprzak, K. S. Higher activity of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) coincides with lower background levels of 8-oxo-2′-deoxyguanosine in DNA of fetal compared with maternal mouse organs. *Free Radic. Biol. Med.* **27:**90–94; 1999.

[19] Bialkowski, K.; Bialkowska, A.; Kasprzak, K. S. Cadmium(II), unlike nickel(II), inhibits 8-oxo-dGTPase activity and increases 8-oxo-dG level in DNA of the rat testis, a target organ for cadmium(II) carcinogenesis. *Carcinogenesis* **20:**1621–1624; 1999.

[20] Hayakawa, H.; Taketomi, A.; Sakumi, K.; Kuwano, M.; Sekiguchi, M. Generation and elimination of 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate, a mutagenic substrate for DNA synthesis, in human cells. *Biochemistry* **34:**89–95; 1995.

[21] Bialkowski K. Metabolism of 8-oxoguanine derivatives. Ph.D. dissertation, The Ludwik Rydygier Medical University, Bydgoszcz, Poland; 1997.

[22] Bialkowski, K.; Kasprzak, K. S. Activity of the antimutagenic enzyme 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) in cultured Chinese hamster ovary cells: effects of cell cycle, proliferation rate, and population density. *Free Radic. Biol. Med.* **28:**337–344; 2000.

[23] Kang, D.; Nishida, J.; Iyama, A.; Nakabeppu, Y.; Furuichi, M.; Fujiwara, T.; Sekiguchi, M.; Takeshige, K. Intracellular localization of 8-oxo-dGTPase in human cells, with special reference to the role of the enzyme in mitochondria. *J. Biol. Chem.* **270:** 14659–14665; 1995.

[24] Traut, T. W. Physiological concentrations of purines and pyrimidines. *Mol. Cell Biochem.* **140:**1–22; 1994.

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Karol Białkowski, Kazimierz S. Kasprzak (2000) Activity of the antimutagenic enzyme, 8-oxo-2'-deoxyguanosine 5'-triphosphate pyrophosphohydrolase (8-oxo-dGTPase), in cultured Chinese hamster ovary cells: Effects of cell cycle, proliferation rate, and population density, *Free Radical Biology & Medicine* 28: 337-344.

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Original Contribution

ACTIVITY OF THE ANTIMUTAGENIC ENZYME 8-OXO-2′-DEOXYGUANOSINE 5′-TRIPHOSPHATE PYROPHOSPHOHYDROLASE (8-OXO-dGTPase) IN CULTURED CHINESE HAMSTER OVARY CELLS: EFFECTS OF CELL CYCLE, PROLIFERATION RATE, AND POPULATION DENSITY

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Abstract—Mammalian 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolases (8-oxo-dGTPases), such as MTH1, are believed to play the same antimutagenic role as their bacterial homologues, like MutT. Both decompose promutagenic 8-oxo-dGTP, a product of active oxygen's attack on dGTP. It is not known how 8-oxo-dGTPase expression and function are regulated. Therefore, we investigated the effect of cell population density, proliferation rate, and cell cycle phase on 8-oxo-dGTPase specific activity in cultured Chinese hamster ovary K1-BH4 (CHO) cells. With increasing cell population density (from 30 to 95% confluence), the activity of 8-oxo-dGTPase per milligram protein decreased by 33% (p = .007 by ANOVA) while cells shifted by 9% into the G_0/G_1 phase, with a 5% drop in cells in S phase. Importantly, inhibition of the cells' proliferation rate by calf serum deprivation caused a more dramatic 23% shift toward the G_0/G_1 phase and a 25% drop in S phase, but had no effect on 8-oxo-dGTPase activity. Likewise, no differences in the enzyme activity were observed within cell populations of different cell cycle phases separated by centrifugal elutriation. Thus, the present results exclude cell cycle–dependent regulation of 8-oxo-dGTPase activity in CHO cells or its simple dependence on proliferation rate. The observed decrease of 8-oxo-dGTPase activity with increasing cell population density might be related to augmentation of cell-to-cell contact. © 2000 Elsevier Science Inc.

Keywords—8-oxo-dGTPase, Antimutagenic enzyme, Free radicals, MTH1, MutT, Nucleotide oxidative damage

INTRODUCTION

The *mutT*⁻ mutant strain of *Escherichia coli*, characterized by a 1000- to 10,000-fold increase in frequency of a specific A → C point mutation, was the first reported mutator organism [1,2]. The product of the wild-type *mutT* gene, an enzyme, has been initially identified as a pyrophosphatase that hydrolyzes canonical ribonucleoside and 2′-deoxyribonucleoside 5′-triphosphates to the corresponding nucleoside monophosphates, with a great preference for dGTP [3,4]. It was subsequently found, however, that this enzyme most effectively hydrolyzed the products of oxidative modification of dGTP and GTP, 8-oxo-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) [5] and 8-oxoguanosine 5′-triphosphate (8-oxo-GTP) [6,7]. These products may be generated by endogenous oxidants arising in normal cell metabolism and under oxidative stress. The high spontaneous mutation rate in *mutT*⁻ *E. coli* is thought to result from the lack of a functional 8-oxo-dGTP pyrophosphatase (8-oxo-dGTPase) that prevents incorporation of promutagenic 8-oxo-dGTP into nascent DNA [reviewed in ref. 8]. The mutagenicity of 8-oxo-dGTP is a consequence of the mispairing properties of 8-oxoguanine, which can be incorporated into DNA opposite cytosine or adenine [5,9–12]. The 8-oxo-G:A mispair may result in an A:T → C:G transversion [13–15]. *E. coli* MutT protein also prevents DNA transcription errors by hydrolyzing 8-oxo-GTP that could be otherwise misincorporated into nascent RNA [7].

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Mammalian homologues of MutT protein share a homology with the bacterial enzymes in terms of kinetic properties and amino acid sequences [16–20]. Eukaryotic 8-oxo-dGTPases have been believed, but not proven, to play the same antimutagenic role as MutT plays in bacteria. It is currently not known why some cell types and tissues (for example fetal and cancerous tissues) demonstrate higher expression of the 8-oxo-dGTPase gene, or how this expression is controlled. Does it depend on a particular tissue or cell type, cell proliferation rate, cell cycle phase, or all of these and other factors? Is this enzyme inducible, e.g., by oxidative stress? Acquiring information about the regulation of eukaryotic 8-oxo-dGTPase activity in relation to different physiological phases of the cell's life seems to be crucial. Therefore, we have investigated the influence of the cell population density, proliferation rate, and cell cycle phase on the specific activity of 8-oxo-dGTPase in cultured CHO cells.

MATERIALS AND METHODS

Chemicals

8-Oxo-2′-deoxyguanosine 5′-triphosphate and 5′-monophosphate were synthesized as described previously [17]. Ham's F-12 nutrient mixture, fetal bovine albumin, 200 mM glutamine solution, penicillin/streptomycin mixture, and 0.25% trypsin solution containing no calcium or magnesium were purchased from Biofluids, Inc. (Rockville, MD, USA). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and harvesting

CHO cells, variant K1-BH4, were maintained at 37°C in 225 cm^2 culture flasks, under air containing 5% CO_2, in 35 ml of Ham's F-12 nutrient mixture supplemented with 5% fetal bovine serum (unless specified otherwise), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). The cells were harvested by trypsinization. After removal of the medium, the cells were treated for 3 min with 5 ml of 0.25% trypsin solution, followed by 15 ml of Ham's complete medium. Detached cells were collected and washed three times with 30 ml of 20 mM Tris-buffered saline, each time with centrifugation at 1000 $\times$ g for 10 min at 4°C. If not used immediately for subsequent procedures, the cell pellets were stored at −70°C.

Cell cultures of different population density

CHO cells were subcultured in 20 flasks (1×10^6 cells/flask). Four cultures were harvested every 24 h, between 48 and 144 h after seeding. The growth medium in cultures intended for harvesting after 120 and 144 h was changed after 96 h to avoid possible starvation effects. The viability of cells was evaluated by the trypan blue exclusion assay. The cells from four flasks were pooled and counted with a hemocytometer. Approximately 1×10^6 cells were collected for flow cytometric analysis. The remaining cells were pelleted by centrifugation and used for determination of 8-oxo-dGTPase activity.

Reduction of cell proliferation rate

CHO cells were subcultured in eight flasks with 1×10^6 cells/flask. When the cultures reached ~70% confluence, the cells from four cultures were harvested, counted, and 1×10^6 cells from one of the cultures were taken for flow cytometry. 8-Oxo-dGTPase activity was measured separately in all four cell samples. The remaining four cultures were starved by removing the complete medium from the flasks and replacing it for the next 48 h with 35 ml of medium lacking fetal calf serum (FCS). The cells were further harvested and processed in the same way as nonstarved cells.

Centrifugal elutriation

Separation of cells at different phases of the cell cycle was accomplished by the elutriation method described by Grdina et al. [21] with some important modifications. The elutriation system consisted of a J6 Beckman elutriation centrifuge with a JE-5.0 rotor equipped with a single standard 5 ml elutriation chamber (Beckman Coulter, Inc., Fullerton, CA, USA), and variable flow L/S high precision Masterflex peristaltic pump (Barnant Company, Barrington, IL, USA). The elutriation medium, Tris-buffered saline (TBS), consisted of 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, maintained at 4°C. The speed and temperature of the rotor were set constant at 1790 rpm and 4°C. Approximately 3×10^8 CHO cells were harvested from cultures at ~80% confluence, washed three times with TBS, and finally suspended in 50 ml of TBS (4°C). Cell suspensions were introduced to the elutriation chamber at a flow rate of 9.4 ml/min. After collecting three 50 ml fractions, the flow rate was adjusted to 12.6 ml/min and then gradually increased to 30.6 ml/min in 1.8 ml/min increments. A 50 ml fraction was collected after each subsequent increase of the flow rate and kept on ice. Finally, the flow rate was set to 70 ml/min to remove all cell aggregates from the system. The cells in each fraction were counted and samples containing 1×10^6 cells were taken for flow cytometry. The remaining cells were pelleted by centrifugation and frozen at −70°C for the 8-oxo-dGTPase assay.

Flow cytometry

The cells (~1 × 10^6) were suspended in 0.6 ml of 20 mM phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS) and fixed by adding 1.4 ml cold absolute ethanol, while shaking, followed by incubation for at least 24 h at −20°C. Fixed cells were pelleted by centrifugation at 500 × *g* for 10 min at 4°C, resuspended in 200 μl PBS plus 200 μl of a mixture containing 0.1 mg/ml DNAse-free RNAse and 50 μg/ml propidium iodide. Cells were finally incubated in the dark at 25°C for 1 h prior to flow cytometric analysis. The propidium iodide–stained cell samples were assayed at 488 nm on an EPICS Profile flow cytometer (Coulter, Hialeah, FL, USA) equipped with an air-cooled 20 mW argon laser. A minimum of 10,000 cells was collected in each run. The cells were selected by pulse-height (doublet elimination) analysis and only the integrated signals were collected (to reject doublets). All histograms were evaluated by Multicycle software (advanced version, Phoenix Flow Systems, San Diego, CA, USA) [22]. The G_0/G_1 and G_2/M peaks were determined using Gaussian curves. Chi-square statistics were used to determine goodness-of-fit between the computer-generated curve and the raw data. Resolution of G_0/G_1 peaks was evaluated by coefficient of variation, which is calculated by dividing the half-peak width by the mean channel number. The S phase fraction was calculated by the area under the zero order polynomial curve between the mean channel number of the G_0/G_1 peak and that of the G_2/M peak. Guidelines from a consensus conference for DNA flow cytometry were followed throughout the study to control analytical variations [23].

Determination of 8-oxo-dGTPase activity

Typically, 1–5 × 10^7 cells were suspended in 0.4–0.6 ml of 20 mM Tris-HCl hypotonic buffer, pH 7.4, and homogenized on ice with a type B tight pestle (50 strokes) in a 2 ml Dounce homogenizer. The resulting homogenates were ultracentrifuged for 1 h at 100,000 × *g* (4°C) and the resulting supernatants, referred to below as "extracts," were collected. Total protein concentration was determined in each extract by the biuret method, using crystalline bovine serum albumin as a standard [24], and then made equal by diluting the extracts with 20 mM Tris-HCl, pH 7.4. Three 100 μl aliquots of each extract were ultrafiltered through a 30 kDa cut-off, low protein–binding ultrafiltration membrane (Ultrafree-MC Filtration Unit, Millipore, Bedford, MA, USA), by centrifugation at 3000 × *g* until complete passage of the sample (typically 20 min). The resulting ultrafiltrates were used for the determination of 8-oxo-dGTPase activity either immediately or after storage at −70°C for several days. The mean 8-oxo-dGTPase activity in the cell extract was calculated as described previously [17] from high-performance liquid chromatography (HPLC) determinations in three separate ultrafiltrates of the same extract. Enzyme activity was expressed in relation to total protein concentration in the extract. An activity unit was defined as the amount of enzyme converting 1 pmol of 8-oxo-dGTP to 8-oxo-dGMP per minute under the reaction conditions previously defined [17].

RESULTS

Effect of cell population density

CHO cell cultures harvested between 48 and 144 h after seeding exhibited an obvious increase in cell number (Fig. 1A) accompanied by a shift in cell distribution among the cell cycle phases (Fig. 1B). For example, at the beginning of the experiment, cell cultures harvested after 48 h of growth were approximately 30% confluent. In these cultures, 54% of cells were in the G_0/G_1 phase, 27% of cells were in S phase, and 19% of cells were in the G_2/M phase. At the end of the study, the cultures harvested after 144 h were approximately 95% confluent and had 63, 22, and 15% of cells in the G_0/G_1, S, and G_2/M phases, respectively (floating cells were not included). The cells harvested at all time points were viable as tested by the trypan blue exclusion assay.

With the time of growth and increasing cell population density, the specific activity of 8-oxo-dGTPase in CHO cell cultures tended to decrease (Fig. 1C). The activity dropped from (mean ± SE) 596 ± 24 U/mg protein at 48 h to 400 ± 33 U/mg protein at 144 h after inoculation of the cells. As tested by ANOVA analysis, the slope of the linear regression curve of the decrease was significant with $p = .007$.

Effect of starvation

CHO cells deprived of fetal calf serum (FCS) for 48 h significantly slowed their proliferation in comparison with control cells. During that time, the number of cells in starving cultures increased by only 32% (on average), while the number of cells cultured in the complete medium increased by at least 55%. Total protein recovered from the starving cultures increased by only 5%, whereas the complete medium allowed the cultures a 39% mean increase in the total protein content. At 48 h, 40.6% of cells in control cultures were in the S phase, compared with only 13% of cells

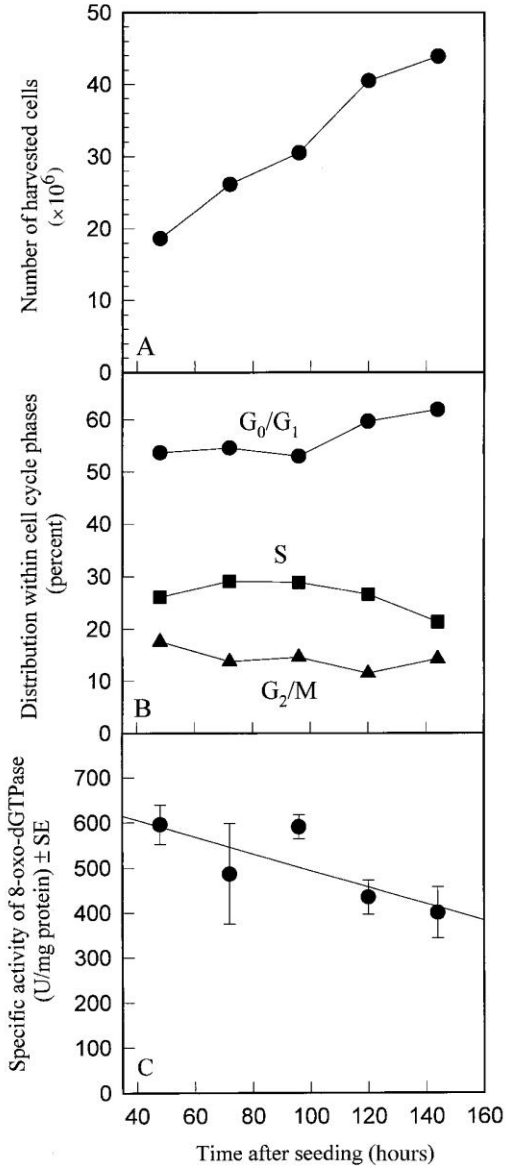

Fig. 1. Decreasing activity of 8-oxo-dGTPase with increasing confluence of CHO cells. CHO cells were harvested every 24 h between 48 and 144 h after inoculation of the cells. (A) Harvested cells were counted to demonstrate the dynamics of proliferation. Cell cultures were approximately 30 and 95% confluent at 48 and 144 h after seeding, respectively. (B) Distributions of cell cycle phases were determined by means of flow cytometric assay of cellular DNA content. (C) The activity of 8-oxo-dGTPase decreased with the time of growth (p = .007 by ANOVA). Error bars represent standard errors.

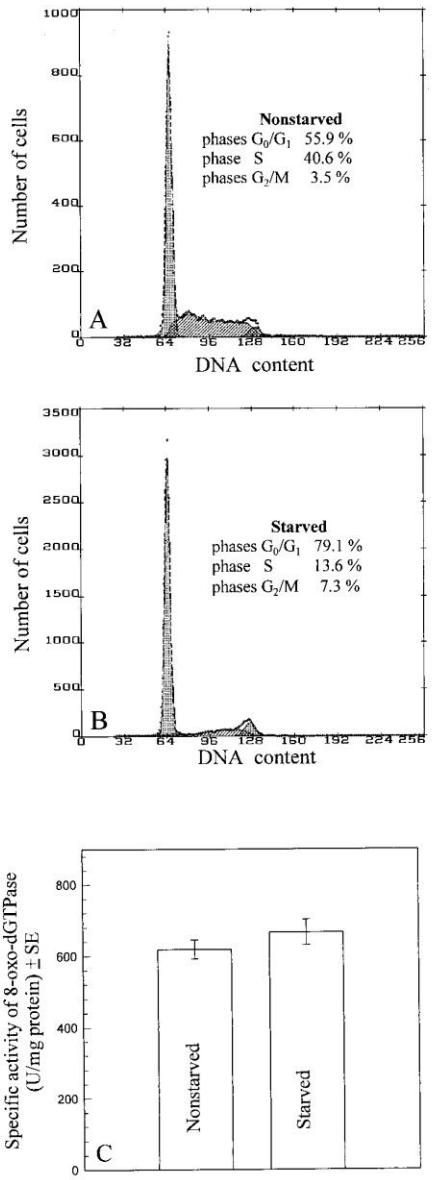

Fig. 2. Fetal calf serum starvation does not affect specific activity of 8-oxo-dGTPase in CHO cells. (A) Cells grown for 48 h in a medium lacking FCS had a substantially lower proliferation rate when compared with control cultures grown in the presence of 5% FCS. Viability of cells was not affected. (B) Flow cytometric analysis revealed a decrease in the proportion of cells in S phase and an increase in the proportion in G_0/G_1 phase in the starved cells vs. the control cells (A vs. B). (C) However, the quiescence that developed in the starved cells did not affect specific activity of 8-oxo-dGTPase.

in the starving cultures (Figs. 2A and 2B). The fraction of cells in G_0/G_1 increased from 56% in the control cultures to 79% in the starving cultures. Starvation did not affect cell viability. In contrast to these profound differences in protein and cell cycle phase, the activity of 8-oxo-dGTPase in the control and starved cells did not differ significantly as tested by the Student's t-test for unpaired samples (Fig. 2C).

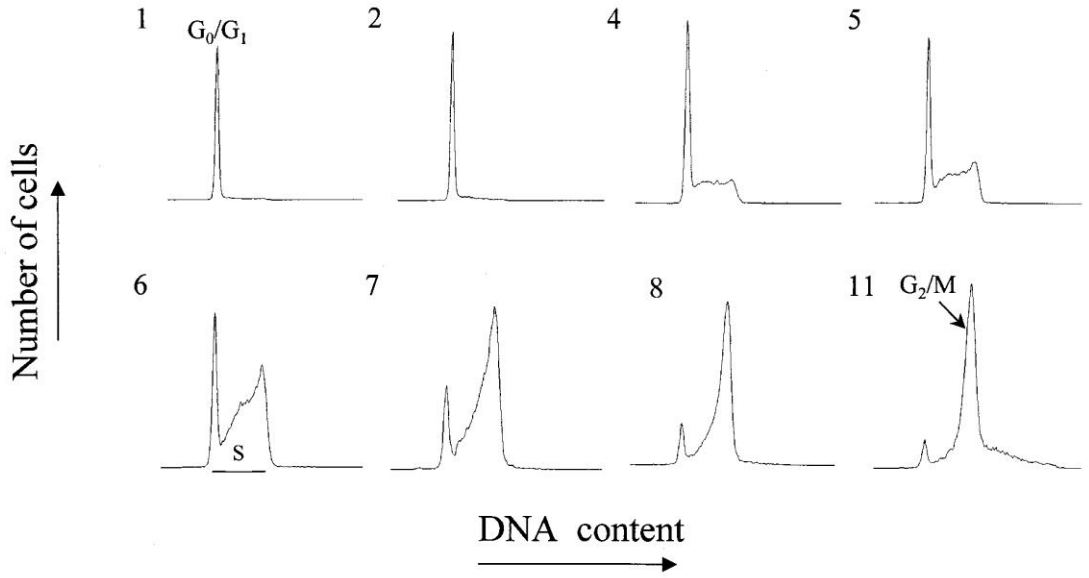

Fig. 3. Flow cytometric assay of DNA content in the fractions of CHO cells obtained by centrifugal elutriation method. Histograms of cell fractions selected for subsequent determination of 8-oxo-dGTPase activity. Fractions 1, 6, and 11 demonstrate a maximum enrichment in G_0/G_1, S, and G_2/M phase cells, respectively. The percentage of cells distributed at different phases among particular elutriation fractions is presented in Fig. 4A.

Effect of the cell cycle

The results of flow cytometric analysis of DNA content in the cell populations constituting elutriation fractions 1, 2, 4–8, and 11 are shown in Fig. 3. Fraction 1 exhibited a maximum 87% enrichment of cells in the G_0/G_1 phase; fraction 6 was maximally enriched in S phase, with 62% of cells in this phase; fraction 11 contained 77% of cells in G_2 and M phases (Fig. 4A). However, despite great differences in cell cycle distribution among these fractions, specific activities of 8-oxo-dGTPase, ranging from 481 ± 34 to 627 ± 39 U/mg of protein, appeared not to be statistically different from each other (Fig. 4), as judged by the Student's *t*-test for unpaired samples.

DISCUSSION

It is commonly believed that mammalian homologues of the bacterial MutT protein have the same function as the bacterial protein itself: to hydrolyze promutagenic 8-oxo-dGTP that may arise by oxidation of dGTP during normal cellular metabolism or under oxidative stress. Although 8-oxo-dGTP is indeed the most effective substrate for mammalian MTH1 protein (a homologue of *E. coli* MutT protein) identified thus far, an antimutagenic capacity of MTH1 in mammalian cells has not yet been proven. Some insight into the role(s) of this protein was acquired by investigating the processes of expression regulation of the *MTH1* gene. The results indicated that certain mammalian cell types contained much higher levels of MTH1 mRNA or 8-oxo-dGTPase activity than others did. For example, Jurkat cells, a human T-cell leukemia cell line, expressed more MTH1 mRNA than normal human lymphocytes [16]. Okamoto et al. [25] observed overexpression of human MTH1 mRNA in renal-cell carcinomas compared to the adjacent nontumorous renal tissue. Likewise, Wani et al. [26] reported higher level of MTH1 mRNA in human breast tumor than in normal breast ductal cells. Hibi et al. [27] found *MTH1* to be one of the most clearly overexpressed genes in human nonsmall cell lung cancer cells (relative to normal bronchial and tracheal epithelial cells). Kennedy et al. [28] observed the same phenomenon in different lung cancer cell lines in comparison with normal human bronchial epithelial cells.

The elevated expression of the *MTH1* gene in human tumors has been most frequently proposed to be due to the increased exposure of cancer tissues to endogenous reactive oxygen species. Some authors even suggest using the overexpression of human *MTH1* gene as a molecular marker of oxidative stress in lung cancer cells [25,28]. However, this suggestion still awaits an experimental confirmation. Although the promoter region of the murine *MTH1* gene contains putative Activator Protein-1 (AP-1) recognition sequences, and AP-1 is a re-

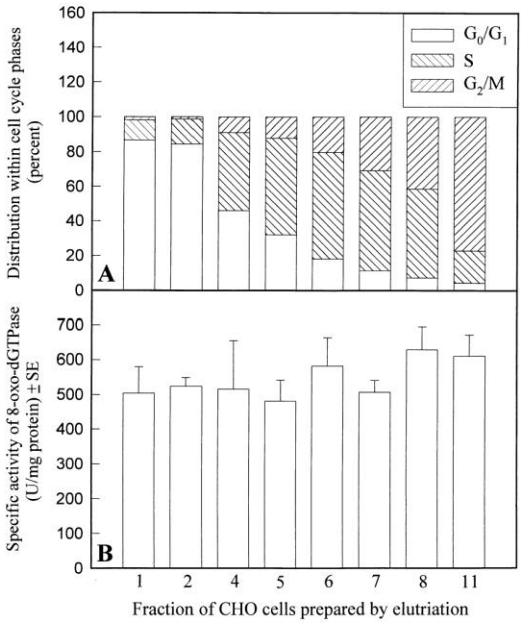

Fig. 4. Specific activity of 8-oxo-dGTPase is not cell cycle regulated in cultured CHO cells. (A) Centrifugal elutriation allowed for separation of CHO cell fractions differing in the proportion of cells in various cell cycle phases. (B) Analysis of 8-oxo-dGTPase activity in those fractions indicated no statistically significant change of the enzyme activity with regard to any particular cell cycle phase.

dox-regulated transcription activator, effects of AP-1 up-regulation or oxidative stress on *MTH1* expression have never been demonstrated. On the other hand, data concerning normal tissues under physiological conditions indicate possible dependence of the level of *MTH1* gene expression on proliferative activity of the tissue (or cultured cell line). Thus, Wani and D'Ambrosio [29] investigated the expression of human *MTH1* in normal skin and found a clear gradient of MTH1 mRNA in the epidermis, ranging from high levels in the replicating basal epithelial cells to undetectable levels in the non-mitotic suprabasal and granular epithelial cell layers. The levels of MTH1 mRNA in mouse organs, observed by Kakuma et al. [19], seemed to reflect differences in cell proliferation rates among those organs (with the exception of liver, where the level was unexpectedly high). Accordingly, the highest levels of this mRNA were found in the thymus and testis, and the lowest in the brain. The same is true for human testis and thymus [30]. The highest relative expression of *MTH1* was, however, noticed in mouse embryonic stem cells, known to have a very high proliferation rate [19,31]. Interestingly, Oda et al. [30] found that human fetal liver and brain displayed higher *MTH1* expression than the corresponding tissues of adult individuals; this pattern might also be associated with differences in proliferation rate. This phenomenon was confirmed by our previous study in which markedly higher specific activity of 8-oxo-dGTPase was observed in fetal lungs and livers, compared with the corresponding maternal organs [32]. The effect of cell proliferation stimulation on the expression of MTH1 mRNA was directly demonstrated by Oda et al. [30] who treated quiescent human lymphocytes with mitogens and observed a 6-fold increase in MTH1 mRNA level.

To further characterize the above phenomena, we studied the specific activity of 8-oxo-dGTPase in CHO cells. Many types of cultured cells become quiescent upon reaching confluence, and the CHO cells in our study were no exception. Under the present experimental conditions, CHO cells slowed proliferation as the cultures gradually became overcrowded, as signified by a 9% shift in cell distribution towards the G_0/G_1 phase, typical for quiescence. This shift was accompanied by a 33% decrease in 8-oxo-dGTPase activity. The potential of CHO cells to react properly to contact inhibition of cell proliferation is limited. This may be why the observed effect on 8-oxo-dGTPase activity is not as dramatic as the opposite effect of mitogens on 8-oxo-dGTPase mRNA level in lymphocytes [30]. Interestingly, the slowed proliferation and cell distribution shift toward the G_0/G_1 phase caused by serum starvation, which were even more extensive than those caused by increasing confluence, produced no significant change in 8-oxo-dGTPase activity. This difference suggests that some aspects of cell population density, i.e., cell-to-cell contact and metabolic cooperation (signaling), may play more important roles in downregulation of 8-oxo-dGTPase activity than do cell cycle phases or mere proliferation rate. Thus, inhibition of cell proliferation does not seem to be sufficient by itself to affect enzymatic activity of MTH1.

Many enzymes of nucleotide metabolism are cell cycle–regulated [reviewed in ref. 33]. Therefore, in the present study, the levels of 8-oxo-dGTPase activity have also been determined in cells of different phases of the cell cycle. Surprisingly, analysis of cell samples enriched in cells at the particular phases did not demonstrate any significant differences in the specific activity of the enzyme. Hence, the present results exclude regulation of 8-oxo-dGTPase activity through cell cycle-dependent signaling, at least in the CHO cells. They also indicate that activity of 8-oxo-dGTPase in proliferating cells is as important in the other phases as it is during DNA synthesis. This is consistent with the postulated physiological role of MTH1 protein in mammalian cells against the promutagenic 8-oxo-dGTP. To hydrolyze 8-oxo-dGTP, MTH1 must be fully functional not only during the DNA replication phase but also when DNA undergoes repair synthesis. This result is also consistent with the guardian role of this enzyme against 8-oxo-GTP to prevent tran-

scription errors in RNA synthesis over the entire cell cycle, as suggested by Taddei et al. [7].

The data presented in this article should help in future studies to distinguish between cell- and tissue-specific and cell proliferation–and cell cycle–dependent differences in 8-oxo-dGTPase activity among various cells and tissues, including normal and neoplastic tissues. They should also help determine whether 8-oxo-dGTPase activity can be induced by reactive oxygen species and if increased expression of the *MTH1* gene can really serve as a marker of oxidative stress. Possible effects of enhanced in vivo proliferation of normal mouse tissue, as well as those of ionizing radiation in CHO cells, on 8-oxo-dGTPase activity, are currently under investigation in our laboratory.

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REFERENCES

[1] Treffers, H. P.; Spinelli, V.; Belser, N. O. A factor (or mutator gene) influencing mutation rates in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **40:** 1064–1071; 1954.

[2] Yanofsky, C.; Cox, E. C.; Horn, V. The unusual mutagenic specificity of an *E. coli* mutator gene. *Proc. Natl. Acad. Sci. USA* **55:** 274–281; 1966.

[3] Bhatnagar, S. K.; Bessman, M. J. Studies on the mutator gene, mutT of *Escherichia*. Molecular cloning of the gene, purification of the gene product, and identification of a novel nucleoside triphosphatase. *J. Biol. Chem.* **263:** 8953–8957; 1988.

[4] Bhatnagar, S. K.; Bullions, L. C.; Bessman, M. J. Characterization of the mutT nucleoside triphosphatase of *Escherichia coli*. *J. Biol. Chem.* **266:** 9050–9054; 1991.

[5] Maki, H.; Sekiguchi, M. MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature* **355:** 273–275; 1992.

[6] Hayakawa, H.; Taketomi, A.; Sakumi, K.; Kuwano, M.; Sekiguchi, M. Generation and elimination of 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate, a mutagenic substrate for DNA synthesis, in human cells. *Biochemistry* **34:** 89–95; 1995.

[7] Taddei, F.; Hayakawa, H.; Bouton, M.; Cirinesi, A.; Matic, I.; Sekiguchi, M.; Radman, M. Counteraction by MutT protein of transcriptional errors caused by oxidative damage. *Science* **278:** 128–130; 1997.

[8] Fowler, R. G.; Schaaper, R. M. The role of the *mutT* gene of *Escherichia coli* in maintaining replication fidelity. *FEMS Microbiol. Rev.* **21:** 43–54; 1997.

[9] Grollman, A. P.; Moriya, M. Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet.* 9:246–249; 1993.

[10] Sekiguchi, M. MutT-related error avoidance mechanism for DNA synthesis. *Genes Cells* 1:139–145; 1996.

[11] Pavlov, Y. I.; Minnick, D. T.; Izuta, S.; Kunkel, T. A. DNA replication fidelity with 8-oxodeoxyguanosine triphosphate. *Biochemistry* **33:** 4695–4701; 1994.

[12] Kamath-Loeb, A. S.; Hizi, A.; Kasai, H.; Loeb, L. A. Incorporation of the guanosine triphosphate analogs 8-oxo-dGTP and 8-NH_2-dGTP by reverse transcriptases and mammalian DNA polymerases. *J. Biol. Chem.* **272:** 5892–5898; 1997.

[13] Tajiri, T.; Maki, H.; Sekiguchi, M. Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat. Res.* **336:** 257–267; 1995.

[14] Cheng, K. C.; Cahill, D. S.; Kasai, H.; Nishimura, S.; Loeb, L. A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G—T and A—C substitutions. *J. Biol. Chem.* **267:** 166–172; 1992.

[15] Inoue, M.; Kamiya, H.; Fujikawa, K.; Ootsuyama, Y.; Murata-Kamiya, N.; Osaki, T.; Yasumoto, K.; Kasai, H. Induction of chromosomal gene mutations in *Escherichia coli* by direct incorporation of oxidatively damaged nucleotides. *J. Biol. Chem.* **273:** 11069–11074; 1998.

[16] Mo, J. Y.; Maki, H.; Sekiguchi, M. Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. *Proc. Natl. Acad. Sci. USA* **89:** 11021–11025; 1992.

[17] Bialkowski, K.; Kasprzak, K. S. A novel assay of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity. *Nucleic Acids Res.* **26:** 3194–3201; 1998.

[18] Furuichi, M.; Yoshida, M. C.; Oda, H.; Tajiri, T.; Nakabeppu, Y.; Tsuzuki, T.; Sekiguchi, M. Genomic structure and chromosome location of the human mutT homologue gene MTH1 encoding 8-oxo-dGTPase for prevention of A:T to C:G transversion. *Genomics* **24:** 485–490; 1994.

[19] Kakuma, T.; Nishida, J.; Tsuzuki, T.; Sekiguchi, M. Mouse MTH1 protein with 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphatase activity that prevents transversion mutation. cDNA cloning and tissue distribution. *J. Biol. Chem.* **270:** 25942–25948; 1995.

[20] Cai, J. P.; Kakuma, T.; Tsuzuki, T.; Sekiguchi, M. cDNA and genomic sequences for rat 8-oxo-dGTPase that prevents occurrence of spontaneous mutations due to oxidation of guanine nucleotides. *Carcinogenesis* **16:** 2343–2350; 1995.

[21] Grdina, D. J.; Meistrich, M. L.; Meyn, R. E.; Johnson, T. S.; White, R. A. Cell synchrony techniques. I. A comparison of methods. *Cell Tissue Kinet.* **17:** 223–236; 1984.

[22] Kallioniemi, O. P.; Visakorpi, T.; Holli, K.; Isola, J. J.; Rabinovitch, P. S. Automated peak detection and cell cycle analysis of flow cytometric DNA histograms. *Cytometry* **16:** 250–255; 1994.

[23] Shankey, T. V.; Rabinovitch, P. S.; Bagwell, B.; Bauer, K. D.; Duque, R. E.; Hedley, D. W.; Mayall, B. H.; Wheeless, L.; Cox, C. Guidelines for implementation of clinical DNA cytometry. International Society for Analytical Cytology. *Cytometry* **14:** 472–477; 1993.

[24] Gornall, A. G.; Bardawill, C. J.; David, M. M. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:** 751–766; 1949.

[25] Okamoto, K.; Toyokuni, S.; Kim, W. J.; Ogawa, O.; Kakehi, Y.; Arao, S.; Hiai, H.; Yoshida, O. Overexpression of human *mutT* homologue gene messenger RNA in renal-cell carcinoma: evidence of persistent oxidative stress in cancer. *Int. J. Cancer* **65:** 437–441; 1996.

[26] Wani, G.; Milo, G. E.; D'Ambrosio, S. M. Enhanced expression of the *8-oxo-7,8-dihydrodeoxyguanosine triphosphatase* gene in human breast tumor cells. *Cancer Lett.* **125:** 123–130; 1998.

[27] Hibi, K.; Liu, Q.; Beaudry, G. A.; Madden, S. L.; Westra, W. H.; Wehage, S. L.; Yang, S. C.; Heitmiller, R. F.; Bertelsen, A. H.; Sidransky, D.; Jen, J. Serial analysis of gene expression in non-small cell lung cancer. *Cancer Res.* **58:** 5690–5694; 1998.

[28] Kennedy, C. H.; Cueto, R.; Belinsky, S. A.; Lechner, J. F.; Pryor, W. A. Overexpression of *hMTH1* mRNA: a molecular marker of oxidative stress in lung cancer cells. *FEBS Lett.* **429:** 17–20; 1998.

[29] Wani, G.; D'Ambrosio, S. M. Cell type-specific expression of human 8-oxo-7,8-dihydroguanosine triphosphatase in normal breast and skin tissues *in vivo*. *Carcinogenesis* **16:** 277–283; 1995.

[30] Oda, H.; Nakabeppu, Y.; Furuichi, M.; Sekiguchi, M. Regulation of expression of the human *MTH1* gene encoding 8-oxo-dGTPase. *J. Biol. Chem.* **272:** 17843–17850; 1997.

[31] Igarashi, H.; Tsuzuki, T.; Kakuma, T.; Tominaga, Y.; Sekiguchi, M. Organization and expression of the mouse *MTH1* gene for preventing transversion mutation. *J. Biol. Chem.* **272:** 3766–3772; 1997.

[32] Bialkowski, K.; Bialkowska, A.; Anderson, L. M.; Kasprzak, K. S. Higher activity of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) coincides with lower background levels of 8-oxo-2′-deoxyguanosine in DNA of fetal compared with maternal mouse organs. *Free Radic. Biol. Med.* **27:** 90–94; 1999.

[33] Kunz, B. A.; Kohalmi, S. E.; Kunkel, T. A.; Mathews, C. K.; McIntosh, E. M.; Reidy, J. A. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. *Mutat. Res.* **318:** 1–64; 1994.

8.

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Original Contribution

CELLULAR 8-OXO-7,8-DIHYDRO-2′-DEOXYGUANOSINE 5′-TRIPHOSPHATE PYROPHOSPHOHYDROLASE ACTIVITY OF HUMAN AND MOUSE MTH1 PROTEINS DOES NOT DEPEND ON THE PROLIFERATION RATE

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Abstract—Mammalian MTH1 proteins, homologs of *Escherichia coli* MutT, are enzymes decomposing 8-oxo-7,8-dihydro-2′deoxyguanosine 5′triphosphate (8-oxo-dGTP) to 8-oxo-7,8-dihydro-2′deoxyguanosine 5′monophosphate and inorganic pyrophosphate. They play an antimutagenic role by preventing the incorporation of promutagenic 8-oxo-dGTP into DNA. *MTH1* gene expression is higher in some physiological types of mammalian cells and in numerous cancer cells, but the mechanism of that upregulation still remains unclear. It has been hypothesized that *MTH1* expression might be associated with a proliferation rate of the cells. Therefore, we tested this hypothesis by comparing the functional levels of *MTH1* gene expression measured as the 8-oxo-dGTPase activity of its protein products in normal mouse livers and hepatectomized regenerating livers. Although the proliferation rate of the hepatocytes in the regenerating livers was much higher than that in control livers, as confirmed by immunohistochemical assay of proliferating cell nuclear antigen, the 8-oxo-dGTPase activity was not different. In a second approach, we used 57 lines of human cancer cells in which 8-oxo-dGTPase activity was measured and confronted with cell population doubling time. No significant correlations between 8-oxo-dGTPase activity and proliferation rate were observed within groups of six leukemia, eight melanoma, nine lung, seven colon, six central nervous system, six ovarian, eight renal, and seven breast cancer cell lines. Thus, we conclude that the *MTH1* expression manifested as the 8-oxo-dGTPase activity of its protein products in mammalian cells is not associated with proliferation rate. Our results will help in further testing of the hypothesis that *MTH1* overexpression may be a specific marker of carcinogenesis and/or oxidative stress.

Keywords—MTH1, 8-Oxo-2′deoxyguanosine 5′triphosphate, 8-Oxo-7,8-dihydro-2′deoxyguanosine 5′triphosphatase pyrophosphohydrolase, Antimutagenic enzyme, MutT homolog, Oxidative DNA damage, DNA repair, Cancer marker, Free radicals

INTRODUCTION

Mammalian MTH1 protein, a homolog of bacterial MutT protein, plays an important antimutagenic role in cells by preventing incorporation of oxidatively modified purine nucleotides into DNA during replication [reviewed in 1]. The most widely characterized function of mammalian MTH1 protein, and probably the most relevant to its antimutagenic properties, is its enzymatic activity toward a promutagenic 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP), the product of oxidative damage to 2′deoxyguanosine 5′triphosphate (dGTP), a substrate for DNA synthesis [reviewed in [2]. 8-Oxo-dGTP is thought to be generated in the cells by reactive oxygen species arising under physiological conditions and predominantly under oxidative stress. It is an erroneous substrate for many DNA polymerases [3–7]. Its mutagenicity results from mispairing properties of 8-oxo-7,8-dihydroguanine, which can pair with cytosine as effectively as with adenine [8,9]. Mammalian MTH1 protein decomposes 8-oxo-dGTP to the corresponding monophosphate (8-oxo-dGMP) and inorganic pyrophosphate [10–13]. Therefore, MTH1 was alternatively named 8-oxo-dGTP pyrophosphohydrolase, or simply 8-oxo-

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dGTPase. However, as several more substrates for human MTH1 protein, such as 8-oxo-dATP, 2-OH-dATP [14], 2-OH-ATP [15], and 8-Cl-dGTP [16], were discovered later, this enzyme recently acquired the more general name "oxidized purine nucleoside triphosphatase" [1].

As *mutT*$^-$ strain of *Escherichia coli* devoid of its 8-oxo-dGTPase activity exhibits a very strong mutator phenotype characterized by a high frequency of AT → CG transversions [17–20], the mammalian homologs of the *mutT* gene (*MTH1)* attracted the attention of carcinogenesis investigators. One of the models of cancer development assumes formation of a mutator cell in an early stage of cell transformation. A knockout mutation of the *MTH1* gene was anticipated to result in a mammalian mutator phenotype. However, such a mutation has not been observed to date in human hereditary nonpolyposis colorectal cancers [21], sporadic ovarian cancers [22], rat mammary carcinomas [23], human sporadic colorectal carcinomas [24], and multiple colorectal adenomas [25]. Surprisingly, instead of *MTH1* disruption, a characteristic overexpression of this gene has been noticed in different cancer cells and tissues as compared with their healthy counterparts (see Discussion) [23,26–31]. These observations have led to formulation of the hypothesis that *MTH1* overexpression is a marker of carcinogenesis. Because *MTH1* overexpression has been most frequently assigned to a state of persistent oxidative stress in cancer cells, it has also been proposed that such overexpression might be a marker of the oxidative stress itself [26,29,31]. However, before *MTH1* can be qualified as a cancer marker it is essential to learn whether its overexpression is triggered by the cell transformation process per se or is just a result of gaining some features by cancer cells that are not unique to cancer. As suggested previously, the proliferation rate of the cells might be such a factor [32]. In our previous paper we demonstrated that 8-oxo-dGTPase activity of MTH1 protein in Chinese hamster ovary (CHO) cells is not cell cycle-dependent and its level is not affected by inhibition of cell proliferation resulting from fetal calf serum starvation. On the other hand, we have observed a significant, progressive decrease in 8-oxo-dGTPase activity in CHO cultures approaching confluence. As the association between 8-oxo-dGTPase activity and the proliferation rate of cells could neither be confirmed nor excluded at that stage, we decided to further investigate the problem using an in vivo approach described in the present paper. Partial dissection of liver (partial hepatectomy) in rodents is known to stimulate a regeneration of the organ that involves proliferation of normally quiescent hepatocytes [reviewed in 33]. Therefore, we decided to use this model to test the possible dependence of 8-oxo-dGTPase activity on cell proliferation rate both in vivo, in regenerating mouse liver, and in vitro, in 57 human cancer cell lines. These cell lines are routinely used for drug screening by the Developmental Therapeutics Program at the National Cancer Institute, National Institutes of Health (Fredrick, MD, USA) (http://www.dtp.nci.nih.gov), and among other features, differ widely in their doubling times.

MATERIALS AND METHODS

Chemicals

8-Oxo-7,8-dihydro-2′deoxyguanosine 5′monophosphate and 5′triphosphate were synthesized as described previously [13]. All other chemicals of the highest available purity were bought from Sigma–Aldrich (St. Louis, MO, USA).

Animals and treatment

Twelve 8-week-old C57BL6 male mice were kept under pathogen-free conditions on corncob bedding, with 12-h fluorescent light/12-h dark cycle, at 24 ± 2°C and 50 ± 5% relative humidity. They were fed NIH-31 diet and had free access to tap water. Six mice were subjected to partial hepatectomy to stimulate regenerative proliferation of the hepatocytes, while the six remaining animals were sham-operated (control group). Animals were anesthetized with metafane. Thoracic and peritoneal regions of their bodies was shaved. A 1.5-cm-long vertical incision was made on the left side below the diaphragm to open the peritoneal cavity. The left lobe of the liver was withdrawn from the cavity and a sterile silk loop was placed over the left lobe to the base and pulled tight to cut the blood supply, and the lobe was excised leaving a 3 mm stump above the silk. The liver was placed back into the peritoneal cavity, the cavity was covered with 5-O dexon, and the skin was closed with 9 mm autoclips. After the incision was closed, mice were placed on a heating pad until recovery was complete. The mice constituting the control group were operated in the same way, with their livers, however, remaining untouched (sham operation).

On Day 3 after surgery, all animals were euthanized with carbon dioxide and their livers immediately collected. Five hundred milligram sections of the livers were fixed in 10% formalin and embedded in paraffin for immunohistochemical localization of proliferating cell nuclear antigen protein (PCNA), and the remaining tissue was frozen in liquid nitrogen for 8-oxo-dGTPase activity determination.

Immunohistochemical localization of PCNA in the liver

PCNA is a nonhistone nuclear protein whose expression is correlated with the mitotic activity of cells and is therefore used as a marker of cell proliferation [34]. The Mouse PCNA Staining Kit was purchased from Zymed

Laboratories, Inc. (San Francisco, CA, USA). The staining procedure followed Zymed protocol (http://www.zymed.com).

Determination of 8-oxo-dGTPase activity in liver tissues

8-Oxo-dGTPase activity was determined using a method developed by us previously [13, 35]. The livers (~500 mg) were homogenized on ice with 2 ml of cold 20 mM Tris–HCl, pH 7.4, in a motor-driven, 10 ml Potter-Elvehjem homogenizer. The homogenates were centrifuged for 15 min at 2500*g* (4°C). Primary supernatants were further ultracentrifuged for 1 h at 100,000*g* (4°C) in a fixed-angle Ti 50 rotor (Beckman Instruments, Fullerton, CA, USA). The resulting supernatants (1 ml), termed below as "extracts," were collected for further processing. Total protein concentrations in the extracts were determined in triplicate by the biuret method [36] using crystalline bovine serum albumin as a standard. The protein concentrations in all liver extracts were then equalized to 24 mg/ml with the homogenization buffer. Three 150-μl aliquots of each extract were ultrafiltered through 30 kDa cutoff, low-protein-binding regenerated cellulose membranes (Ultrafree-MC Filtration Units, Millipore, Bedford, MA, USA). This step separated the 8-oxo-dGTP pyrophosphohydrolase activity from the other interfering phosphohydrolases that decompose 8-oxo-7,8-dihydro-2′deoxyguanosine 5′tri-, di-, and monophosphate [37]. The determinations of 8-oxo-dGTPase activity in the ultrafiltrates were carried out in 0.5 ml capped polypropylene tubes, as follows. A 60 μl volume of the reaction solution, containing 40 μM 8-oxo-dGTP, 5 mM $MgCl_2$, 100 mM Tris–HCl (pH 8.5), and ultrafiltrate, was incubated at 37°C for 90 min. The reaction was initiated by addition of the ultrafiltrate (5 μl) and terminated with 20 μl of 50 mM Na_2EDTA (disodium ethylenediamine tetraacetate). Blank samples were prepared with Na_2EDTA added before the ultrafiltrate, followed by incubation at 37°C. The reaction solutions were analyzed by high-performance liquid chromatography with UV absorbance detection (HPLC-UV) for the amount of 8-oxo-dGMP formed. Our Waters HPLC (Waters Co., Milford, MA, USA) consisted of two pumps (Model 510), an autosampler (Model 717 plus), a UV–VIS photodiode array detector (Model 996), and a Luna C18(2) column (250 × 4.6 mm, 5 μm grain; Phenomenex, Torrance, CA, USA). The system was controlled by a Millennium^{32} Chromatography Manager (Waters Co.). Twenty microliter aliquots of the reaction mixtures was chromatographed isocratically with 100 mM NaH_2PO_4–NaOH buffer (pH 5.5)/ methanol (93/7), at a flow rate of 1 ml/min. Solutions of known concentrations of 8-oxo-dGMP, ranging from 1 to 20 μM, were used for calibration. For quantification of the reaction product, chromatograms acquired at 295 nm were integrated. The enzymatic activity unit (U) was defined as the amount of enzyme converting 1 pmol of 8-oxo-dGTP to 8-oxo-dGMP per minute under the above reaction conditions. The mean 8-oxo-dGTPase activity in the tissue extract was calculated from determinations of three separate ultrafiltrates of a given extract. To compare different organ extracts, their activities determined in ultrafiltrates were expressed as number of activity units per milligram of total protein contained in the corresponding extracts.

Determination of 8-oxo-dGTPase activity in cultured cells

Fifty-seven human cancer cell lines were used in this study that were routinely cultured by the Developmental Therapeutics Program at the National Cancer Institute, National Institutes of Health; among them were six leukemia (CCRF-CEM, HL-60(TB), K-562, MOLT-4, RPMI 8226, SR) and eight melanoma (LOX IMVI, MALME-3M, M14, SK-MEL-2, SK-MEL-5, SK-MEL-28, UACC-62, UACC-257) cell lines, as well as nine lung (A549/ATCC, EKVX, HOP-62, HOP-92, NCI-H23, NCI-H226, NCI-H322M, NCI-H460, NCI-H522), seven colon (COLO-205, HCC-2998, HCT-15, HCT-116, HT-29, KM-12, SW620), six central nervous system (SF-268, SF-295, SF-539, SNB-19, SNB-75, U251), six ovarian (IGROV1, SK-OV-3, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8), eight renal (786-0, A498, ACHN, CAKI-1, RXF-393, SN12C, TK-10, UO-31), and seven breast (MCF-7, NCI/ADR-RES, MDA-MB-231/ATCC, HS 578T, MDA-MB-435, BT-549, T-47D) cancer cell lines. The cells were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum and 2 mM l-glutamine as described elsewhere [38]. They were harvested by scraping and washed three times with 20 mM Tris–HCl, 150 mM NaCl, pH 7.4. A pellet of 6 to 8 million cells was suspended in 1 ml hypotonic 20 mM Tris–HCl buffer, pH 7.4, and homogenized on ice by ultrasonication for 10 s. The homogenate was ultracentrifuged for 1 h at 100,000*g* (4°C). One milliliter of the resulting supernatant (referred to as an "extract") located between the pellet and fatty upper layer, was gently collected. Total protein concentration in the extracts was determined by the biuret method [36] using crystalline bovine serum albumin as standard. Individual cell extracts were diluted with 20 mM Tris–HCl buffer, pH 7.4, so that their total protein concentrations were equalized to the level of 1 mg/ml. Four 100 μl portions of each extract were ultrafiltered at 4°C through 30 kDa cutoff, low-protein-binding regenerated cellulose membranes (Ultrafree-MC Filtration Units, Millipore, Bedford, MA, USA). The 8-oxo-dGTPase activity in all ultrafiltrates was determined essentially as described above except that the enzymatic reaction was carried

out for 50 min, and 30 μl aliquots of the reaction solution were analyzed. 8-Oxo-dGTPase activity reported for each cell line in the figures constitutes a mean value of determinations performed in four separate ultrafiltrates and expressed as activity units per milligram of protein in the original extract. Statistica 6.0 software (StatSoft Inc., Tulsa, OK, USA) was used for all statistical calculations.

RESULTS

PCNA levels in regenerating and control mouse livers

Immunohistochemical staining for PCNA in both liver groups 3 days after PH or sham surgery revealed a high, greater than 25-fold increase in the number of PCNA-positive nuclei in the regenerating livers as compared with those in sham-operated animals. Representative sections of the regenerating livers and the control livers immunostained for PCNA are shown in Fig. 1.

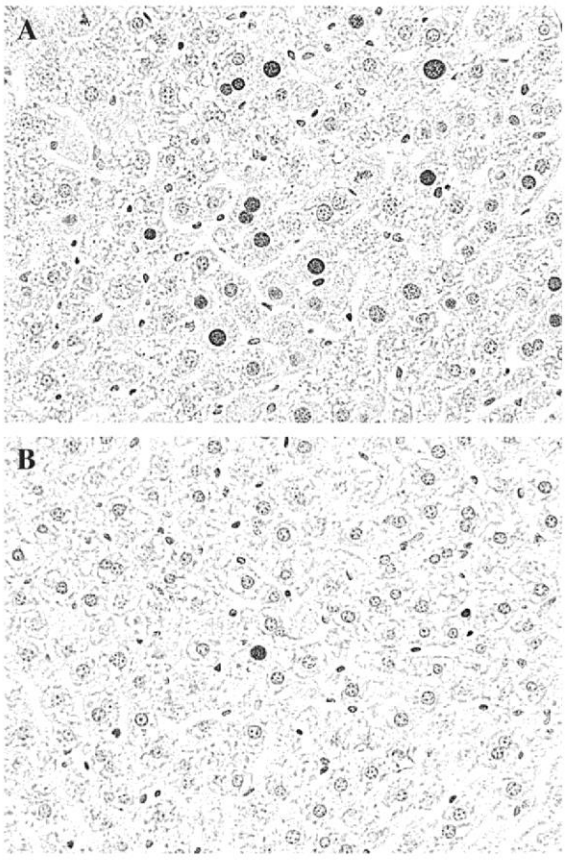

Fig. 1. Immunohiostochemical localization of proliferating cell nuclear antigen (PCNA) in mouse livers. PCNA-positive nuclei are the brown-stained cell nuclei clearly visible on the blue, hematoxylin-counter-stained, tissue background. Representative sections of mouse livers: (A) 3 days after partial hepatectomy, (B) 3 days after sham operation. Magnification, 750×.

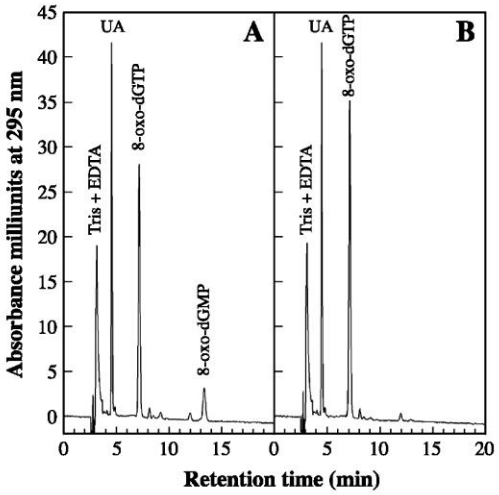

Fig. 2. HPLC-UV analysis of a typical 8-oxo-dGTPase assay reaction mixture prepared and chromatographed as specified under Materials and Methods. (A) The 8-oxo-dGMP peak represents the sole product of 8-oxo-dGTP hydrolysis catalyzed by the ≤30 kDa ultrafiltrate of mouse liver extract. (B) The 8-oxo-dGMP peak is absent in a blank sample prepared by adding Na_2EDTA to the 8-oxo-dGTP substrate solution before introduction of the ultrafiltrate. The large peak of uric acid (UA) and other minor peaks are produced by compounds contained in the ultrafiltrate of the tissue extract. Tris and EDTA, highly concentrated reagents present in the reaction mixture, elute unretained as a dead-volume peak.

8-Oxo-dGTPase activity in regenerating and control mouse livers

Activity of 8-oxo-dGTPase in livers of six control (sham-operated) mice ranged from 25.1 to 38.9 U/mg protein, thus showing marked individual differences with a mean value of 33.9 U/mg protein. 8-Oxo-dGTPase activity in six regenerating mouse livers ranged from 23.7 to 38.1 U/mg protein, showing a mean value of 29.5 U/mg protein. Typical HPLC-UV analysis of enzymatic reaction solution is presented in Fig. 2. The enzymatic activity data are presented in Fig. 3. For clarity, only mean values of three separate determinations are presented in the graph. Multiple analysis of variance (MANOVA) revealed that there was no significant difference between 8-oxo-dGTPase activity in both investigated groups.

8-Oxo-dGTPase activity in human cancer cell lines

Fifty-seven human cancer cell lines, representing leukemia, melanoma, lung, colon, central nervous system, ovarian, renal, and breast cancers, revealed substantial differences in their activities of 8-oxo-dGTPase that ranged from 395 to 5314 U/mg protein. Activities of the particular cell lines are not identified by name in this article as this study will be described and discussed in full detail in a separate article devoted to human 8-oxo-dGTPase activity as a potential marker of

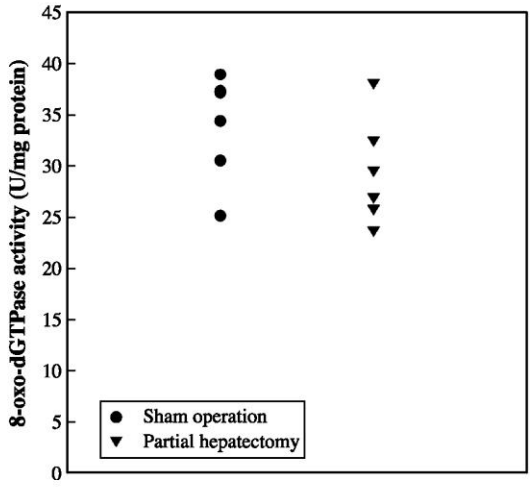

Fig. 3. 8-Oxo-dGTPase activity in livers of hepatectomized and sham-operated mice measured 3 days after the treatment. Each point represents mean activity level ($N = 3$) determined in individual livers.

carcinogenesis. Doubling times of the investigated cell lines ranged from 17.4 to 79.5 h. If all 57 cell lines are pooled, no correlation is observed between 8-oxo-dGTPase activity and proliferation rate of the cells (correlation coefficient $r = -.021$ at $p = .926$). The correlational analysis for both variables, however, is best done within groups of cancer cell types (Fig. 4) as opposed to all groups combined. The reason for this is obvious from the individual within-group correlations. Of the eight such correlations, five are positive and three are negative. They range from r values as high as .54 in the case of renal cancer cell lines to r values as low as $-.66$ for cancer cells originating from central nervous system. Given the wide range of within-group correlations and the high p value of the overall correlation, it is rather not judicious to pool them. Correlational analysis of individual cancer cell line groups reveals that none of the computed correlation coefficients (r) is high enough to imply a direct association between 8-oxo-dGTPase activity and doubling time of the cell lines (Fig. 4), and that none of those correlations is significant at the $p < .05$ level. We thus conclude that our data do not provide any evidence of a direct or inverse relationship between human 8-oxo-dGTPase activity and proliferation rate of the cells.

DISCUSSION

The results reported to date concerning physiological tissues indicated a possible association between the level of *MTH1* gene expression and the proliferative activity of tissue or particular cell type. Thus, Wani et al. [39] investigated the expression of human *MTH1* in normal skin and found a clear gradient of *MTH1* mRNA in the epidermis, ranging from a high level in the replicating basal epithelial cells to an undetectable level in the nonmitotic suprabasal and granular epithelial cell layers. The levels of *MTH1* mRNA in mouse organs, observed by Kakuma et al. [11], seemed to reflect differences in cell proliferation rates among those organs. Accordingly, the highest levels of this mRNA were found in actively proliferating organs like testis and thymus, and the lowest in brain, which is composed mostly of quiescent neurons. As demonstrated by Oda et al. [32], human testis and thymus also exhibit the highest *MTH1* mRNA levels among different human organs. The highest relative expression of *MTH1* gene was, however, noted in mouse embryonic stem cells, known to have a high

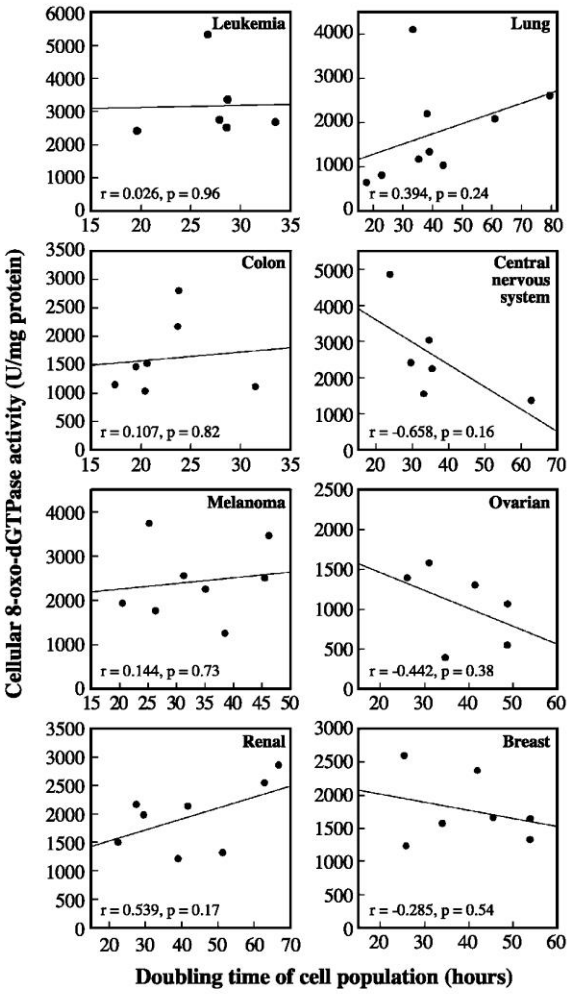

Fig. 4. 8-Oxo-dGTPase activity as a function of proliferation rate in different human cancer cell lines grouped according to cancer type. Each point represents the average of four determinations for each cell line sample. The lines were obtained by regression analysis. The correlation coefficients (r) and corresponding p values were calculated for each group of cell lines.

proliferation rate [11,40]. It was found that human fetal liver and brain displayed higher *MTH1* mRNA levels than the corresponding tissues of adult individuals [32], which might also reflect higher proliferation rate in young organs. This phenomenon was confirmed by our previous study in which markedly higher specific activity of 8-oxo-dGTPase was observed in fetal lungs and livers, compared with the corresponding maternal organs [35]. Most interestingly, Oda et al. [32] have shown a 6-fold increase in *MTH1* mRNA level in human lymphocytes after concomitant treatment with phytohemagglutinin and interleukin-2, which are well-known mitogens. That observation was strongly suggestive of a direct association between proliferation rate of the cells and *MTH1* gene expression. To further characterize the above phenomena, we previously studied the specific activity of 8-oxo-dGTPase in cultured CHO cells in the context of proliferation rate and cell cycle phase [41]. Changes in the proliferation rate of a cell population are associated with the concurrent changes in cell distribution within the cell cycle phases. As we have shown, 8-oxo-dGTPase activity is not cell cycle-dependent. Also, inhibition of proliferative activity of CHO cells by fetal calf serum starvation did not affect their 8-oxo-dGTPase activity. On the other hand, we observed a slight decrease (by 33%) in the activity in CHO cultures approaching confluence and becoming more quiescent. It suggested that some aspects of cell population density, i.e., cell-to-cell contact and metabolic cooperation (signaling), may play more important roles in down- or upregulation of 8-oxo-dGTPase activity than do cell cycle phases or mere proliferation rate. As our results were rather inconclusive at that stage, we decided to further investigate the influence of proliferation rate on mammalian *MTH1* gene expression, measured as a cellular 8-oxo-dGTPase activity of MTH1 protein. Although such activity does not necessarily mean the levels of mRNA and MTH1 protein expression, as determined in other studies, it provides data best reflecting the functional level of *MTH1* expression, i.e., the parameter that is most relevant to the biological role of this gene.

A model of regenerating liver seemed very attractive in terms of massive stimulation of the proliferation under in vivo conditions. Partial dissection of the liver is well known to trigger proliferative activity of almost all hepatocytes of the regenerating organ. During the regeneration process that follows 70% hepatectomy, most hepatocytes divide once or twice and return to quiescence [33]. A mouse liver regenerates to its normal mass within 8 days after surgical excision [42]. DNA synthesis reaches the maximal level 42 h after partial hepatectomy and the mitotic activity of the hepatocytes shows an increase 8 h after the DNA synthesis peak, i.e., 50 h after the hepatectomy [43]. In the present experiment, we allowed dissected livers to regenerate for 72 h to ensure upregulation of all potential downstream genes related to proliferative activity. The much larger numbers of PCNA-positive nuclei in regenerating liver hepatocytes (Fig. 1) indicates that at 72 h after hepatectomy, many cells were still synthesizing DNA and dividing to repopulate dissected organ. Meanwhile, 8-oxo-dGTPase activity of MTH1 protein in regenerating livers remained at the same level as in the control organs of sham-operated mice. This clearly shows that changes in the proliferative activity of the cells are not sufficient to affect cellular activity of the MTH1 protein. A similar experiment was performed by Takahashi et al. [44], who treated rats with carbon tetrachloride, a hepatotoxic compound that also initiates liver regeneration. These authors reported a slight increase in hepatic *MTH1* mRNA level 2 and 3 days after CCl_4 treatment. However, this increase was most likely caused by the massive infiltration of necrotic liver tissue by blood mononuclear cells that express *MTH1* more intensely than hepatocytes.

Besides some types of normal cells, also some cancer cells and/or tissues frequently show characteristic overexpression of the *MTH1 gene*. For example:

1. Okamoto et al. observed significantly higher levels of *MTH1* mRNA in the majority of 40 primary renal cell carcinomas than in adjacent nontumorous kidney tissues [26].
2. Wani et al. found that 30–85% of breast ductal carcinoma cells in 11 individual human breast tumors exhibited relatively high *MTH1* mRNA levels, in contrast with the undetectable level in normal breast ductal cells [28].
3. Hibi et al. reported a lack of detectable *MTH1* transcription in normal human lung tissue as opposed to easily measurable *MTH1* mRNA levels in 7 of 8 cultured non-small cell lung cancer cell lines and 6 of 12 primary lung tumors [27].
4. Kennedy et al. noted 1.3 to 2.8-fold higher expression of human *MTH1* mRNA in seven of eight investigated lung cancer cell lines compared with normal bronchial epithelial cell line [29].
5. A significantly higher level of human MTH1 protein was found by Kennedy at al. in 13 of 20 primary non-small cell lung carcinomas, as compared with the corresponding histologically normal surrounding tissues [31].
6. Iida et al. observed much higher MTH1 protein levels in four of five human autopsied brain tumors, as well as moderate to high MTH1 levels in 29 of 45 investigated postoperative brain tumors as compared with the surrounding normal brain tissues [45].

7. Nine rat mammary primary carcinomas were found by Okochi et al. to have higher MTH1 mRNA levels than those detected in three normal mammary glands [23].

However, whether or not the overexpression of *MTH1* gene measured at the mRNA or protein level always translates into an enzymatically functional protein remains an open question. Its answer requires more extensive comparative investigations of *MTH1* expression in the same cells, as measured at the mRNA, MTH1 protein, and 8-oxo-dGTPase activity levels, which we plan to pursue.

MTH1 upregulation is most often suggested to result from persistent oxidative stress which likely develops in cancer cells [26,28,29,31]. In other words, many investigators anticipate the existence of a cellular signaling mechanism that induces MTH1 expression in response to reactive oxygen species (ROS) to counteract the increased formation of oxidized purine nucleotides. Although a slight induction of *MTH1* expression by very high concentrations of hydrogen peroxide [46] and ROS-generating crocidolite asbestos [47] has been demonstrated, this kind of a feedback mechanism is still rather hypothetical and requires further, more direct experimental evidence. As long as we know nothing about the mechanism of this cancer-associated upregulation, different features of cancer cells should be considered as contributing to *MTH1* overexpression. As many cancer cells proliferate faster than the cells they originate from, it was likely that MTH1 could be a mere marker of proliferation rather than a marker of carcinogenesis and/or oxidative stress. As we have shown in this study, however, there is no correlation between MTH1 8-oxo-dGTPase activity and proliferation rate of 57 different human cancer cell lines. We believe that this conclusion is of primary importance for all investigators who try to relate *MTH1* gene expression to carcinogenesis and eventually turn it into a tumor marker.

According to Cai et al. [48], MTH1 may not be the only mammalian protein with 8-oxo-dGTP pyrophosphohydrolase activity. Based on gene homology, these authors identified mouse *MTH2* gene and then cloned in bacteria a 19.6 kDa MTH2 protein, having a K_m of 32 μM toward 8-oxo-dGTP. However, the level of MTH2 expression in mammalian cells, if any, was not determined. In comparison, the K_m value for human MTH1 8-oxo-dGTPase activity, 12 μM, was determined for pure protein [10] and found to be identical to that of cellular ultrafiltrates [49]. This could not be the case if MTH2 were also present in the ultrafiltrates. Therefore, the possible contribution of MTH2, if any, to the 8-oxo-dGTPase activity measured in the present experiment does not seem to be significant.

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REFERENCES

[1] Nakabeppu, Y. Molecular genetics and structural biology of human MutT homolog, MTH1. *Mutat. Res.* **477:**59–70; 2001.
[2] Sekiguchi, M.; Tsuzuki, T. Oxidative nucleotide damage: consequences and prevention. *Oncogene* **21:**8895–8904; 2002.
[3] Cheng, K. C.; Cahill, D. S.; Kasai, H.; Nishimura, S.; Loeb, L. A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G–T and A–C substitutions. *J. Biol. Chem.* **267:**166–172; 1992.
[4] Minnick, D. T.; Pavlov, Y. I.; Kunkel, T. A. The fidelity of the human leading and lagging strand DNA replication apparatus with 8-oxodeoxyguanosine triphosphate. *Nucleic Acids Res.* **22:** 5658–5664; 1994.
[5] Pavlov, Y. I.; Minnick, D. T.; Izuta, S.; Kunkel, T. A. DNA replication fidelity with 8-oxodeoxyguanosine triphosphate. *Biochemistry* **33:**4695–4701; 1994.
[6] Kamath-Loeb, A. S.; Hizi, A.; Kasai, H.; Loeb, L. A. Incorporation of the guanosine triphosphate analogs 8-oxo-dGTP and 8-NH2-dGTP by reverse transcriptases and mammalian DNA polymerases. *J. Biol. Chem.* **272:**5892–5898; 1997.
[7] Einolf, H. J.; Schnetz-Boutaud, N.; Guengerich, F. P. Steady-state and pre-steady-state kinetic analysis of 8-oxo-7,8-dihydroguanosine triphosphate incorporation and extension by replicative and repair DNA polymerases. *Biochemistry* **37:**13300–13312; 1998.
[8] Oda, Y.; Uesugi, S.; Ikehara, M.; Nishimura, S.; Kawase, Y.; Ishikawa, H.; Inoue, H.; Ohtsuka, E. NMR studies of a DNA containing 8-hydroxydeoxyguanosine. *Nucleic Acids Res.* **19:**1407–1412; 1991.
[9] Kouchakdjian, M.; Bodepudi, V.; Shibutani, S.; Eisenberg, M.; Johnson, F.; Grollman, A. P.; Patel, D. J. NMR structural studies of the ionizing radiation adduct 7-hydro-8-oxodeoxyguanosine (8-oxo-7H-dG) opposite deoxyadenosine in a DNA duplex: 8-oxo-7H-dG(syn).dA(anti) alignment at lesion site. *Biochemistry* **30:**1403–1412; 1991.
[10] Mo, J. Y.; Maki, H.; Sekiguchi, M. Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool. *Proc. Natl. Acad. Sci. USA* **89:**11021–11025; 1992.
[11] Kakuma, T.; Nishida, J.; Tsuzuki, T.; Sekiguchi, M. Mouse MTH1 protein with 8-oxo-7,8-dihydro-2′deoxyguanosine 5′triphosphatase activity that prevents transversion mutation. cDNA cloning and tissue distribution. *J. Biol. Chem.* **270:**25942–25948; 1995.
[12] Cai, J. P.; Kakuma, T.; Tsuzuki, T.; Sekiguchi, M. cDNA and genomic sequences for rat 8-oxo-dGTPase that prevents occurrence of spontaneous mutations due to oxidation of guanine nucleotides. *Carcinogenesis* **16:**2343–2350; 1995.
[13] Bialkowski, K.; Kasprzak, K. S. A novel assay of 8-oxo-2′deoxyguanosine 5′triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity. *Nucleic Acids Res.* **26:** 3194–3201; 1998.
[14] Fujikawa, K.; Kamiya, H.; Yakushiji, H.; Fujii, Y.; Nakabeppu, Y.; Kasai, H. The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein. *J. Biol. Chem.* **274:**18201–18205; 1999.
[15] Fujikawa, K.; Kamiya, H.; Yakushiji, H.; Nakabeppu, Y.; Kasai, H. Human MTH1 protein hydrolyzes the oxidized ribonucleotide, 2-hydroxy-ATP. *Nucleic Acids Res.* **29:**449–454; 2001.
[16] Fujikawa, K.; Yakushiji, H.; Nakabeppu, Y.; Masuda, M.; Ohshima, H.; Kasai, H. 8-Chloro-dGTP, a hypochlorous acid-modified nucleotide, is hydrolyzed by hMTH1, the human MutT homolog. *FEBS Lett.* **512:**149–151; 2002.

[17] Treffers, H. P.; Spinelli, V.; Belser, N. O. A. A factor (or mutator gene) influencing mutation rates in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **40:**1064–1071; 1954.
[18] Yanofsky, C.; Cox, E. C.; Horn, V. The unusual mutagenic specificity of an E. coli mutator gene. *Proc. Natl. Acad. Sci. USA* **55:**274–281; 1966.
[19] Schaaper, R. M.; Dunn, R. L. *Escherichia coli* mutT mutator effect during in vitro DNA synthesis: enhanced A.G replicational errors. *J. Biol. Chem.* **262:**16267–16270; 1987.
[20] Watanabe, T.; van Geldorp, G.; Najrana, T.; Yamamura, E.; Nunoshiba, T.; Yamamoto, K. Miscoding and misincorporation of 8-oxo-guanine during leading and lagging strand synthesis in Escherichia coli. *Mol. Gen. Genet.* **264:**836–841; 2001.
[21] Wu, C.; Nagasaki, H.; Maruyama, K.; Nakabeppu, Y.; Sekiguchi, M.; Yuasa, Y. Polymorphisms and probable lack of mutation in a human mutT homolog, hMTH1, in hereditary nonpolyposis colorectal cancer. *Biochem. Biophys. Res. Commun.* **214:**1239–1245; 1995.
[22] Takama, F.; Kanuma, T.; Wang, D.; Nishida, J. I.; Nakabeppu, Y.; Wake, N.; Mizunuma, H. Mutation analysis of the hMTH1 gene in sporadic human ovarian cancer. *Int. J. Oncol.* **17:**467–471; 2000.
[23] Okochi, E.; Ichimura, S.; Sugimura, T.; Ushijima, T. The absence of Mth1 inactivation and DNA polymerase kappa overexpression in rat mammary carcinomas with frequent A:T to C:G transversions. *Jpn. J. Cancer Res.* **93:**501–506; 2002.
[24] Halford, S. E.; Rowan, A. J.; Lipton, L.; Sieber, O. M.; Pack, K.; Thomas, H. J.; Hodgson, S. V.; Bodmer, W. F.; Tomlinson, I. P. Germline mutations but not somatic changes at the MYH locus contribute to the pathogenesis of unselected colorectal cancers. *Am. J. Pathol.* **162:**1545–1548; 2003.
[25] Sieber, O. M.; Lipton, L.; Crabtree, M.; Heinimann, K.; Fidalgo, P.; Phillips, R. K.; Bisgaard, M. L.; Orntoft, T. F.; Aaltonen, L. A.; Hodgson, S. V.; Thomas, H. J.; Tomlinson, I. P. Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in MYH. *N. Engl. J. Med.* **348:**791–799; 2003.
[26] Okamoto, K.; Toyokuni, S.; Kim, W. J.; Ogawa, O.; Kakehi, Y.; Arao, S.; Hiai, H.; Yoshida, O. Overexpression of human mutT homologue gene messenger RNA in renal-cell carcinoma: evidence of persistent oxidative stress in cancer. *Int. J. Cancer* **65:**437–441; 1996.
[27] Hibi, K.; Liu, Q.; Beaudry, G. A.; Madden, S. L.; Westra, W. H.; Wehage, S. L.; Yang, S. C.; Heitmiller, R. F.; Bertelsen, A. H.; Sidransky, D.; Jen, J. Serial analysis of gene expression in non-small cell lung cancer. *Cancer Res.* **58:**5690–5694; 1998.
[28] Wani, G.; Milo, G. E.; D'Ambrosio, S. M. Enhanced expression of the 8-oxo-7,8-dihydrodeoxyguanosine triphosphatase gene in human breast tumor cells. *Cancer Lett.* **125:**123–130; 1998.
[29] Kennedy, C. H.; Cueto, R.; Belinsky, S. A.; Lechner, J. F.; Pryor, W. A. Overexpression of hMTH1 mRNA: a molecular marker of oxidative stress in lung cancer cells. *FEBS Lett.* **429:**17–20; 1998.
[30] Liu, Z.; Wang, L. E.; Strom, S. S.; Spitz, M. R.; Babaian, R. J.; DiGiovanni, J.; Wei, Q. Overexpression of hMTH in peripheral lymphocytes and risk of prostate cancer: a case–control analysis. *Mol. Carcinog.* **36:**123–129; 2003.
[31] Kennedy, C. H.; Pass, H. I.; Mitchell, J. B. Expression of human MutT homologue (hMTH1) protein in primary non-small-cell lung carcinomas and histologically normal surrounding tissue. *Free Radic. Biol. Med.* **34:**1447–1457; 2003.
[32] Oda, H.; Nakabeppu, Y.; Furuichi, M.; Sekiguchi, M. Regulation of expression of the human MTH1 gene encoding 8-oxo-dGTPase: alternative splicing of transcription products. *J. Biol. Chem.* **272:**17843–17850; 1997.
[33] Fausto, N. Liver regeneration. *J. Hepatol.* **32:**19–31; 2000.
[34] Linden, M. D.; Torres, F. X.; Kubus, J.; Zarbo, R. J. Clinical application of morphologic and immunocytochemical assessments of cell proliferation. *Am. J. Clin. Pathol.* **97:**S4–S13; 1992.
[35] Bialkowski, K.; Bialkowska, A.; Anderson, L. M.; Kasprzak, K. S. Higher activity of 8-oxo-2′deoxyguanosine 5′triphosphate pyrophosphohydrolase (8-oxo-dGTPase) coincides with lower background levels of 8-oxo-2′deoxyguanosine in DNA of fetal compared with maternal mouse organs. *Free Radic. Biol. Med.* **27:**90–94; 1999.
[36] Gornall, A. G.; Bardawill, C. J.; David, M. M. Determination of serum proteins by means of biuret reaction. *J. Biol. Chem.* **177:**751–766; 1949.
[37] Kasprzak, K. S.; Bialkowski, K. Inhibition of antimutagenic enzymes, 8-oxo-dGTPases, by carcinogenic metals: recent developments. *J. Inorg. Biochem.* **79:**231–236; 2000.
[38] Monks, A.; Scudiero, D.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J. Natl. Cancer Inst.* **83:**757–766; 1991.
[39] Wani, G.; D'Ambrosio, S. M. Cell type-specific expression of human 8-oxo-7,8-dihydroguanosine triphosphatase in normal breast and skin tissues in vivo. *Carcinogenesis* **16:**277–283; 1995.
[40] Igarashi, H.; Tsuzuki, T.; Kakuma, T.; Tominaga, Y.; Sekiguchi, M. Organization and expression of the mouse MTH1 gene for preventing transversion mutation. *J. Biol. Chem.* **272:**3766–3772; 1997.
[41] Bialkowski, K.; Kasprzak, K. S. Activity of the antimutagenic enzyme 8-oxo-2′deoxyguanosine 5′triphosphate pyrophosphohydrolase (8-oxo-dGTPase) in cultured Chinese hamster ovary cells: effects of cell cycle, proliferation rate, and population density. *Free Radic. Biol. Med.* **28:**337–344; 2000.
[42] Greene, A. K.; Wiener, S.; Puder, M.; Yoshida, A.; Shi, B.; Perez-Atayde, A. R.; Efstathiou, J. A.; Holmgren, L.; Adamis, A. P.; Rupnick, M.; Folkman, J.; O'Reilly, M. S. Endothelial-directed hepatic regeneration after partial hepatectomy. *Ann. Surg.* **237:**530–535; 2003.
[43] Furnus, C. C.; Inda, A. M.; Andrini, L. B.; Garcia, M. N.; Garcia, A. L.; Badran, A. F.; Errecalde, A. L. Chronobiology of the proliferative events related to angiogenesis in mice liver regeneration after partial hepatectomy. *Cell Biol. Int.* **27:**383–386; 2003.
[44] Takahashi, S.; Hirose, M.; Tamano, S.; Ozaki, M.; Orita, S.; Ito, T.; Takeuchi, M.; Ochi, H.; Fukada, S.; Kasai, H.; Shirai, T. Immunohistochemical detection of 8-hydroxy-2′deoxyguanosine in paraffin-embedded sections of rat liver after carbon tetrachloride treatment. *Toxicol. Pathol.* **26:**247–252; 1998.
[45] Iida, T.; Furuta, A.; Kawashima, M.; Nishida, J.; Nakabeppu, Y.; Iwaki, T. Accumulation of 8-oxo-2′-deoxyguanosine and increased expression of hMTH1 protein in brain tumors. *Neuro.-oncol.* **3:**73–81; 2001.
[46] Meyer, F.; Fiala, E.; Westendorf, J. Induction of 8-oxo-dGTPase activity in human lymphoid cells and normal fibroblasts by oxidative stress. *Toxicology* **146:**83–92; 2000.
[47] Kim, H. N.; Morimoto, Y.; Tsuda, T.; Ootsuyama, Y.; Hirohashi, M.; Hirano, T.; Tanaka, I.; Lim, Y.; Yun, I. G.; Kasai, H. Changes in DNA 8-hydroxyguanine levels, 8-hydroxyguanine repair activity, and hOGG1 and hMTH1 mRNA expression in human lung alveolar epithelial cells induced by crocidolite asbestos. *Carcinogenesis* **22:**265–269; 2001.
[48] Cai, J. P.; Ishibashi, T.; Takagi, Y.; Hayakawa, H.; Sekiguchi, M. Mouse MTH2 protein which prevents mutations caused by 8-oxoguanine nucleotides. *Biochem. Biophys. Res. Commun.* **305:**1073–1077; 2003.
[49] Bialkowski, K.; Kasprzak, K. S. Inhibition of 8-oxo-2′deoxyguanosine 5′triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity of the antimutagenic human MTH1 protein by nucleoside 5′diphosphates. *Free Radic. Biol. Med.* **35:**595–602; 2003.

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Review

Oxidative DNA damage in cancer patients: a cause or a consequence of the disease development?

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Abstract

A wide variety of oxidative DNA lesions are present in living cells. One of the best known lesions of this type is 8-oxoguanine (8-oxoGua) which has been shown to have mutagenic properties. An influence of antioxidative vitamins and labile iron pool on the background level of 8-oxoGua in cellular DNA is discussed and oxidative damage to free nucleotide pool as a possible source of 8-oxo-2′-deoxyguanosine in DNA and urine is described. An involvement of 8-oxoGua in the origin and/or progression of cancer is reviewed. It is concluded that a severe oxidative stress manifested as a high level of 8-oxoGua in cellular DNA as well as in urine of cancer patients is a consequence of development of many types of cancer. Although at present it is impossible to answer directly the question concerning involvement of oxidative DNA damage in cancer etiology it is likely that oxidative DNA base modifications may serve as a source of mutations that initiate carcinogenesis (i.e. they may be causal factors responsible for the process).

Keywords: Cancer; Oxidative DNA damage; MTH1

1. Introduction

In living cells, there is a steady formation of DNA lesions. A substantial number of these lesions are formed by endogenous factors that damage DNA on a continuous basis. These include reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) derived from oxidative respiration. Free radical attack upon DNA generates a whole series of DNA damage, among them modified DNA bases. Hydroxyl radical causes the formation of a large number of pyrimidine- and purine-derived lesions in DNA (reviewed in [1]). Some of these modified DNA bases have considerable potential to damage the integrity of the genome (reviewed in [2,3]). 8-Oxo-7,8-dihydroguanine (8-oxoGua) is one of the most widely studied lesions. The presence of 8-oxoGua residues in DNA leads to GC → TA transversions unless repaired prior to DNA replication [4]. Therefore, the presence of 8-oxoGua in cells may lead to point mutations.

Moreover, many observations indicate a direct correlation between 8-oxoGua formation and carcinogenesis in vivo [2,5] and that oxygen-derived radicals are known to induce mutagenesis in hotspot codons of the human *p53* and *Ha-ras* genes [6,7]. Therefore, the

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background oxidative damage to DNA may be critical to the appreciation of the importance of the oxidative stress in the development of cancer.

This article reviews recent data concerning involvement of oxidative DNA damage, particularly 8-oxoGua, in the initiation and/or progression of cancer.

2. Analyses of 8-oxoGua in human samples

The background level of 8-oxoGua in cellular DNA represent a dynamic equilibrium between rate of oxidative DNA damage and rate of repair of the damage in that specific tissue/cells. That repair enzymes which specifically recognize and remove 8-oxoGua evolved is a clear indication of the biological meaning of the lesion, but exactly how much 8-oxoGua is present at the background level is the subject of controversy. The European Standards Committee on Oxidative DNA Damage (ESCODD) was set up to resolve the problems associated with the measurement of background levels of oxidative DNA damage (in particular 8-oxoGua) in human cells. As a result of this endeavors the analyses have become more precise and accurate [8,9].

Alternative approach to asses oxidative DNA damage is the measurement of urinary excretion of 8-oxoGua and 8-oxodG (8-oxo-7,8-dihydro-2′-deoxyguanosine). It is generally accepted that the products of repair of 8-oxoGua in cellular DNA are excreted into the urine without further metabolism [10,11]. Some data suggest that 8-oxodG may derive from nucleotide excision repair (NER) [12–14]. However, oxidatively damaged DNA bases are mostly repaired by the base excision repair (BER) pathway although the NER pathway may also play a role in the repair of some oxidized DNA bases [15,16]. Therefore, simultaneous determination of the level of 8-oxodG as well as 8-oxoGua in urine, may better reflect oxidative damage of cellular DNA.

The analysis of 8-oxoGua in urine presents particular difficulties [17] and until recently there has been no reliable assay for its detection. Recently, new techniques based on mass spectrometric detection (MS) of the analytes were developed which allowed for simultaneous determination of 8-oxodG and 8-oxoGua in the same urine sample [18–20]. One of the methods involved HPLC prepurification followed by gas chromatography with isotope dilution MS detection [18,19]. Using this method we have found that urinary excretion of 8-oxoGua and 8-oxodG does not depend on diet in the case of humans and may reflect involvement of different repair mechanisms (respectively BER and NER) [19]. We cannot entirely exclude a possibility that other than repair processes can contribute to 8-oxoGua and 8-oxodG level in human urine, e.g. 8-oxodG may derives from dead cells [21]. On the other hand, the excretion of 8-oxoGua may also include a contribution from oxidized RNA. However, this possibility is less likely for the following reasons:

(i) No enzymatic activity which can recognize and liberate 8-oxoGua from RNA has been demonstrated up to date.
(ii) It is also unlikely that 8-oxoGua can be liberated from RNA *via* spontaneous hydrolysis of *N*-glycosylic bond of the nucleoside (depurination) since it was demonstrated that the bond is very stable (much more stable than in unmodified nucleoside) [22].

3. Endogenous factors that shape the background level of 8-oxoGua in cellular DNA and their relevance to carcinogenesis

3.1. Antioxidant vitamins, uric acid versus 8-oxoGua level in cellular DNA

Many epidemiological studies have reported an inverse association between vegetable and fruit consumption and occurrence of cancer and other degenerative diseases [23–25]. One of the possible mechanisms of this protective effect is by exerting the antioxidative activities of such plant food constituents as Vitamins A, C and E. These antioxidant vitamins are effective free radical scavengers. Therefore, they should protect biomolecules such as proteins, lipids and nucleic acids from oxidative damage. Another effective scavenger of ROS is uric acid [26]. Uric acid at physiological concentration is regarded as a main antioxidant and not only does it efficiently scavenge free radicals but it has also been shown to stabilize ascorbic acid in human serum [27] and reduce consumption of α-tocopherol and β-carotene [26].

Table 1
Biomarkers of oxidative stress in the study groups, data from [114]

	Mean ± S.D.		*P*-value (cancer vs. control)
	Colon cancer group ($n = 45$)	Control group ($n = 55$)	
Plasma ascorbic acid concentration (μM)	29.45 ± 27.41	49.76 ± 29.24	0.0006[a]
Plasma α-tocopherol concentration (μM)	18.87 ± 14.50	24.69 ± 14.55	0.0490[a]
Plasma retinol concentration (μM)	0.80 ± 0.75	1.23 ± 0.61	0.0021[a]
Plasma uric acid concentration (mg/dl)	3.73 ± 1.32	4.28 ± 1.13	0.0280[a]
8-oxodGuo/10^6 dG in lymphocytes	13.76 ± 7.19	9.57 ± 3.95	0.0034[a]

[a] Statistically significant differences (Student's *t*-test, $P < 0.05$).

One of the possible mechanisms of protective effect of antioxidant vitamins against cancer development may be by decreasing the amount of potentially mutagenic oxidatively modified DNA bases. Duthie et al. [28] using single cell gel electrophoreses (comet assay) found that supplementation of healthy volunteers with Vitamin C (100 mg per day) Vitamin E (280 mg per day) and β-carotene (25 mg per day) significantly reduced base damage in lymphocyte DNA. Collins et al. [29] demonstrated significant negative correlation between basal concentration of serum carotenoids and oxidatively modified pyrimidines. Supplementation of patients with carotenoids did not influence oxidative DNA damage. The authors did not find any correlation between the damage and concentration of Vitamins E and C. Another studies investigating oxidative DNA damage after supplementation with carotenoids, Vitamin C or E demonstrated no effect (for detailed discussion of this subject, see [30]). Therefore, at present it is difficult to reach firm conclusion whether supplementation of antioxidant vitamins protects against oxidative DNA damage. However, it is also possible that a preventive effect of the vitamins can be only seen when their basal level is very low possibly due to severe oxidative stress. Indeed, we demonstrated that the supplementation of HIV infected patients, who had very low levels of antioxidant vitamins and significantly increased amount of 8-oxoGua (and other base modifications), resulted in the restoration of the vitamin levels characteristic for the control subjects. Vitamin supplementation resulted in the significant decrease in the levels of the modified bases as compared to the patients who received placebo [31].

In another study, we have found that the endogenous levels of the analyzed antioxidant vitamins in plasma of colon cancer patients were significantly lower than that in the control group (Table 1) [32]. The simple explanation of this finding might be differences in feeding habits between both studied groups. However, it is noteworthy that the members of the studied groups were chosen randomly and, according to the interview, the groups were consisted of in such a way that the members of the both could match feeding habits and living conditions. Therefore, it is rather unlikely that the different concentration of vitamins in their blood was a result of lifestyle. Presumably, severe oxidative stress, characteristic for colon cancer, resulting in the production of ROS is responsible for consumption of the antioxidant vitamins. The decreased amount of uric acid in blood plasma of colon cancer patients also supports this assumption (Table 1). This prooxidative environment resulted in increased amount of 8-oxodG in lymphocytes DNA of cancer patients. The level of this lesion in DNA of colon cancer patients was significantly higher ($P = 0.0034$) than in DNA of control group [31]. These findings suggest that oxidative stress may be characteristic not only for the diseased tissue but for some other tissues of the cancer patients (for further discussion of this issue, see Section 5.2).

Our results suggest that people with advanced stages of cancer may be helped by treatment with antioxidants. Supplementation with antioxidants may slow dawn the progression of the disease.

However, we cannot entirely exclude that the vitamins are surrogate markers for other factors and aspects of healthy lifestyle.

4. Iron as a possible cause of 8-oxoGua accumulation

As it was mentioned above there are several studies, which have demonstrated that antioxidant

vitamins could decrease the amount of 8-oxodG in cellular DNA. On the other hand, almost nothing is known about endogenous oxidants which may intensify oxidative stress and raise the background level of 8-oxodG in cellular DNA. One such important element in the establishment of pro-oxidative status of the cell is iron.

Iron has the capacity to accept and donate electrons easily, changing between ferric (Fe^{2+}) and ferrous (Fe^{3+}) iron. Due to this feature, it is useful component of cytochroms and oxygen binding molecules like hemoglobin and myoglobin. However, inside the cell iron can exist in another form, as a "free" or "labile" iron (LIP, iron not bound to proteins). LIP-associated iron is in dynamic equilibrium with other sequestered iron forms in the cell and is bound to cytosolic low molecular weight ligands that have not yet been identified. This iron form is catalytically active and participates in the reaction involved in the production of harmful ROS (the Fenton reaction) [33]. Therefore, proteins sequester iron to reduce this threat. Iron ions circulate bound to plasma transferrin whereas ferritin serves to accumulate it. In our study, we analyzed the broad spectrum of the components that affect iron metabolism as well as their possible association with the endogenous level of 8-oxodG [34,35]. No correlation has been found between plasma concentration of ferritin or transferrin saturation and the amount of 8-oxodG in the DNA of lymphocytes [35]. On the other hand, a positive correlation has been determined between LIP and the oxidatively modified nucleoside (Fig. 1) [35]. This in turn suggests that under physiological conditions LIP is available for catalyzing the Fenton type reaction in a close proximity to cellular DNA. However, neither the exact chemical nature of the complex between iron and DNA is known, nor it is established how iron can get into the nucleus.

There are experimental data which demonstrate the existence of free iron pool in sera of patients with hemochromatosis [36]. This disease predisposes to cancer. Epidemiological data also indicate that elevation of the body iron level may increase the risk of cancer [37,38]. Our results suggest a mechanism that may directly link iron overload and carcinogenesis. Specifically, iron overload may favor the persistence of harmful LIP, which may catalyze generation of the potentially carcinogenic 8-oxodG moiety in cellular DNA.

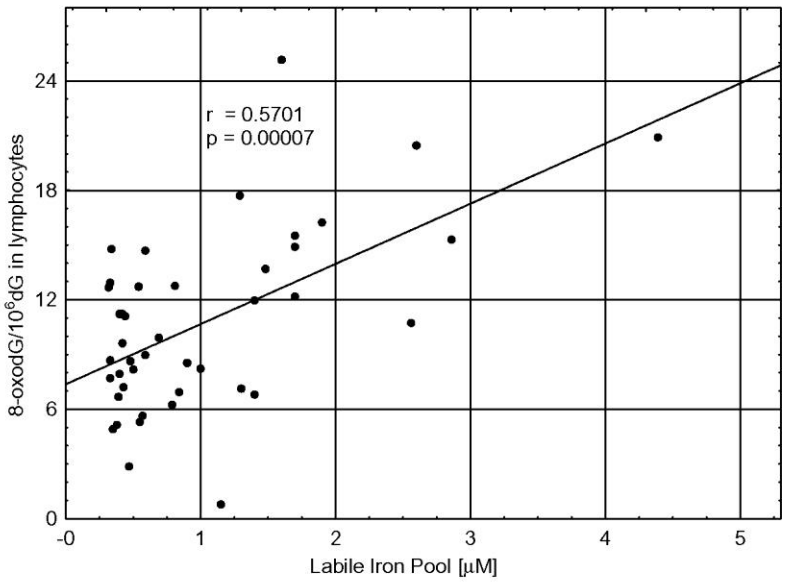

Fig. 1. Correlation between 8-oxodG levels and labile iron pool in lymphocytes, data from [35].

5. Accumulation of 8-oxoGua in cancer patients

5.1. Cancer tissues

We and others have demonstrated that elevated levels of typical free radical-induced DNA base modifications, including 8-oxoGua, exist in human cancerous tissues when compared with the cancer-free surrounding tissue [39–42].

It is not known whether these elevated levels of DNA lesions play a causative role in carcinogenesis or are merely the result of the disease. However, a treatment of laboratory animals with carcinogenic agents causes a similar pattern of oxidative base modification in their target organs before tumor formation occurs [43]. As previously mentioned, several lesions which have been found in higher amounts in cancerous tissues [39–42], possess mutagenic properties. These data suggest an important role for oxidative DNA base damage in carcinogenesis.

Our recent investigations of benign tumors showed that oxidative DNA damage might be a causative factor in cancer development. A higher endogenous level of 8-oxoGua in uterine myoma tissues was observed when compared to their respective tumor-free tissues [44]. Uterine myomas are common gynecological diseases. They are monoclonal, benign tumors derived from a single mutated myometrial cell. One of the factors that may predispose malignant transformation is the greater size of the tumor [45]. The positive correlation found in our work between the size of the tumor and the amount of 8-oxoGua (Fig. 2) [44] suggests that the higher level of 8-oxoGua and possibly other base lesions in benign tumors may be a risk factor that may determine the transformation of benign tumors to malignant tumors. Conversely, the increased levels of modified DNA bases may contribute to the genetic instability and metastatic potential of tumor cells in fully developed cancer.

It has been estimated that most human cancers contain a large number of mutations. At least 11,000 individual DNA mutations exist in a single carcinoma cell of colorectal tumors [46,47]. It is possible that a part of these mutations may arise during the development of the disease and may contribute to the metastatic potential of tumor cells. Our results suggest that one potential source of this unusually large number of mutations may be a damage to DNA by ROS. This explanation has also been suggested by Malins et al. who showed that the potential of metastasis of breast cancer tissues increased together with the increases in the level of oxidatively modified DNA bases [40].

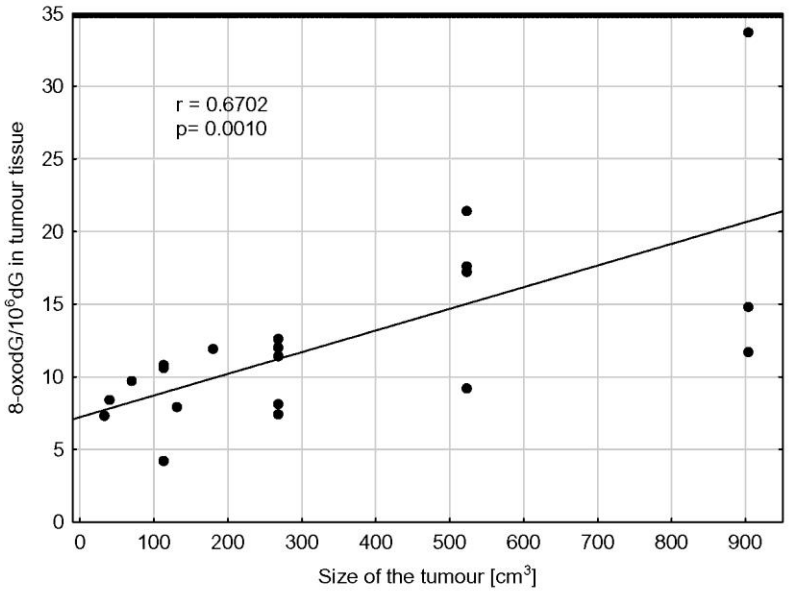

Fig. 2. Correlation coefficient between the background level of 8-oxodG in lymphocytes DNA and the tumor size, data from [44].

5.2. Urinary excretion of 8-oxoGua and 8-oxodG in cancer patients

Since the level of the modified nucleosides/bases in urine may be an indicator of oxidative insult on DNA and a general marker of oxidative stress, we investigated whether the amount of 8-oxoGua and its nucleoside form (8-oxodG) excreted into urine was higher in cancer patients than in the control group. It was found that the amount of the modified base, but not the nucleoside, excreted into urine was approximately 50% higher in cancer patients than in the control group [48].

The level of the modified bases in urine can depend on oxidative DNA insult and can be reflective of an involvement of different repair mechanism(s). It is rather unlikely that DNA repair capacity might differ in cancer patients in comparison with healthy subjects. Therefore, the higher level of 8-oxoGua in urine of cancer patients may be explained, at least partially, by the reported oxidative stress in cancer tissue [39,42,49,50]. However, the amount of the modified base/nucleoside excreted into urine should represent the average rate of DNA damage in the whole body [12,14]. Therefore, it is doubtful that the elevated level of the base product in cancerous cells alone could account for the observed 50% increase of 8-oxoGua in urine. Our results suggest rather that oxidative stress, represented by the increased amount of the compound in urine, may be characteristic not only for the diseased tissue but also for some other tissues (or the whole organism) of cancer patients (see also Section 3.1). The precise mechanism(s) of the oxidative stress is still unknown. However, some mechanisms may be suggested:

(i) It has been recently documented that cancer patients showed signs of extensive granulocyte activation with a release of ROS followed by a dramatic increase of 8-isoprostane, one of the biomarkers of oxidative stress [51].
(ii) It has been shown that malignant cells can produce hydrogen peroxide at levels as large as those characteristic for stimulated polymorfonuclear leukocytes [52]. Therefore, one of the reasons for the observed oxidative stress in advanced stages of cancer may be a release of the large number of cancer cells into the blood stream [53] and their penetration into other tissues. Interestingly, it has been demonstrated that exposure to the activated leukocytes causes oxidative DNA base modifications (among them 8-oxoGua) in target cells [54].
(iii) Still another reason for the observed phenomenon could be that some tumors may stimulate the defense systems of the body so that they react against the tumor to produce cytokines [55]. Some of the cytokines can be responsible for ROS production [56,57]. It has been shown that elevated plasma level of TNF is responsible for increased oxidative DNA damage of CD 34^+ cells [58].

It is also possible that prooxidant environment is characteristic for advanced stages of cancer and that oxidative stress is rather a result of the disease development.

6. Oxidative damage to free nucleotide pool as a possible source of 8-oxodG in cellular DNA and urine

It is widely accepted that guanine moieties present in free nucleotides may be equally or even more susceptible to oxidative damage than those constituting cellular DNA. Many in vitro experiments have shown that 8-oxo-dGTP (a product of 2′-deoxyguanosine 5′-triphosphate (dGTP) modification), has mutagenic properties [59]. This damaged nucleotide may be misincorporated into nascent DNA opposite adenine or cytosine moieties [4,60]. 8-OxoGua:A and 8-oxoGua:C mispairs [61], if not repaired by DNA repair enzymes, may give rise to AT → CG, and less frequently, GC → TA point mutations during subsequent replication rounds [62–68].

6.1. A role of MTH1 protein (8-oxodGTPase)

Living organisms posses an enzyme 8-oxodGTP pyrophosphohydrolase (8-oxodGTPase) that very effectively degrades 8-oxodGTP moieties [59]. *Escherichia coli* strain carrying a knockout mutation in the *mutT* gene coding for this enzyme demonstrates very strong mutator phenotype [69] characterized by at least 1000-fold increase in the frequency of AT →

CG point mutations [70,71]. A wild type *mutT* gene was cloned [72,73] and its final product identified as 15 kDa protein capable of enzymatic hydrolysis of canonical deoxyribonucleoside- and ribonucleoside 5′-triphosphates [73,74] to their corresponding 5′-monophosphates and inorganic pyrophosphate [75]. Maki and Sekiguchi discovered that MutT protein most effectively hydrolyzes 8-oxodGTP thereby preventing its incorporation into DNA during DNA replication process [60]. Thus, an antimutagenic function of MutT protein has been attributed to decomposing 8-oxodGTP which otherwise may cause AC → CG transversions. It is still not clear, however, whether the entire antimutagenic effect of MutT protein is exclusively associated with 8-oxodGTP-hydrolyzing activity. In order to account for such a high mutation rate in *mutT*$^-$ strain the concentration of free 8-oxodGTP as well as 8-oxoguanine level in DNA should rather increase massively. However, the level of 8-oxodG in DNA is only slightly higher in *mutT*$^-$ mutant cells as compared to wild type strain [76]. Also the cellular concentrations of 8-oxodGTP in both strains are unknown. The description of physiological function of MutT protein, presented above, has been recently questioned [77].

Mammalian homologues of bacterial *mutT* gene were cloned, characterized, and designated *MTH1* genes (***mutT* h**omologue **1**) [78]. Transfection of the human *MTH1* gene into *mutT*$^-$ *E. coli* resulted in partial reversal [79], and similar transfection of mouse and rat genes resulted in a complete reversal [80,81] of the high AT → CG point mutation rate, typical for incorporational mutagenicity of 8-oxodGTP observed in these mutants. Therefore, it has been postulated that mammalian MTH1 proteins coded by these genes play the same role in sanitizing free nucleotide pools. Indeed, human [82], mouse [80,83], rat [81,84], and hamster [85,86] MTH1 proteins are nucleoside 5′-triphophate pyrophosphohyrolases that very effectively decompose 8-oxodGTP. That is why these mammalian enzymes are most frequently called 8-oxodGTPases. A human *MTH1* gene (*hMTH1*) is located on the chromosome 7p22 [87]. It consists of 5 exons and can produce up to seven types (1, 2A, 2B, 3A, 3B, 4A and 4B) of mRNAs as a result of the existence of multiple sites of transcription initiation and alternative RNA splicing processes [88]. Type 1 mRNA and the18 kDa hMTH1 protein variant are predominant products of *hMTH1* [89]. The enzymatic activity of purified 18 kDa human 8-oxodGTPase is Mg^{2+}-dependent [82,90]. The enzyme shows its maximum activity under slightly alkaline conditions [82] and demonstrates a broad specificity towards nucleoside 5′-triphosphates. Although hMTH1 decomposes most effectively 2-hydroxy-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP) [91], 2-hydroxyadenosine 5′-triphosphate (2-OH-ATP) [92], 8-oxodGTP [82], and 8-oxo-2′-deoxyadenosine 5′-triphosphate (8-oxodATP) [91], it is also capable to hydrolyze less effectively 8-oxoguanosine 5′-triphosphate (8-oxoGTP) [93], 8-chloro-2′-deoxyguanosine 5′-triphosphate (8-Cl-dGTP) [94] and canonical deoxyribonucleoside and ribonucleoside 5′-triphosphates, such as dGTP [82]. Four isolated forms of hMTH1 protein (p18, p21, p22 and p26) demonstrate equal activities towards 8-oxodGTP [89]. Human 18 kDa 8-oxodGTPase was shown to be located mostly in cytosolic and mitochondrial soluble fractions [95], although rat tissues revealed also an apparent nuclear localization of MTH1 protein [96].

6.2. *MTH1 and carcinogenesis*

Models of the cancer development assume a formation of the mutator phenotype in the early stage of the cell transformation. A knockout of *MTH1* gene was anticipated to generate a mammalian mutator phenotype. However, such a knockout mutation in *MTH1* gene has not been discovered in mammalian cancer cells. Surprisingly, instead of *MTH1* mutation, a characteristic overexpression of *MTH1* has been noticed in different cancer cells and tissues as compared to their healthy counterparts [97–101]. Since this overexpression in the cancer cells was most frequently assigned to a state of persistent oxidative stress in cancer cells, it has also been proposed that *MTH1* overexpression might be a marker of the oxidative stress [97,99]. Although an induction of *MTH1* expression by high concentrations of hydrogen peroxide [102] and crocidolite asbestos [103] has been recently demonstrated, different aspects of *MTH1* gene expression regulation in normal and cancer cells still remain unclear.

It is not known how many of 8-oxoGua molecules present in cellular DNA are incorporated from 8-oxodGTP and how many of them are formed as a result of direct DNA oxidation. Higher activity of

8-oxodGTPase has been demonstrated to coincide with lower background levels of 8-oxodG in DNA of fetal compared with maternal mouse organs [83], albeit there might be no causative relationship between both measured parameters. Since bacterial and mammalian 8-oxodGTPases are inhibited by cadmium(II) ions in vitro, it has been hypothesized that this inhibition might increase the level of 8-oxodG in DNA what, in turn, may lead to carcinogenesis [90]. In vivo inhibitory effect of 0.3–3 μM cadmium acetate has been confirmed in cultured CHO cells by means of a novel method of 8-oxodGTPase activity determination in cell-free extracts [85]. Also rats treated with a single injection of 20 μmol Cd(II) acetate revealed 50% loss of 8-oxodGTPase activity in testes, target organs for cadmium(II) carcinogenesis in rats. Interestingly, this decrease of 8-oxodGTPase activity was accompanied with a two-fold rise in 8-oxodG level in testicular DNA [84,104]. It implies again a potential influence of 8-oxodGTPase on 8-oxodG content in DNA.

A better insight into the role of MTH1 protein has been acquired by the creation of *MTH1* nullizygous cell lines and mice [105]. These transgenic mice, defective in *MTH1* gene and devoid of 8-oxodGTP pyrophosphohydrolase activity, demonstrated higher incidence of lung, liver, and stomach cancers accompanied by two-fold increase in spontaneous mutation frequency in *Hprt* gene, as compared to wild type mice [106]. Nevertheless, this slightly higher mutation rate has not been confirmed in a more recent study that showed the same level of mutation frequency in *rpsL* reporter gene of *E. coli*, introduced into both $MTH1^{+/+}$ and $MTH1^{-/-}$ mice strains [107]. Unfortunately, a comparison of 8-oxodG levels in DNA of wild type versus MTH1-deficient mice has not been reported to date. Hence, it is not sure whether MTH1 protein deficiency may indeed bring about a detectable increase in the level of this DNA lesion, although an expression of the human *MTH1* in mouse cells has been recently demonstrated to reduce steady-state and H_2O_2-induced DNA 8-oxodG levels [108]. A lack of 8-oxodGTPase activity in the cell must not necessarily result in the increase of 8-oxodG level in DNA, since it depends also on a concerted action of all 8-oxoGua-glycosylases and mismatch repair enzymes that may counterbalance an elevated 8-oxodGTP incorporation into DNA.

6.3. Metabolism of 8-oxodGTP

Since neither 8-oxodGMP can be phosphorylated to 8-oxodGDP by the human guanylate kinase nor 8-oxoGDP (8-oxoguanosine-5′-diphosphate) can be reduced to 8-oxodGDP by the human nucleoside 5′-diphosphate reductase [93], the only likely origins of cellular 8-oxodGTP seem to be (1) a direct oxidation of dGTP or (2) enzymatic phosphorylation of 8-oxodGDP (from dGDP oxidation) by nucleoside 5′-diphosphate kinase (Fig. 3). Mammalian MTH1 proteins hydrolyze 8-oxodGTP to 8-oxodGMP. This catabolic step is irreversible because 8-oxodGMP cannot be phosphorylated back to 8-oxodGDP [109]. In human tissues 8-oxodGMP is further degraded to 8-oxodG by Mg^{2+}-dependent 5′-nucleotidase that can also hydrolyze dGMP, TMP, and dAMP though less efficiently [109]. Apparently, 8-oxodG is the end-product of this catabolic pathway in mammalian tissues. A decomposition of 8-oxodG by cell-free extracts of mammalian tissues is undetectable [109,110]. Under physiological conditions unmodified 2′-deoxyguanosine is decomposed by cellular purine nucleoside phosphorylase (PNP-ase) that catalyses phosphorolysis of *N*-glycosidic bond to generate free guanine and deoxyribose 1′-monophosphate. A commercial preparation of the rabbit PNPase is totally inactive towards 8-oxodG [110]. 8-OxodG, as an uncharged molecule, may readily leave the cell to be excreted into urine. Therefore, it is very likely that at least a part of 8-oxodG pool observed in urine comes from degradation of 8-oxodG phosphates.

It is likely that MTH1 is not the only one mammalian enzyme responsible for 8-oxodGTP catabolism. At the concentrations exceeding 20 μM, 8-oxodGTP is mainly degraded to 8-oxodGDP by the extracts of mammalian tissues [82,85]. 8-oxodGDP completely inhibits MTH1 protein activity [85,91]. This activity is only partially Mg^{2+}-dependent suggesting an involvement of more than one enzyme in the reaction (Bialkowski, unpublished results). Our unpublished results performed on rat tissue extracts indicate that at higher concentrations (20 μM or more) 8-oxodGTP is sequentially decomposed to 8-oxodGDP, 8-oxodGMP, and finally to 8-oxodG without participation of MTH1 protein, since this enzyme is inhibited by an initial formation of 8-oxodGDP. An existence of 8-oxodGDP phoshohydrolase

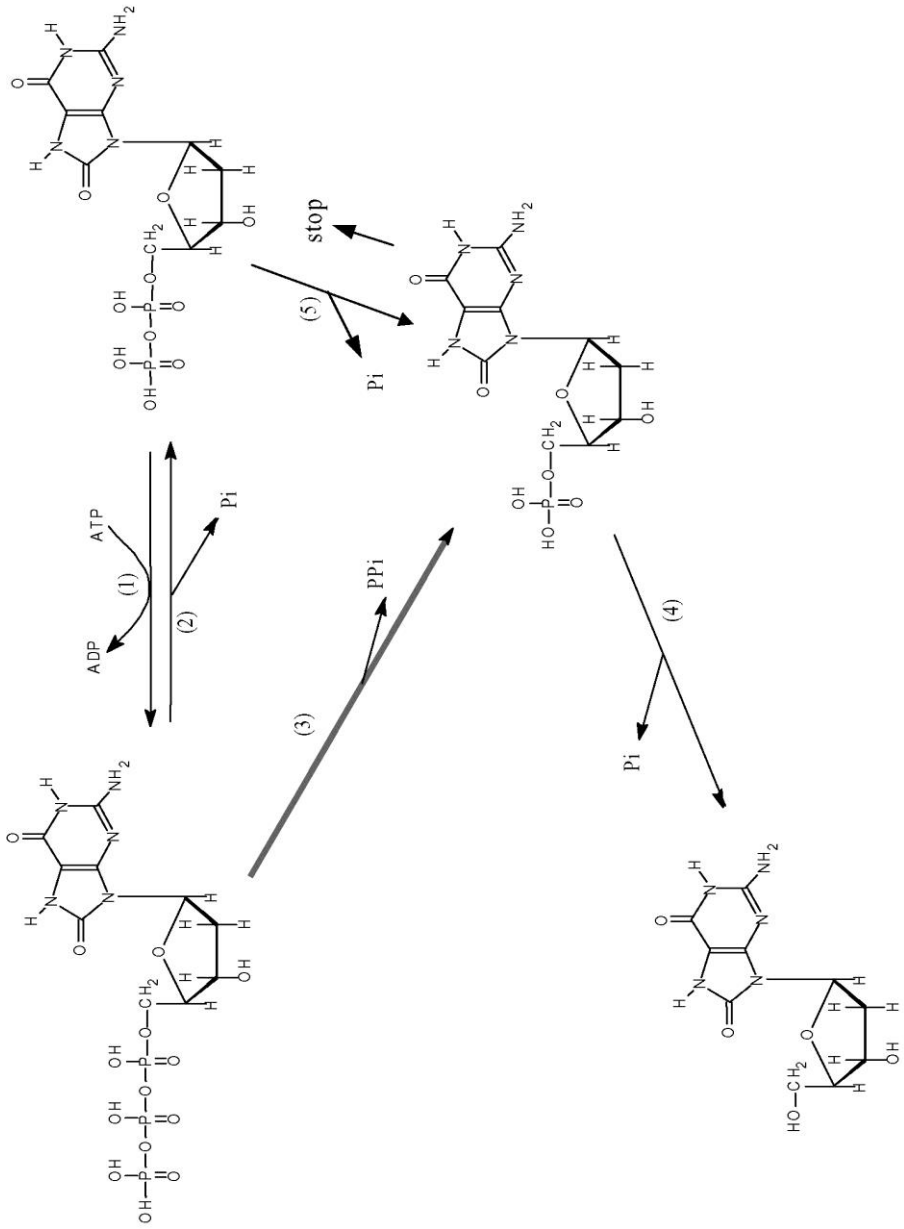

Fig. 3. 8-OxodGTP catabolic pathway: (1) 5′-diphosphonucleoside kinase, (2) 8-oxodGTP phosphatase, (3) 8-oxodGTP pyrophosphatase (8-oxodGTPase), (4) 8-oxodGMP 5′-nucleotidase, (5) 8-oxodGDP phosphatase. Pi: inorganic monophosphate; PPi: inorganic pyrophosphate.

activity (that has not been previously reported in the literature) was confirmed by reacting genuine 8-oxodGDP with the same tissue extracts. In this case, 8-oxodGDP is also sequentially decomposed to 8-oxodGMP and 8-oxodG. MTH1 protein may not be involved in this pathway, since 8-oxodGDP is not a substrate for this enzyme. However, at lower 8-oxodGTP concentrations ($C \leq 5\,\mu M$), 8-oxodGMP appears to be the main initial product of degradation, and this suggests the involvement of MTH1 protein (8-oxodGTP pyrophosphohydrolase) in this process. It means that 8-oxodGTP degradation pathway involving MTH1 protein is most likely predominant at hypothetically very low concentration of cellular 8-oxodGTP.

7. Conclusions

There is increasing evidence that most human cancers contain large number of mutations [46,47]. This in turn suggests that they are generated continuously during tumor progression. Endogenous cellular processes (oxidative phosphorylation, peroxisomal fatty acid metabolism, cytochrome P-450 reactions or "respiratory burst" of phagocytic cells) are efficient source of harmful ROS that may be responsible for oxidative DNA base modifications and may serve as a source of mutations that initiate carcinogenesis. Since severe oxidative stress is also characteristic for advanced stages of cancer (see above), these modifications may also serve as an efficient source of mutations which arise during tumor progression and thus contribute to tumor aggressiveness. In order to contribute to mutations, oxidative DNA damage would need to occur at a sufficiently high frequency to exceed the capacity of the cell for DNA repair. There are more than 20 different oxidative DNA base modifications [111]. Therefore, the extent of oxidative DNA base damage appears to be very high. As a consequence the background level might contain not only the damages that initiate carcinogenesis but might also contribute to ongoing tumor progression. In this context it is also noteworthy that in our study a combine amount of 8-oxoGua and 8-oxodG excreted into urine of healthy subjects was 2.5 nmol/kg per day, corresponding to about 2000 oxidative modifications of guanine per cell per day [112].

Summing up; in the light of the presented data it is likely that severe oxidative stress is a consequence of development of many types of cancer. However, at present it is impossible to answer directly the question concerning involvement of oxidative stress in cancer origin since full development of the disease in response to carcinogen exposure takes 20–40 years. Therefore, it is very difficult to prove directly that DNA lesion responsible for carcinogenic process is the lesion present in tumors many generations later. Nevertheless, it should be remembered that ROS are one of the most attractive factors to explain the high incidence of sporadic cancer because they have well established mutagenic potential and also are products of endogenous processes (see above) as well as their production may be strongly influenced by environmental factors.

It is also noteworthy that oxidative stress can influence carcinogenesis not only *via* inducing random damage to DNA (or other biomolecules). The prooxidative environment may confer a growth advantage to tumor cells and more generally it may influence carcinogenic transformation by stimulating specific signaling cascades that regulate cell growth and apoptosis [113].

References

[1] M. Dizdaroglu, Oxidative damage to DNA in mammalian chromatin, Mutat. Res. 275 (1992) 331–342.
[2] R.A. Floyd, The role of 8-hydroxyguanine in carcinogenesis, Carcinogenesis 11 (1990) 1447–1450.
[3] A.L. Jackson, L.A. Loeb, The contribution of endogenous sources of DNA damage to the multiple mutations in cancer, Mutat. Res. 477 (2001) 7–21.
[4] K.C. Cheng, D.S. Cahill, H. Kasai, S. Nishimura, L.A. Loeb, 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G → T and A → C substitutions, J. Biol. Chem. 267 (1992) 166–172.
[5] D.I. Feig, T.M. Reid, L.A. Loeb, Reactive oxygen species in tumorigenesis, Cancer Res. 54 (1994) 1890s–1894s.
[6] M.Q. Du, P.L. Carmichael, D.H. Phillips, Induction of activating mutations in the human c-Ha-ras-1 proto-oncogene by oxygen free radicals, Mol. Carcinog. 11 (1994) 170–175.
[7] D. Yu, J.A. Berlin, T.M. Penning, J. Field, Reactive oxygen species generated by PAH o-quinones cause change-in-function mutations in p53, Chem. Res. Toxicol. 15 (2002) 832–842.
[8] Comparison of different methods of measuring 8-oxoguanine as a marker of oxidative DNA damage. European Standards Committee on Oxidative DNA Damage (ESCODD), Free Radic. Res. 32 (2000) 333–341.

[9] Inter-laboratory validation of procedures for measuring 8-oxo-7,8-dihydroguanine/8-oxo-7,8-dihydro-2′-deoxyguanosine in DNA, Free Radic. Res. 36 (2002) 239–245.
[10] M.K. Shigenaga, C.J. Gimeno, B.N. Ames, Urinary 8-hydroxy-2′-deoxyguanosine as a biological marker of in vivo oxidative DNA damage, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 9697–9701.
[11] J. Suzuki, Y. Inoue, S. Suzuki, Changes in the urinary excretion level of 8-hydroxyguanine by exposure to reactive oxygen-generating substances, Free Radic. Biol. Med. 18 (1995) 431–436.
[12] M.S. Cooke, M.D. Evans, K.E. Herbert, J. Lunec, Urinary 8-oxo-2′-deoxyguanosine—source, significance and supplements, Free Radic. Res. 32 (2000) 381–397.
[13] S. Loft, A. Fischer-Nielsen, I.B. Jeding, K. Vistisen, H.E. Poulsen, 8-Hydroxydeoxyguanosine as a urinary biomarker of oxidative DNA damage, J. Toxicol. Environ. Health 40 (1993) 391–404.
[14] S. Loft, H.E. Poulsen, Estimation of oxidative DNA damage in man from urinary excretion of repair products, Acta Biochim. Pol. 45 (1998) 133–144.
[15] P.M. Girard, S. Boiteux, Repair of oxidized DNA bases in the yeast *Saccharomyces cerevisiae*, Biochimie 79 (1997) 559–566.
[16] G. Dianov, C. Bischoff, J. Piotrowski, V.A. Bohr, Repair pathways for processing of 8-oxoguanine in DNA by mammalian cell extracts, J. Biol. Chem. 273 (1998) 33811–33816.
[17] H.J. Helbock, K.B. Beckman, M.K. Shigenaga, P.B. Walter, A.A. Woodall, H.C. Yeo, B.N. Ames, DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 288–293.
[18] J.L. Ravanat, P. Guicherd, Z. Tuce, J. Cadet, Simultaneous determination of five oxidative DNA lesions in human urine, Chem. Res. Toxicol. 12 (1999) 802–808.
[19] D. Gackowski, R. Rozalski, K. Roszkowski, A. Jawien, M. Foksinski, R. Olinski, 8-Oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine levels in human urine do not depend on diet, Free Radic. Res. 35 (2001) 825–832.
[20] A. Weimann, D. Belling, H.E. Poulsen, Quantification of 8-oxo-guanine and guanine as the nucleobase, nucleoside and deoxynucleoside forms in human urine by high-performance liquid chromatography–electrospray tandem mass spectrometry, Nucleic Acids Res. 30 (2002) E7.
[21] T. Lindahl, Instability and decay of the primary structure of DNA, Nature 362 (1993) 709–715.
[22] K. Bialkowski, P. Cysewski, R. Olinski, Effect of 2′-deoxyguanosine oxidation at C8 position on *N*-glycosidic bond stability, Z. Naturforsch. [C.] 51 (1996) 119–122.
[23] G. Block, B. Patterson, A. Subar, Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence, Nutr. Cancer 18 (1992) 1–29.
[24] B.N. Ames, L.S. Gold, W.C. Willett, The causes and prevention of cancer, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 5258–5265.
[25] W.C. Willett, Diet and health: what should we eat? Science 264 (1994) 532–537.
[26] V. Schlotte, A. Sevanian, P. Hochstein, K.U. Weithmann, Effect of uric acid and chemical analogues on oxidation of human low density lipoprotein in vitro, Free Radic. Biol. Med. 25 (1998) 839–847.
[27] A. Sevanian, K.J. Davies, P. Hochstein, Conservation of Vitamin C by uric acid in blood, J. Free Radic. Biol. Med. 1 (1985) 117–124.
[28] S.J. Duthie, A. Ma, M.A. Ross, A.R. Collins, Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes, Cancer Res. 56 (1996) 1291–1295.
[29] A.R. Collins, B. Olmedilla, S. Southon, F. Granado, S.J. Duthie, Serum carotenoids and oxidative DNA damage in human lymphocytes, Carcinogenesis 19 (1998) 2159–2162.
[30] P. Moller, S. Loft, Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies, Am. J. Clin. Nutr. 76 (2002) 303–310.
[31] P. Jaruga, B. Jaruga, D. Gackowski, A. Olczak, W. Halota, M. Pawlowska, R. Olinski, Supplementation with antioxidant vitamins prevents oxidative modification of DNA in lymphocytes of HIV-infected patients, Free Radic. Biol. Med. 32 (2002) 414–420.
[32] D. Gackowski, M. Kruszewsk, Z. Banaszkiewicz, A. Jawien, R. Olinski, Lymphocyte labile iron pool, plasma iron, transferrin saturation and ferritin levels in colon cancer patients, Acta Biochim. Pol. 49 (2002) 269–272.
[33] B. Halliwell, J.M. Gutteridge, Free Radicals in Biology and Medicine, Oxford University Press, New York, NY, 1999.
[34] D. Gackowski, M. Kruszewski, A. Jawien, M. Ciecierski, R. Olinski, Further evidence that oxidative stress may be a risk factor responsible for the development of atherosclerosis, Free Radic. Biol. Med. 31 (2001) 542–547.
[35] D. Gackowski, M. Kruszewski, T. Bartlomiejczyk, A. Jawien, M. Ciecierski, R. Olinski, The level of 8-oxo-7,8-dihydro-2′-deoxyguanosine is positively correlated with the size of the labile iron pool in human lymphocytes, J. Biol. Inorg. Chem. 7 (2002) 548–550.
[36] B. de Valk, M.A. Addicks, I. Gosriwatana, S. Lu, R.C. Hider, J.J. Marx, Non-transferrin-bound iron is present in serum of hereditary haemochromatosis heterozygotes, Eur. J. Clin. Invest. 30 (2000) 248–251.
[37] R.L. Nelson, F.G. Davis, E. Sutter, L.H. Sobin, J.W. Kikendall, P. Bowen, Body iron stores and risk of colonic neoplasia, J. Natl. Cancer Inst. 86 (1994) 455–460.
[38] R.G. Stevens, B.I. Graubard, M.S. Micozzi, K. Neriishi, B.S. Blumberg, Moderate elevation of body iron level and increased risk of cancer occurrence and death, Int. J. Cancer 56 (1994) 364–369.
[39] R. Olinski, T. Zastawny, J. Budzbon, J. Skokowski, W. Zegarski, M. Dizdaroglu, DNA base modifications in chromatin of human cancerous tissues, FEBS Lett. 309 (1992) 193–198.
[40] D.C. Malins, E.H. Holmes, N.L. Polissar, S.J. Gunselman, The etiology of breast cancer. Characteristic alteration in hydroxyl radical-induced DNA base lesions during oncogenesis with potential for evaluating incidence risk, Cancer 71 (1993) 3036–3043.
[41] K. Okamoto, S. Toyokuni, K. Uchida, O. Ogawa, J. Takenewa, Y. Kakehi, H. Kinoshita, Y. Hattori-Nakakuki,

H. Hiai, O. Yoshida, Formation of 8-hydroxy-2′-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins in human renal-cell carcinoma, Int. J. Cancer 58 (1994) 825–829.

[42] P. Jaruga, T.H. Zastawny, J. Skokowski, M. Dizdaroglu, R. Olinski, Oxidative DNA base damage and antioxidant enzyme activities in human lung cancer, FEBS Lett. 341 (1994) 59–64.

[43] K.S. Kasprzak, P. Jaruga, T.H. Zastawny, S.L. North, C.W. Riggs, R. Olinski, M. Dizdaroglu, Oxidative DNA base damage and its repair in kidneys and livers of nickel(II)-treated male F344 rats, Carcinogenesis 18 (1997) 271–277.

[44] M. Foksinski, R. Kotzbach, W. Szymanski, R. Olinski, The level of typical biomarker of oxidative stress 8-hydroxy-2′-deoxyguanosine is higher in uterine myomas than in control tissues and correlates with the size of the tumor, Free Radic. Biol. Med. 29 (2000) 597–601.

[45] L.B. Schwartz, M.P. Diamond, P.E. Schwartz, Leiomyosarcomas: clinical presentation, Am. J. Obstet. Gynecol. 168 (1993) 180–183.

[46] B. Vogelstein, E.R. Fearon, S.R. Hamilton, S.E. Kern, A.C. Preisinger, M. Leppert, Y. Nakamura, R. White, A.M. Smits, J.L. Bos, Genetic alterations during colorectal-tumor development, N. Engl. J. Med. 319 (1988) 525–532.

[47] Y. Ionov, M.A. Peinado, S. Malkhosyan, D. Shibata, M. Perucho, Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis, Nature 363 (1993) 558–561.

[48] R. Rozalski, D. Gackowski, K. Roszkowski, M. Foksinski, R. Olinski, The level of 8-hydroxyguanine, a possible repair product of oxidative DNA damage, is higher in urine of cancer patients than in control subjects, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 1072–1075.

[49] D.C. Malins, R. Haimanot, Major alterations in the nucleotide structure of DNA in cancer of the female breast, Cancer Res. 51 (1991) 5430–5432.

[50] S. Toyokuni, K. Okamoto, J. Yodoi, H. Hiai, Persistent oxidative stress in cancer, FEBS Lett. 358 (1995) 1–3.

[51] J. Schmielau, O.J. Finn, Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients, Cancer Res. 61 (2001) 4756–4760.

[52] T.P. Szatrowski, C.F. Nathan, Production of large amounts of hydrogen peroxide by human tumor cells, Cancer Res. 51 (1991) 794–798.

[53] V.T. De Vita Jr., S. Hellman, S.A. Rosenberg, Cancer: Principles & Practice of Oncology, Lippincott Wiliams & Wilkins, Philadelphia, 2001.

[54] M. Dizdaroglu, R. Olinski, J.H. Doroshow, S.A. Akman, Modification of DNA bases in chromatin of intact target human cells by activated human polymorphonuclear leukocytes, Cancer Res. 53 (1993) 1269–1272.

[55] L.M. Franks, N.M. Teich, Introduction to the Cellular and Molecular Biology of Cancer, Oxford University Press, Oxford, New York, Tokyo, 1997.

[56] M. Ohba, M. Shibanuma, T. Kuroki, K. Nose, Production of hydrogen peroxide by transforming growth factor-beta 1 and its involvement in induction of egr-1 in mouse osteoblastic cells, J. Cell Biol. 126 (1994) 1079–1088.

[57] Y. Kayanoki, J. Fujii, K. Suzuki, S. Kawata, Y. Matsuzawa, N. Taniguchi, Suppression of antioxidative enzyme expression by transforming growth factor-beta 1 in rat hepatocytes, J. Biol. Chem. 269 (1994) 15488–15492.

[58] C.M. Peddie, C.R. Wolf, L.I. McLellan, A.R. Collins, D.T. Bowen, Oxidative DNA damage in CD34+ myelodysplastic cells is associated with intracellular redox changes and elevated plasma tumor necrosis factor-alpha concentration, Br. J. Haematol. 99 (1997) 625–631.

[59] M. Sekiguchi, T. Tsuzuki, Oxidative nucleotide damage: consequences and prevention, Oncogene 21 (2002) 8895–8904.

[60] H. Maki, M. Sekiguchi, MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis, Nature 355 (1992) 273–275.

[61] S. Shibutani, M. Takeshita, A.P. Grollman, Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG, Nature 349 (1991) 431–434.

[62] Y.I. Pavlov, D.T. Minnick, S. Izuta, T.A. Kunkel, DNA replication fidelity with 8-oxodeoxyguanosine triphosphate, Biochemistry 33 (1994) 4695–4701.

[63] A.A. Purmal, Y.W. Kow, S.S. Wallace, 5-Hydroxypyrimidine deoxynucleoside triphosphates are more efficiently incorporated into DNA by exonuclease-free Klenow fragment than 8-oxopurine deoxynucleoside triphosphates, Nucleic Acids Res. 22 (1994) 3930–3935.

[64] H. Miller, R. Prasad, S.H. Wilson, F. Johnson, A.P. Grollman, 8-oxodGTP incorporation by DNA polymerase beta is modified by active-site residue Asn279, Biochemistry 39 (2000) 1029–1033.

[65] D.T. Minnick, Y.I. Pavlov, T.A. Kunkel, The fidelity of the human leading and lagging strand DNA replication apparatus with 8-oxodeoxyguanosine triphosphate, Nucleic Acids Res. 22 (1994) 5658–5664.

[66] A.S. Kamath-Loeb, A. Hizi, H. Kasai, L.A. Loeb, Incorporation of the guanosine triphosphate analogs 8-oxo-dGTP and 8-NH2-dGTP by reverse transcriptases and mammalian DNA polymerases, J. Biol. Chem. 272 (1997) 5892–5898.

[67] M. Inoue, H. Kamiya, K. Fujikawa, Y. Ootsuyama, N. Murata-Kamiya, T. Osaki, K. Yasumoto, H. Kasai, Induction of chromosomal gene mutations in Escherichia coli by direct incorporation of oxidatively damaged nucleotides. New evaluation method for mutagenesis by damaged DNA precursors in vivo, J. Biol. Chem. 273 (1998) 11069–11074.

[68] H.J. Einolf, N. Schnetz-Boutaud, F.P. Guengerich, Steady-state and pre-steady-state kinetic analysis of 8-oxo-7,8-dihydroguanosine triphosphate incorporation and extension by replicative and repair DNA polymerases, Biochemistry 37 (1998) 13300–13312.

[69] H.P. Treffers, V. Spinelli, N.O.A. Belser, A factor (or mutator gene) influencing mutation rates in *Escherichia coli*, Proc. Natl. Acad. Sci. U.S.A. 40 (1954) 1064–1071.

[70] C. Yanofsky, E.C. Cox, V. Horn, The unusual mutagenic specificity of an *E. coli* mutator gene, Proc. Natl. Acad. Sci. U.S.A. 55 (1966) 274–281.

[71] R.M. Schaaper, R.L. Dunn, Escherichia coli mutT mutator effect during in vitro DNA synthesis. Enhanced A.G. replicational errors, J. Biol. Chem. 262 (1987) 16267–16270.
[72] M. Akiyama, T. Horiuchi, M. Sekiguchi, Molecular cloning and nucleotide sequence of the mutT mutator of Escherichia coli that causes A:T to C:G transversion, Mol. Gen. Genet. 206 (1987) 9–16.
[73] S.K. Bhatnagar, M.J. Bessman, Studies on the mutator gene, purification of the gene product, and identification of a novel nucleoside triphosphatase, J. Biol. Chem. 263 (1988) 8953–8957.
[74] S.K. Bhatnagar, L.C. Bullions, M.J. Bessman, Characterization of the mutT nucleoside triphosphatase of *Escherichia coli*, J. Biol. Chem. 266 (1991) 9050–9054.
[75] D.J. Weber, S.K. Bhatnagar, L.C. Bullions, M.J. Bessman, A.S. Mildvan, NMR and isotopic exchange studies of the site of bond cleavage in the MutT reaction, J. Biol. Chem. 267 (1992) 16939–16942.
[76] T. Tajiri, H. Maki, M. Sekiguchi, Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*, Mutat. Res. 336 (1995) 257–267.
[77] M.L. Tassotto, C.K. Mathews, Assessing the metabolic function of the MutT 8-oxodeoxyguanosine triphosphatase in *Escherichia coli* by nucleotide pool analysis, J. Biol. Chem. 277 (2002) 15807–15812.
[78] Y. Nakabeppu, Molecular genetics and structural biology of human MutT homolog, MTH1, Mutat. Res. 477 (2001) 59–70.
[79] K. Sakumi, M. Furuichi, T. Tsuzuki, T. Kakuma, S. Kawabata, H. Maki, M. Sekiguchi, Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis, J. Biol. Chem. 268 (1993) 23524–23530.
[80] T. Kakuma, J. Nishida, T. Tsuzuki, M. Sekiguchi, Mouse MTH1 protein with 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphatase activity that prevents transversion mutation. cDNA cloning and tissue distribution, J. Biol. Chem. 270 (1995) 25942–25948.
[81] J.P. Cai, T. Kakuma, T. Tsuzuki, M. Sekiguchi, cDNA and genomic sequences for rat 8-oxo-dGTPase that prevents occurrence of spontaneous mutations due to oxidation of guanine nucleotides, Carcinogenesis 16 (1995) 2343–2350.
[82] J.Y. Mo, H. Maki, M. Sekiguchi, Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein: sanitization of nucleotide pool, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 11021–11025.
[83] K. Bialkowski, A. Bialkowska, L.M. Anderson, K.S. Kasprzak, Higher activity of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) coincides with lower background levels of 8-oxo-2′-deoxyguanosine in DNA of fetal compared with maternal mouse organs, Free Radic. Biol. Med. 27 (1999) 90–94.
[84] K. Bialkowski, A. Bialkowska, K.S. Kasprzak, Cadmium(II), unlike nickel(II), inhibits 8-oxo-dGTPase activity and increases 8-oxo-dG level in DNA of the rat testis, a target organ for cadmium(II) carcinogenesis, Carcinogenesis 20 (1999) 1621–1624.
[85] K. Bialkowski, K.S. Kasprzak, A novel assay of 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) activity in cultured cells and its use for evaluation of cadmium(II) inhibition of this activity, Nucleic Acids Res. 26 (1998) 3194–3201.
[86] K. Bialkowski, K.S. Kasprzak, Activity of the antimutagenic enzyme 8-oxo-2′-deoxyguanosine 5′-triphosphate pyrophosphohydrolase (8-oxo-dGTPase) in cultured chinese hamster ovary cells: effects of cell cycle, proliferation rate, and population density, Free Radic. Biol. Med. 28 (2000) 337–344.
[87] M. Furuichi, M.C. Yoshida, H. Oda, T. Tajiri, Y. Nakabeppu, T. Tsuzuki, M. Sekiguchi, Genomic structure and chromosome location of the human mutT homologue gene MTH1 encoding 8-oxo-dGTPase for prevention of A:T to C:G transversion, Genomics 24 (1994) 485–490.
[88] H. Oda, Y. Nakabeppu, M. Furuichi, M. Sekiguchi, Regulation of expression of the human MTH1 gene encoding 8-oxo-dGTPase. Alternative splicing of transcription products, J. Biol. Chem. 272 (1997) 17843–17850.
[89] H. Oda, A. Taketomi, R. Maruyama, R. Itoh, K. Nishioka, H. Yakushiji, T. Suzuki, M. Sekiguchi, Y. Nakabeppu, Multi-forms of human MTH1 polypeptides produced by alternative translation initiation and single nucleotide polymorphism, Nucleic Acids Res. 27 (1999) 4335–4343.
[90] D.W. Porter, H. Yakushiji, Y. Nakabeppu, M. Sekiguchi, M.J. Fivash Jr., K.S. Kasprzak, Sensitivity of *Escherichia coli* (MutT) and human (MTH1) 8-oxo-dGTPases to in vitro inhibition by the carcinogenic metals, nickel(II), copper(II), cobalt(II) and cadmium(II), Carcinogenesis 18 (1997) 1785–1791.
[91] K. Fujikawa, H. Kamiya, H. Yakushiji, Y. Fujii, Y. Nakabeppu, H. Kasai, The oxidized forms of dATP are substrates for the human MutT homologue, the hMTH1 protein, J. Biol. Chem. 274 (1999) 18201–18205.
[92] K. Fujikawa, H. Kamiya, H. Yakushiji, Y. Nakabeppu, H. Kasai, Human MTH1 protein hydrolyzes the oxidized ribonucleotide, 2-hydroxy-ATP, Nucleic Acids Res. 29 (2001) 449–454.
[93] H. Hayakawa, A. Hofer, L. Thelander, S. Kitajima, Y. Cai, S. Oshiro, H. Yakushiji, Y. Nakabeppu, M. Kuwano, M. Sekiguchi, Metabolic fate of oxidized guanine ribonucleotides in mammalian cells, Biochemistry 38 (1999) 3610–3614.
[94] K. Fujikawa, H. Yakushiji, Y. Nakabeppu, T. Suzuki, M. Masuda, H. Ohshima, H. Kasai, 8-Chloro-dGTP, a hypochlorous acid-modified nucleotide, is hydrolyzed by hMTH1, the human MutT homolog, FEBS Lett. 512 (2002) 149–151.
[95] D. Kang, J. Nishida, A. Iyama, Y. Nakabeppu, M. Furuichi, T. Fujiwara, M. Sekiguchi, K. Takeshige, Intracellular localization of 8-oxo-dGTPase in human cells, with special reference to the role of the enzyme in mitochondria, J. Biol. Chem. 270 (1995) 14659–14665.
[96] K.S. Kasprzak, Y. Nakabeppu, T. Kakuma, Y. Sakai, K. Tsuruya, M. Sekiguchi, J.M. Ward, B.A. Diwan, K. Nagashima, B.H. Kasprzak, Intracellular distribution of the

antimutagenic enzyme MTH1 in the liver, kidney and testis of F344 rats and its modulation by cadmium, Exp. Toxicol. Pathol. 53 (2001) 325–335.

[97] K. Okamoto, S. Toyokuni, W.J. Kim, O. Ogawa, Y. Kakehi, S. Arao, H. Hiai, O. Yoshida, Overexpression of human mutT homologue gene messenger RNA in renal-cell carcinoma: evidence of persistent oxidative stress in cancer, Int. J. Cancer 65 (1996) 437–441.

[98] E. Okochi, S. Ichimura, T. Sugimura, T. Ushijima, The absence of Mth1 inactivation and DNA polymerase kappa overexpression in rat mammary carcinomas with frequent A:T to C:G transversions, Jpn. J. Cancer Res. 93 (2002) 501–506.

[99] C.H. Kennedy, R. Cueto, S.A. Belinsky, J.F. Lechner, W.A. Pryor, Overexpression of hMTH1 mRNA: a molecular marker of oxidative stress in lung cancer cells, FEBS Lett. 429 (1998) 17–20.

[100] K. Hibi, Q. Liu, G.A. Beaudry, S.L. Madden, W.H. Westra, S.L. Wehage, S.C. Yang, R.F. Heitmiller, A.H. Bertelsen, D. Sidransky, J. Jen, Serial analysis of gene expression in non-small cell lung cancer, Cancer Res. 58 (1998) 5690–5694.

[101] G. Wani, G.E. Milo, S.M. D'Ambrosio, Enhanced expression of the 8-oxo-7,8-dihydrodeoxyguanosine triphosphatase gene in human breast tumor cells, Cancer Lett. 125 (1998) 123–130.

[102] F. Meyer, E. Fiala, J. Westendorf, Induction of 8-oxo-dGTPase activity in human lymphoid cells and normal fibroblasts by oxidative stress, Toxicology 146 (2000) 83–92.

[103] H.N. Kim, Y. Morimoto, T. Tsuda, Y. Ootsuyama, M. Hirohashi, T. Hirano, I. Tanaka, Y. Lim, I.G. Yun, H. Kasai, Changes in DNA 8-hydroxyguanine levels, 8-hydroxyguanine repair activity, and hOGG1 and hMTH1 mRNA expression in human lung alveolar epithelial cells induced by crocidolite asbestos, Carcinogenesis 22 (2001) 265–269.

[104] K.S. Kasprzak, K. Bialkowski, Inhibition of antimutagenic enzymes, 8-oxo-dGTPases, by carcinogenic metals. Recent developments, J. Inorg. Biochem. 79 (2000) 231–236.

[105] T. Tsuzuki, A. Egashira, S. Kura, Analysis of *MTH1* gene function in mice with targeted mutagenesis, Mutat. Res. 477 (2001) 71–78.

[106] T. Tsuzuki, A. Egashira, H. Igarashi, T. Iwakuma, Y. Nakatsuru, Y. Tominaga, H. Kawate, K. Nakao, K. Nakamura, F. Ide, S. Kura, Y. Nakabeppu, M. Katsuki, T. Ishikawa, M. Sekiguchi, Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 11456–11461.

[107] A. Egashira, K. Yamauchi, K. Yoshiyama, H. Kawate, M. Katsuki, M. Sekiguchi, K. Sugimachi, H. Maki, T. Tsuzuki, Mutational specificity of mice defective in the MTH1 and/or the MSH2 genes, DNA Rep. (Amst.) 1 (2002) 881–893.

[108] C. Colussi, E. Parlanti, P. Degan, G. Aquilina, D. Barnes, P. Macpherson, P. Karran, M. Crescenzi, E. Dogliotti, M. Bignami, The mammalian mismatch repair pathway removes DNA 8-oxodGMP incorporated from the oxidized dNTP pool, Curr. Biol. 12 (2002) 912–918.

[109] H. Hayakawa, A. Taketomi, K. Sakumi, M. Kuwano, M. Sekiguchi, Generation and elimination of 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate, a mutagenic substrate for DNA synthesis, in human cells, Biochemistry 34 (1995) 89–95.

[110] K. Bialkowski, Metabolism of 8-oxoguanine derivatives, Ph.D. dissertation, The Ludwik Rudygier Medical University, Bydgoszcz, Poland, 1997.

[111] M. Dizdaroglu, Mechanism of free radical damage to DNA, in: O.I. Aruoma, B. Halliwell (Eds.), DNA and Free Radicals: Techniques, Mechanisms & Applications, OICA International, Saint Lucia, London, 1998, pp. 3–26.

[112] R. Olinski, D. Gackowski, M. Foksinski, R. Rozalski, K. Roszkowski, P. Jaruga, Oxidative DNA damage: assessment of the role in carcinogenesis, atherosclerosis, and acquired immunodeficiency syndrome(1), Free Radic. Biol. Med. 33 (2002) 192–200.

[113] M. Benhar, D. Engelberg, A. Levitzki, ROS, stress-activated kinases and stress signaling in cancer, EMBO Rep. 3 (2002) 420–425.

[114] D. Gackowski, Z. Banaszkiewicz, R. Rozalski, A. Jawien, R. Olinski, Persistent oxidative stress in colorectal carcinoma patients, Int. J. Cancer 101 (2002) 395–397.

IV. PODSUMOWANIE

Bakteryjny gen *mutT* jest historycznie pierwszym odkrytym genem mutatorowym, opisanym w literaturze naukowej już w roku 1954. Jego mutatorowy potencjał w komórkach *E. coli* linii *mutT*$^{-}$ jest ogromny i przejawia się podwyższeniem tempa mutacji spontanicznych nawet o 10,000 razy. Brak funkcjonalnego białka MutT w tych komórkach wywołuje niemal wyłącznie mutacje punktowe typu transwersji AT $\rightarrow$ CG. Badania nad przyczyną tego wyjątkowego zjawiska, prowadzone na przełomie lat osiemdziesiątych i dziewięćdziesiątych ubiegłego wieku, doprowadziły do ustalenia sekwencji genu *mutT* oraz do izolacji i scharakteryzowania jego białkowego produktu. Ku zaskoczeniu badaczy tej materii białko MutT nie pełniło żadnych funkcji w procesach replikacji lub naprawy DNA, jak w przypadku typowych produktów genów mutatorowych. Zostało natomiast zidentyfikowane jako enzym z grupy fosfohydrolaz o dość szerokiej specyficzności substratowej wobec różnych 5'-trifosforanów rybo- i 2'-deoksyrybonukleozydów, które degraduje do nieorganicznego pirofosforanu oraz 5'-monofosforanu nukleozydu. Przełomem w badaniach nad antymutagennym działaniem białka MutT stało się odkrycie z początku lat dziewięćdziesiątych, kiedy wykazano, iż enzym ten degraduje z dużą specyficznością wysoce mutagenny nukleotyd, 8-oksy-2'-deoksyguanozyno-5'-trifosforan (8-oxo-dGTP). Równocześnie udowodniono, że 8-oxo-dGTP, który może powstawać w komórkach na skutek wolnorodnikowego uszkodzenia 2'-deoksyguanozno-5'-trifosforanu (dGTP), jest efektywnym substratem dla licznych polimeraz DNA, które wbudowują monofosforanową resztę tego nukleotydu do nowosyntetyzowanych nici DNA. Z uwagi na zdolność reszty 8-oksyguaniny do parowania zarówno z cytozyną jak i z adeniną, inkorporacja 8-oxo-dGTP do DNA wywołuje mutacje punktowe w kolejnych rundach replikacyjnych i są to głównie transwersje typu AT $\rightarrow$ CG. Rezultatem powiązania tych obserwacji było powstanie hipotezy na temat zasadniczej biologicznej funkcji białka MutT, którego główną rolę określono jako oczyszczanie puli wolnych nukleotydów komórkowych z mutagennych produktów oksydacji trifosforanów nukleozydów purynowych i tym samym utrzymywanie niskiego tempa mutacji punktowych.

Odkrycia te zaowocowały eksplozją zainteresowania genem i białkiem MutT ze strony badaczy procesów nowotworzenia. Zidentyfikowano bowiem podobne do MutT geny i białka w organizmach ssaków, w tym człowieka. Ssacze homologi białka MutT, nazwane MTH1 (skrót określenia „**M**ut**T** **h**omolog **1”**), okazały się również enzymami efektywnie hydrolizującymi 8-oxo-dGTP, a także inne mutagenne nukleotydy, jak 2-oxo-dATP lub 8-oxo-dATP. Zachodziło podejrzenie, że inicjalna, spontaniczna mutacja w genie *MTH1* może spowodować powstanie ssaczych komórek o silnie mutatorowym fenotypie. Zgodnie z jedną z teorii karcynogenezy pojawienie się takiej komórki, wykazującej wysoką niestabilność genetyczną, jest inicjalnym etapem powstawania pierwotnego ogniska nowotworowego. Komórka o fenotypie mutatorowym daje początek linii komórek potomnych, w których po kolejnych podziałach lawinowo wzrasta ilość mutacji w różnych genach, co warunkuje zajście pełnej transformacji nowotworowej. Do dnia dzisiejszego nie potwierdzono jednak istnienia powstałych spontanicznie komórek nowotworowych, które posiadałyby mutację typu „knockout” w genie *MTH1*.

Badania dotyczące fizjologicznej roli białka MTH1 oraz jego wpływu na procesy zachodzące w komórce wymagały uprzedniego opracowania metodyki pomiarów jego enzymatycznej aktywności w komórkach i tkankach. Osiągnięcie tego celu stało się pierwszym wyzwaniem dla autora niniejszej rozprawy. Opracowano kompletną procedurę oznaczania specyficznej aktywności 8-oxo-dGTPazy w lizatach komórek i różnych tkanek ssaków (publikacje 2, 3, 4, 6, 8), na którą składają się:

a) relatywnie tania i szybka metoda syntezy oraz izolacji fosforanowych pochodnych 8-oksy-2'-deoksyguanozyny o wysokiej czystości, związków niezbędnych do przeprowadzenia oznaczeń aktywności 8-oxo-dGTPazy,
b) metoda separacji aktywności 8-oxo-dGTPazowej białka MTH1 od obecnych w ekstraktach komórkowych aktywności niespecyficznych fosfataz, degradujących 8-oxo-dGTP, 8-oxo-dGDP i 8-oxo-dGMP, które interferują z oznaczeniami specyficznej aktywności 8-oxo-dGTPazy,
c) metoda ilościowego oznaczania produktu enzymatycznej hydrolizy 8-oxo-dGTP przez białko MTH1, oparta na wysokosprawnej chromatografii cieczowej z detekcją absorpcji promieniowania ultrafioletowego.

Procedurę oznaczania aktywności 8-oxo-dGTPazy optymalizowano dla poszczególnych rodzajów komórek i tkanek, testując wpływ na odzysk aktywności 8-oxo-dGTPazowej różnych metod homogenizacji, składu buforu

homogenizacyjnego, detergentów, związków stabilizujących białka, inhibitorów proteaz, związków redukujących. Cała procedura analityczna została poddana procesowi walidacji na każdym z etapów w celu określenia jej wiarygodności i ustalenia standardowego błędu jaki wprowadzać mogą poszczególne etapy procedury.

Za pomocą opracowanej metody scharakteryzowano pirofosfohydrolazową aktywność wobec 8-oxo-dGTP, która obecna jest w trzydziestokilodaltonowych ultrafiltratach ekstraktów komórek chomiczych i ludzkich (publikacje 2 i 6). Aktywność obu 8-oxo-dGTPaz jest całkowicie zależna od obecności jonów magnezu i osiąga maksimum przy pH = 8,5. Wyznaczono stałą Michaelisa-Menten dla hydrolizy 8-oxo-dGTP przez 8-oxo-dGTPazę chomiczą i ludzką, które wynoszą odpowiednio 9,3 μM i 12,1 μM. Wartości powyższych parametrów kinetycznych tej aktywności zgodne są z literaturowymi wartościami oznaczonymi dla oczyszczonych preparatów białka MTH1, co pozwala założyć, iż aktywność 8-oxo-dGTPazowa mierzona w ultrafiltratach ekstraktów komórek ssaków należy do tego właśnie białka.

Hipoteza dotycząca fizjologicznej funkcji białka MTH1 jako enzymu zapobiegającego wbudowywaniu mutagennej 8-oksyguaniny do DNA z puli wolnych nukleotydów była szeroko akceptowana od momentu jej sformułowania. Jednak do roku 1999 nie opublikowano żadnych danych eksperymentalnych, które przemawiałyby za tym, iż obecność tego enzymu w komórkach ssaków może w dostrzegalny sposób wpływać na zawartość 8-oksyguaniny w DNA. Pierwszą próbę eksperymentalnej weryfikacji tej hipotezy zaprezentowano w publikacji nr 3. Zaobserwowano znacznie wyższy poziom 8-oksy-2'-deoksyguanozyny (8-oxo-dG) w DNA płuc i wątrób myszy ciężarnych niż w analogicznych narządach ich osiemnastodniowych płodów. Obserwacja ta zbieżna jest z koncepcją akumulacji 8-oxo-dG w DNA wraz z wiekiem. Jednocześnie w tych samych narządach odnotowano znacznie wyższą aktywność 8-oxo-dGTPazy w przypadku narządów płodowych niż matczynych. Chociaż koincydencja ta nie może być dowodem na bezpośredni wpływ wyższej aktywności białka MTH1 na utrzymywanie niskiego poziomu 8-oxo-dG w DNA, to jednak wskazała, że rzeczywiście jest to możliwe.

W połowie lat 90’ zrodziła się hipoteza, iż czynniki obniżające ekspresję genu *MTH1* lub hamujące *in vivo* aktywność 8-oxo-dGTPazową białka MTH1 mogą wykazywać działanie mutagenne i karcynogenne, zwiększając ilość 8-oxo-dG inkorporowanej do DNA z puli wolnych nukleotydów, które ulegają

nieuchronnej oksydacji w warunkach fizjologicznego funkcjonowania organizmów aerobowych. W ten sposób potencjalne inhibitory ekspresji lub aktywności tego enzymu, które bezpośrednio nie przejawiają właściwości prooksydacyjnych, mogłyby pośrednio podwyższać ilość oksydacyjnych uszkodzeń DNA w komórkach. Opisane w literaturze hamowanie aktywności enzymatycznej oczyszczonych preparatów bakteryjnej i ludzkiej 8-oxo-dGTPazy przez niektóre jony metali dało impuls do przebadania wpływu karcynogennych jonów kadmu i niklu na aktywność 8-oxo-dGTPazy *in vivo*. Jak wykazano w publikacji nr 2, przyżyciowe traktowanie hodowanych komórek CHO chomika chińskiego octanem kadmu(II) w stężeniach 0,3-3 μM przez okres do 24 godzin wywołuje obniżenie aktywności 8-oxo-dGTPazy do 70% wartości kontrolnej wprost proporcjonalnie do zastosowanego stężenia soli kadmu. W celu potwierdzenia zaobserwowanego efektu przeprowadzono analogiczny eksperyment z użyciem zwierząt doświadczalnych, opisany w publikacji nr 4. Jony kadmu(II) są efektywnym inhibitorem 8-oxo-dGTPaz *in vitro* oraz silnym karcynogenem dla szczurów, wywołującym głównie nowotwory jąder po podaniu podskórnym. Natomiast jony niklu(II) nie wywołują nowotworów jąder u szczura i są wielokrotnie słabszym inhibitorem 8-oxo-dGTPazowej aktywności MTH1. Kierując się powyższymi przesłankami, trzem grupom samców szczurzych wstrzyknięto podskórnie jednorazową dawkę odpowiednio: 20 μmoli octanu kadmu(II), 90 μmoli octanu niklu(II) i 180 μmoli octanu sodu (grupa kontrolna). Jądra zwierząt pobierano po czasie 2, 8, 24 i 48 godzin od iniekcji i oznaczano w nich aktywność 8-oxo-dGTPazową białka MTH1 oraz poziom 8-oxo-dG w DNA. Jony kadmu powodowały systematyczny spadek aktywności 8-oxo-dGTPazy w badanym czasie, aż do poziomu 50% wartości kontrolnej po 48 godzinach. Jednocześnie zawartość 8-oxo-dG w DNA szczurów traktowanych octanem kadmu wzrastała aż do 48 godziny, kiedy osiągnęła 200% wartości kontrolnej. Jony niklu(II) natomiast spowodowały jedynie nieznaczny spadek aktywności 8-oxo-dGTPazy i wzrost poziomu 8-oxo-dG w DNA, który nie osiągnął znamienności statystycznej. Wyniki tego eksperymentu wskazują, że przynajmniej w części karcynogenny potencjał jonów Cd^{2+} może wynikać z ich zdolności do hamowania aktywności białka MTH1, co wywołuje podwyższoną inkorporację do DNA mutagennej 8-oksyguaniny z puli wolnych nukleotydów. To z kolei prowadzić może do akumulacji mutacji punktowych w genomie komórki i w konsekwencji do jej transformacji nowotworowej. Należy zaznaczyć, iż jądra wykazują w warunkach fizjologicznych szczególnie wysoką aktywność 8-oxo-dGTPazy na tle innych narządów szczura (dane

nieopublikowane), co sugeruje duże znaczenie tego enzymu dla utrzymania niskiego tempa mutacji w intensywnie proliferujących komórkach tego narządu.

Badania kinetyki degradacji 8-oxo-dGTP przez ekstrakty komórek ssaków doprowadziły autora do odkrycia nowej klasy inhibitorów aktywności białka MTH1, 5'-difosforanów nukleozydów. Bardzo efektywnym inhibitorem aktywności 8-oxo-dGTPazowej białka MTH1 okazał się difosforanowy analog substratu, 8-oksy-2'-deoksyguanozyno-5'-difosforan (8-oxo-dGDP), którego stała inhibicji wynosi około 0,5 μM (publikacja nr 2).

Obserwacja ta nasunęła wątpliwość, czy aktywność 8-oxo-dGTPazowa białka MTH1 nie jest hamowana *in vivo* przez różne difosforany nukleozydów obecne fizjologicznie w komórce. Dlatego też zbadano właściwości kanonicznych 5'-difosforanów 2'-deoksyrybonukleozydów i rybonukleozydów (publikacja nr 6), których fizjologiczne stężenia w komórce przekraczają hipotetyczne stężenie 8-oxo-dGTP o 5 do 7 rzędów wielkości. Wykazano, iż najsilniejszymi inhibitorami kompetycyjnymi 8-oxo-dGTPazy ludzkiej pośród tej grupy związków są 2'-deoksyguanozyno-5'-difosforan (stała inhibicji *Ki* = 74 μM), 2'-deoksyadenozyno-5'-difosforan (*Ki* = 147 μM) oraz guanozyno-5'-difosforan (*Ki* = 502 μM). Pozostałe 5'-difosforany nukleozydów kanonicznych, czyli 2'-deoksycytydyny, 2'-deoksytymidyny, adenozyny, cytydyny i urydyny, są znacznie słabszymi inhibitorami enzymu, których wyznaczone stałe inhibicji lokują się w zakresie milimolowym. Uzyskane wyniki pozwoliły stworzyć teoretyczny model opisujący wpływ najsilniejszych inhibitorów z tej grupy na antymutagenną aktywność białka MTH1 w komórkach ludzkich. Obliczenia wykazały, że przy założeniu wewnątrzkomórkowego stężenia 8-oxo-dGTP poniżej 500 pM, średnim stężeniu dGDP i dADP rzędu 5 μM oraz 30 μM GDP, szybkość degradacji 8-oxo-dGTP przez białko MTH1 może zostać obniżona przez wymienione inhibitory jedynie o 15%. Oznacza to, iż obecność w komórce fizjologicznych stężeń difosfonukleozydowych inhibitorów enzymu MTH1 nie hamuje znacząco jego antymutagennej aktywności 8-oxo-dGTPazowej, tym samym nie stanowią one przeszkody w efektywnym oczyszczaniu przez to białko puli wolnych nukleotydów z promutagennych produktów uszkodzeń oksydacyjnych.

Dotychczasowe badania nie potwierdziły istnienia powstałych spontanicznie komórek i tkanek nowotworowych zawierających znokautowany gen *MTH1*, czyli nie zawierających funkcjonalnego białka MTH1. Nieoczekiwanie jednak

badania te doprowadziły do ciekawej obserwacji, że większość tkanek nowotworowych wykazuje podwyższony poziom ekspresji genu *MTH1* (zarówno na poziomie $mRNA_{MTH1}$ jak i białka MTH1) w porównaniu do tkanek nie zmienionych nowotworowo, z których się wywodzą. Na tej podstawie wykreowano hipotezę, że wysoki poziom ekspresji genu *MTH1* mógłby być markerem transformacji nowotworowej komórki. Niektórzy autorzy sugerują, iż bezpośrednim powodem tej nadekspresji jest permanentny stan stresu oksydacyjnego, jakiemu podlegają ogniska nowotworowe *in vivo*, sugerując tym samym, iż ekspresja genu *MTH1* jest regulowana przez reaktywne formy tlenu w procesie sprzężenia zwrotnego. Innymi słowy zakłada się, że wysoka ekspresja genu *MTH1* mogłaby być jednocześnie molekularnym markerem stresu oksydacyjnego. Jednocześnie zróżnicowany poziom ekspresji genu *MTH1* w zdrowych tkankach i różnych typach komórek ssaków nasuwał przypuszczenie, że poziom tej ekspresji może zależeć od funkcjonalnych stanów komórki, takich jak np. tempo proliferacji, faza cyklu komórkowego, czy też jakaś forma metabolicznej kooperacji komórek w populacjach. Gdyby tak było, podwyższona ekspresja MTH1 w komórkach nowotworowych mogłaby być związana z wyższym tempem proliferacji tych komórek, a MTH1 jako potencjalny marker transformacji nowotworowej lub stresu oksydacyjnego mógłby okazać się zwykłym markerem wysokiej aktywności proliferacyjnej lub przewagi jednej z faz cyklu komórkowego w populacji komórek. Badania poświęcone tym zagadnieniom opisano w publikacjach nr 7 i 8. Pierwszą serię eksperymentów przeprowadzono w oparciu o kultury komórek pochodzących z jajnika chomika chińskiego (CHO K1-BH4), hodowanych *in vitro*. Podobnie jak większość niestransformowanych komórek fibroblastycznych, komórki CHO po umieszeniu w medium hodowlanym proliferują, pokrywając jednowarstwowo powierzchnię pojemnika hodowlanego, aż do momentu osiągnięcia stanu pełnej konfluencji. Ścisły kontakt pomiędzy komórkami i fizyczny brak miejsca na kolejne ich generacje wywołuje zahamowanie podziałów komórkowych i stabilizację komórek fazie G0 cyklu komórkowego. Zjawisko to wykorzystano do zbadania wpływu dynamiki podziałów komórkowych i kontaktu międzykomórkowego na aktywność 8-oxo-dGTPazy w kulturach komórek CHO o różnym stopniu konfluencji (30 – 95%). Zanotowano niewielki (30%), ale systematyczny spadek aktywności enzymu wraz ze zbliżaniem się populacji komórek do stanu pełnej konfluencji. Jednoczesne oznaczenia ilościowe DNA, przeprowadzone metodą cytometrii przepływowej, potwierdziły tendencję populacji komórek do przechodzenia w fazy G0/G1 kosztem fazy S oraz G2 i M. Wyniki tak

skonstruowanego doświadczenia nie dawały jednak jasnej odpowiedzi na pytanie, czy tendencja do spadku aktywności 8-oxo-dGTPazy związana jest bezpośrednio z obniżeniem tempa proliferacji. W celu potwierdzenia ewentualnego wpływu zatrzymania podziałów komórkowych na aktywność 8-oxo-dGTPazy, skorzystano z innego, charakterystycznego zjawiska zachodzącego w populacjach komórek hodowanych, zwanego efektem głodzenia. Polega ono na tym, że przeniesienie dynamicznie proliferującej populacji komórek do medium hodowlanego nie zawierającego cielęcej surowicy płodowej wywołuje gwałtowne zahamowanie tempa podziałów komórkowych, któremu towarzyszy masowe zatrzymywanie się komórek w fazie G0. Eksperyment taki przeprowadzono na komórkach CHO, porównując dystrybucję poszczególnych faz cyklu komórkowego oraz aktywność 8-oxo-dGTPazy w populacjach komórek głodzonych przez 48 godzin oraz tych utrzymywanych w kompletnym medium hodowlanym. Pomimo dramatycznego zahamowania tempa proliferacji komórek głodzonych, związanego z wejściem 80% komórek w fazę G0/G1, nie zanotowano zmian aktywności 8-oxo-dGTPazy. Wynik taki sugerował, że aktywność proliferacyjna komórek nie wpływa w istotny sposób na funkcjonalną ekspresję genu *MTH1*, mierzoną na poziomie aktywności białka MTH1. Dane te przemawiały także za tym, że komórkowa aktywność 8-oxo-dGTPazy może pozostawać na tym samym poziomie w kolejnych fazach cyklu komórkowego, odmiennie niż w przypadku licznych enzymów metabolizmu nukleotydów. W celu ostatecznego potwierdzenia braku zależności aktywności 8-oxo-dGTPazy od faz cyklu komórkowego przeprowadzono kolejne doświadczenie. Za pomocą metody przeciwprądowej elutriacji wirowniczej (ang. centrifugal elutriation) z populacji komórek CHO wyizolowano frakcje komórek znajdujących się w różnych fazach cyklu komórkowego. Efektywne wzbogacenie wyizolowanych frakcji w komórki znajdujące się w fazach G0/G1, S oraz G2/M potwierdzono za pomocą cytometrii przepływowej. W uzyskanych frakcjach komórek aktywność 8-oxo-dGTPazy pozostawała na niemal identycznym poziomie, co dowiodło braku regulacji aktywności tego enzymu w zależności od fazy cyklu komórkowego.

Wyniki doświadczeń opisanych powyżej nie dostarczyły jednoznacznej odpowiedzi na pytanie, czy indukcja proliferacji komórek (zwłaszcza *in vivo*) związana jest z automatycznym podwyższeniem ekspresji genu *MTH1*. Aby rozwiać te wątpliwości przeprowadzono kolejne serie eksperymentów opisanych w publikacji nr 8. W tym celu wykorzystano model regenerującej wątroby gryzoni. Częściowa resekcja wątroby wywołuje procesy regeneracyjne, które polegają na masowej stymulacji podziałów mitotycznych hepatocytów,

znajdujących się w fazie G0 cyklu komórkowego. Grupy myszy poddano częściowej resekcji wątroby lub jedynie operacji otwarcia i zamknięcia powłok brzusznych (grupa kontrolna). Trzeciego dnia po operacji pobrano wątroby wszystkich zwierząt w celu oznaczenia w nich funkcjonalnego poziomu ekspresji genu *MTH1*, mierzonego jako aktywność 8-oxo-dGTPazy. Pomimo silnej stymulacji podziałów komórkowych w wątrobach regenerujących, potwierdzonej za pomocą immunohistochemicznego oznaczenia białka PCNA (proliferating cell nuclear antigen), średnia aktywność 8-oxo-dGTPazy w wątrobach regenerujących pozostała na niezmienionym poziomie względem aktywności oznaczonej w wątrobach nie poddanych częściowej resekcji.

Kolejnych danych na temat zależności pomiędzy tempem proliferacji komórek i aktywnością 8-oxo-dGTPazową białka MTH1 dostarczyły badania nad panelem nowotworowych komórek ludzkich, hodowanych rutynowo w ramach projektu *„Developmental Therapeutics Program”*, prowadzonego przez Narodowy Instytut Raka, Narodowych Instytutów Zdrowia USA. Aktywność 8-oxo-dGTPazy oznaczono w pięćdziesięciu siedmiu różnych liniach komórek, z których 6 stanowiły linie komórek białaczki, 8 czerniaka złośliwego, 9 raka płuca, 7 raka jelita grubego, 6 raka centralnego układu nerwowego, 6 raka jajnika, 8 raka nerki i 7 raka piersi. Dzięki oznaczeniu czasu potrzebnego do podwajania się liczby komórek w populacjach poszczególnych linii hodowanych *in vitro*, możliwe było zbadanie potencjalnej korelacji pomiędzy charakterystycznym tempem proliferacji poszczególnych linii i specyficzną aktywnością 8-oxo-dGTPazy. Wyniki tej analizy wykazały, iż nie istnieje żadna znamienna statystycznie korelacja pomiędzy oboma parametrami zarówno w całej grupie 57 linii, jak i w obrębie poszczególnych grup linii o tym samym pochodzeniu tkankowym.

Obie z powyżej opisanych serii eksperymentów wskazują zatem, że aktywność 8-oxo-dGTPazy nie jest zależna od tempa proliferacji komórek ssaków i nie ulega zmianom ani na skutek zahamowania, ani indukcji podziałów komórkowych.

Istotą badań opisanych w publikacjach nr 7 i 8 była próba weryfikacji hipotez sugerujących, że podwyższony, funkcjonalny poziom ekspresji genu *MTH1* ssaków jest specyficznym markerem molekularnym transformacji nowotworowej i/lub stresu oksydacyjnego. Choć wyniki przedstawionych badań nie mogły potwierdzić wprost tych hipotez, to jednak wykazały, że ich prawdziwość jest prawdopodobna. W szerszym kontekście zagadnienia dotyczące roli i znaczenia białka MTH1 w procesie nowotworzenia przedstawiono w końcowym artykule przeglądowym (publikacja nr 9).

V. STRESZCZENIE

Rozprawa ta stanowi podsumowanie opublikowanych wcześniej prac badawczych poświęconych antymutagennemu białku MTH1 ssaków, jego biologicznej roli, regulacji aktywności enzymatycznej oraz znaczeniu w procesie nowotworzenia. Białko MTH1 jest enzymem z grupy pirofosfohydrolaz, degradującym 5'-trifosforany nukleozydów purynowych do 5'-monofosforanów nukleozydów oraz nieorganicznego pirofosforanu. Enzym ten wykazuje szczególnie wysokie powinowactwo do niektórych produktów oksydacyjnych uszkodzeń 2'-deoksyguanozyno-5'-trifosforanu (dGTP) oraz 2'-deoksyadenozyno-5'-trifosforanu (dATP), które są substratami w procesie syntezy DNA. Jednym z najlepiej opisanych mutagennych nukleotydów, które mogą powstawać w komórce pod wpływem reaktywnych form tlenu, jest 8-oksy-2'-deoksyguanozyno-5'-trifosforan (8-oxo-dGTP). Jest on substratem dla licznych polimeraz DNA, które inkorporują go w postaci monofosfonukleozydu do nowosyntetyzowanej nici DNA w trakcie procesu replikacji. Z uwagi na zdolność parowania 8-oksy-2'-deoksyguanozny z 2'-deoksyadenozyną i 2'-deoksycytydyną, inkorporacja taka powodować może powstawanie mutacji punktowych po kolejnych rundach replikacyjnych (głównie transwersji AT $\rightarrow$ CG). Obecnie zakłada się, że zasadnicza funkcja biologiczna białka MTH1 polega na zapobieganiu mutacjom punktowym poprzez oczyszczanie puli wolnych nukleotydów z powstającego w komórkach 8-oxo-dGTP, który jest szczególnie efektywnie degradowany przez MTH1.

W ramach przedstawionej rozprawy opracowano metodykę oznaczeń specyficznej aktywności 8-oxo-dGTPazowej białka MTH1 w komórkach i tkankach ssaków. Praktyczne zastosowanie tej metody umożliwiło:

a) przeprowadzenie kinetycznej charakterystyki degradacji 8-oxo-dGTP przez białko MTH1 człowieka i chomika,
b) poszukiwanie zależności pomiędzy poziomem aktywności 8-oxo-dGTPazowej białka MTH1 a zawartością promutagennej 8-oksy-2'-deoksyguanozyny w DNA tkanek mysich,
c) zbadanie wpływu karcynogennych jonów kadmu i niklu *in vivo* na aktywność 8-oxo-dGTPazową białka MTH1 i zawartość 8-oksy-2'-deoksyguanozny w DNA narządów szczura oraz zaproponowanie nowego

mechanizmu karcynogenezy wywoływanej przez jony Cd^{2+} w jądrach gryzoni,

d) identyfikację całej klasy inhibitorów aktywności enzymatycznej białka MTH1 (5'-difosforanów nukleozydów), kinetyczną charakterystykę ich właściwości oraz analizę ich potencjalnego wpływu na efektywność hydrolizy 8-oxo-dGTP *in vivo*,

e) poszukiwanie wyjaśnienia dla zróżnicowanego poziomu ekspresji białka MTH1 w różnych typach komórek fizjologicznych oraz jego nadekspresji w komórkach nowotworowych; w ramach tych poszukiwań określono wpływ różnych stanów fizjologicznych komórek w populacji na poziom aktywności 8-oxo-dGTPazy, w tym: faz cyklu komórkowego, fizycznych oddziaływań międzykomórkowych oraz tempa proliferacji.

VI. ABSTRACT

Antimutagenic MTH1 protein – biological role, regulation of its enzymatic activity and involvement in carcinogenesis

This D.Sc. dissertation summarizes the results of the research devoted to antimutagenic MTH1 protein, its biological role, regulation of enzymatic activity, and involvement in carcinogenesis. The MTH1 protein is a pyrophosphohydrolase that degrades purine nucleoside 5'-triphosphates to the corresponding nucleoside 5'-monophosphate and inorganic pyrophosphate. The enzyme hydrolyzes most specifically some products of oxidative damage to 2'-deoxyguanosine 5'-triphosphate (dGTP) and 2'-deoxyadenosine 5'-triphosphate which are substrates for DNA synthesis. One of the best characterized mutagenic nucleotides is 8-oxo-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP) which likely forms in the cells under attack of reactive oxygen species. It is an erroneous substrate for many DNA polymerases that incorporate its monophosphate residue to a nascent DNA strand during DNA replication. Such an incorporation causes point mutations during subsequent replication rounds due to mispairing properties of 8-oxo-2'-deoxyguanosine that may form stable pairs with deoxycytidine as well as with deoxyadenosine. As MTH1 protein very effectively hydrolyzes 8-oxo-dGTP, its widely accepted function is assigned to preventing point mutations by sanitization of this promutagenic nucleotide out of cellular free nucleotide pool.

Within the framework of this dissertation an assay of specific 8-oxo-dGTPase activity of MTH1 protein in biological material was developed and validated. The elaborated method allowed subsequently for the following research to be carried out:

a) a kinetic characteristics of 8-oxo-dGTP hydrolysis by murine and human MTH1 proteins,
b) investigations on the possible impact of 8-oxo-dGTPase activity on 8-oxo-2'-deoxyguanosine (8-oxo-dG) content in DNA of mouse organs,

c) *in vivo* investigations on the effects of cadmium(II) and nickel(II) ions on the 8-oxo-dGTPase activity and 8-oxo-dG level in DNA of rat organs that resulted in a proposal of a new mechanism of cadmium testicular carcinogenesis in rodents,
d) an identification and kinetic description of a whole class of mammalian 8-oxo-dGTPase activity inhibitors (nucleoside 5'-diphosphates) and the analysis of their potential influence on the effectiveness of 8-oxo-dGTP decomposition *in vivo*,
e) a search for explanation to diverse MTH1 expression levels in different physiological cell types and MTH1 overexpression in cancer cells; in order to partially elucidate those phenomena different experimental approaches were used to relate cellular 8-oxo-dGTPase activity of MTH1 protein to cell cycling, proliferation rate and cell-to-cell contact.