Generation and Elimination of 8-Oxo-7,8-dihydro-2′-deoxyguanosine 5′-Triphosphate, a Mutagenic Substrate for DNA Synthesis, in Human Cells†

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ABSTRACT: 8-Oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP) is a potent mutagenic substrate for DNA synthesis. The present study deals with generation and degradation of 8-oxo-dGTP in the nucleotide pool of human cells. (1) 8-Oxo-dGTP can be generated not only by direct oxidation of dGTP but also by phosphorylation of 8-oxo-dGDP by nucleoside diphosphate kinase. (2) 8-Oxo-dGTP is rapidly degraded to 8-oxo-dGMP by cellular 8-oxo-dGTPase activity. 8-Oxo-dGMP thus produced cannot be rephosphorylated; guanylate kinase, which phosphorylates both GMP and dGMP to the corresponding nucleoside diphosphates, is totally inactive for 8-oxo-dGMP. (3) 8-Oxo-dGMP is further degraded to 8-oxo-deoxyguanosine by a nucleotidase. The enzyme was partially purified from an extract of human Jurkat cells, and the mode of action was elucidated. 8-Oxo-dGMP is the most preferred substrate of the enzyme, and other nucleoside monophosphates are cleaved at significantly lower rates: K_m for 8-oxo-dGMP is 10 times lower than that for dGMP, the second best substrate for the enzyme. The enzyme appears to convert 8-oxo-dGMP, which accumulates in the cellular nucleotide pool, to a form readily excretable to the cell exterior.

Oxygen radicals are produced through normal cellular metabolism and formation of such radicals is further enhanced by ionizing radiation and by various chemicals (Ames & Gold, 1991). The oxygen radicals attack nucleic acids and generate various modified bases in DNA (Gajewski et al., 1990; Boiteux et al., 1992). Among them, 8-oxo-7,8-dihydroguanine (8-oxoguanine)[1] is the most abundant and appears to play critical roles in carcinogenesis and in aging (Kasai et al., 1986; Ames & Gold, 1991; Bessho et al., 1992). 8-Oxoguanine can pair with both cytosine and adenine during DNA synthesis, and as a result G·C to T·A transversion is induced (Shibutani et al., 1991; Moriya, 1993). Oxidation of guanine also occurs in the cellular nucleotide pool. 8-Oxo-dGTP thus formed is a potent mutagenic substrate for DNA synthesis since it can be incorporated opposite adenine as well as cytosine in DNA, at almost equal efficiencies (Maki & Sekiguchi, 1992). In this case, both of transversions, A·T to C·G and G·C to T·A, would be induced (Cheng et al., 1992).

Studies with *Escherichia coli* mutator mutants revealed that cells possess elaborate mechanisms which prevent mutations caused by oxidation of the guanine base, in both DNA and free nucleotide forms. 8-Oxoguanine residues in DNA can be removed by an enzyme that is coded by the *mutM* gene of *E. coli* (Bessho et al., 1992; Chung et al., 1991; Michaels et al., 1991). The *mutM* mutant cells deficient in the enzyme activity show a 10-fold higher frequency of G·C to T·A transversion, as compared with the wild-type strain (Cabrera et al., 1988). Another mutator gene, named *mutY,* also suppresses specifically G·C to T·A transversion (Nghiem et al., 1988; Au et al., 1988). MutY protein removes adenine from a site of A:8-oxoguanine mismatch (Au et al., 1989; Michaels & Miller, 1992). Thus, two proteins, MutM and MutY, act consecutively at the site of oxidized guanine residue in the DNA to prevent occurrence of mutations (Michaels & Miller, 1992; Tchou & Grollman, 1993). On the other hand, mutations due to misincorporation of 8-oxo-dGTP can be prevented by the *mutT* gene product. MutT protein possesses an enzyme activity which hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP (Maki & Sekiguchi, 1992). The *mutT* mutant induces specifically A·T to C·G transversion (Yanofsky et al., 1966), and this mutational specificity occurs through the concerted actions of the MutM and MutY proteins. 8-Oxoguanine-related mutagenesis may account for a considerable number of spontaneous mutations in mammalian cells. A significant amount of 8-oxoguanine is formed in mammalian DNA, and most of the modified bases are excised from the DNA (Ames & Gold, 1991). Enzyme activities which cleave DNA at the site of 8-oxoguanine were detected in extracts of mammalian cells (Bessho et al., 1993a,b; Yeh et al., 1991); hence, some are likely to be mammalian counterparts of the *E. coli* MutM and MutY proteins. An enzyme similar to the MutT protein found in human cells (Mo et al., 1992)

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[1] Abbreviations: 8-oxo-dGTP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate; 8-oxoguanine, 8-oxo-7,8-dihydroguanine; 8-oxoguanosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxo-dGTPase, 8-oxo-7,8-dihydrodeoxyguanosine triphosphatase; 8-oxo-dGMP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-monophosphate; 8-oxo-dGMPase, 8-oxo-7,8-dihydrodeoxyguanosine monophosphatase; DTT, dithiothreitol; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; TEAB, triethylammonium hydrogencarbonate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline.

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apparently has the capability to suppress the mutator phenotype of *E. coli mutT* mutant cells (Sakumi et al., 1993). These results suggest that mechanisms similar to those found in *E. coli* appear to function in mammalian cells to prevent the occurrence of mutations.

On the basis of studies with bacteria, it is generally accepted that organisms possess enzyme systems to phosphorylate nucleoside monophosphates to di- and triphosphates (Kornberg & Baker, 1992). Conversion of the nucleoside monophosphates to diphosphates is catalyzed by kinases that are specific for each base but indifferent to the sugar moiety; then nucleoside diphosphates are converted to triphosphates through the action of a ubiquitous and nonspecific nucleoside diphosphate kinase. This raises the question of whether or not 8-oxo-dGMP generated by the action of the antimutagenic 8-oxo-dGTPase can be rephosphorylated by these enzymes. The present study was designed to answer this and related questions.

EXPERIMENTAL PROCEDURES

Cells and Reagents. Exponentially growing cells of the Jurkat strain (a human T-cell leukemia cell line) were collected by centrifugation and washed twice with PBS. The cells were rapidly frozen in liquid nitrogen and stored at −80 °C until use. 8-Oxo-dGTP and 8-oxo-GTP were generated by oxidation of dGTP and GTP, respectively, according to the method of Mo et al. (1992). To obtain nucleoside diphosphate samples, the corresponding nucleoside triphosphates were either digested with bacterial alkaline phosphatase or hydrolyzed by treatment with 1 N HCl at 37 °C for 3 h. Radioactive 8-oxo-dGMP and other nucleoside monophosphates were prepared by digestion of the corresponding α-^{32}P-labeled nucleoside triphosphates with a purified preparation of 8-oxo-dGTPase or venom phosphodiesterase I. All the nucleotide samples were purified by HPLC, and the concentrations of the nucleotides were determined on the basis of UV absorbance or specific radioactivity. These oxidized nucleotide samples were defined by the electrochemical method, using the following HPLC−ECD system: apparatus, IRICA Σ871; column, TOSOH TSK-GEL ODS-80T (4.6 × 150 mm); eluant, 12.5 mM citric acid/25 mM sodium acetate/30 mM NaOH/10 mM acetic acid containing 0−10% methanol; flow rate, 1 mL/min; UV detector, Waters 490E, 254 nm; EC detector, IRICA Σ875, 700 mV (oxidation); analysis soft, Waters 820J.

Nucleoside Diphosphate Kinase Assay. The reaction mixture (10 μL) contained 50 mM Tris-HCl, pH 7.5, 2 mM ATP, 25 mM $MgCl_2$, 10 mM KCl, 1 nmol of labeled dGDP or 8-oxo-dGDP, and an extract (Mourad & Parks, 1966). After incubation at 37 °C for various times, 0.5−1-μL aliquots of the mixture were withdrawn and the reaction was terminated by addition of 1 μL of 50 mM EDTA. The samples were applied to a PEI-cellulose F plate and developed in 1 M LiCl. The radioactivity was quantified with the use of a BAS2000 image analyzer.

Guanylate Kinase Assay. Reaction mixtures (10 μL) contained 0.1 M Tris-HCl, pH 8.0, 0.25 M KCl, 20 mM $MgCl_2$, 5 mM ATP, an extract, and 8 nmol of either dGMP or 8-oxo-dGMP labeled with ^{32}P (Oeschger & Bessman, 1966). After incubation at 37 °C for various times, the reaction mixtures were applied to TLC plates as described above. One unit of enzyme activity was defined as the amount catalyzing phosphorylation of 1 nmol of nucleotide per minute at 37 °C.

8-Oxo-dGMPase and Other Nucleotidase Assay. Nucleotidase activities were assayed by measuring the formation of inorganic phosphate. The reaction mixture (12.5 μL) contained 20 mM MES−NaOH, pH 6.5, 4 mM $MgCl_2$, 0.25 nmol of 8-oxo-dGMP or other nucleotides, 1 μg of bovine serum albumin, 8 mM DTT, 2% glycerol, and an extract. The reaction was carried out at 37 °C for 20 min and stopped by adding 2.5 μL of 50 mM EDTA. An aliquot (0.5−1 μL) of the reaction mixture was spotted onto a PEI-cellulose F plate and developed with 1 M LiCl (0.7 M for the TMPase assay). Amounts of inorganic phosphate were quantified as described above. One unit of 8-oxo-dGMPase activity was defined as the amount of enzyme that produces 1 pmol of inorganic phosphate per minute at 37 °C.

Purification of 8-Oxo-dGMPase. All operations were carried out at 4 °C. Forty liters of the Jurkat cell culture (3.4×10^{10} cells) was used to prepare a cell-free extract. Frozen cells were suspended in 64 mL of hypotonic buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM DTT and left on ice for 20 min. After protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride and 0.5 μg/mL each of leupeptin, pepstatin, and chymostatin) were added, the suspension was homogenized in a Dounce homogenizer (B pestle). Then 64 mL of ice-cold solution containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose, and 50% glycerol was added, followed by the addition of 16 mL of neutralized saturated ammonium sulfate (pH 7.5), with gentle stirring. The lysate was stirred on ice for 30 min and centrifuged at 330000*g* for 3 h at 2 °C. The supernatant was collected as fraction I (155 mL). The proteins were precipitated with 0.33 g/mL ammonium sulfate and dissolved in 23 mL of buffer A (25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM DTT, and 15% glycerol), and the solution was dialyzed against 4 L of the same buffer. The dialyzed fraction was collected as fraction II (41 mL). Fraction II was applied to a DEAE-Bio-Gel A column (200-mL bed volume; Bio-Rad). After being washed with 530 mL of buffer A, the proteins were eluted with 625 mL of buffer A containing a linear gradient (0−0.5 M) of NaCl. Fractions containing 8-oxo-dGMPase activity were collected as fraction III and dialyzed against two 1-L changes of buffer A. Dialyzed fraction III was applied to a HiTrap heparin column (bed volume, 15 mL). The flow-through fraction (fraction IV) was loaded onto a Q-Sepharose HP column (bed volume, 20 mL). After being washed with 75 mL of buffer A, the proteins were eluted with 75 mL of buffer A with a linear gradient (0−0.5 M) of NaCl. Fractions containing 8-oxo-dGMPase activity were collected as fraction V (3.5 mL) and dialyzed against 1 L of buffer A. Fraction V was further applied to a column of Mono Q HR5/5 (bed volume, 1 mL) and eluted with 30 mL of buffer A with a linear gradient (0−0.4 M) of NaCl. Fractions containing 8-oxo-dGMPase were collected as fraction VI and used for characterization of the enzyme.

Product Analysis. To identify nucleotides, aliquots (45 μL) of the reaction mixture were applied to a Mono Q column and eluted with 20 mL of water with a linear gradient (0−100%) of TEAB at flow rate of 1 mL/min. A Waters μBondapak C18 column (3.9 × 300 mm) was also used for identification of nucleosides. The samples were applied to the column and eluted with 10 mL of water with a linear

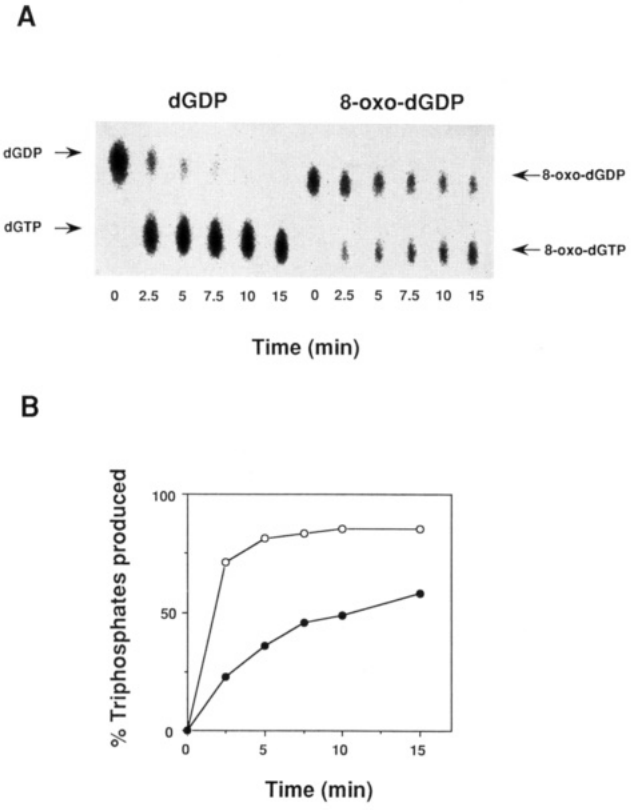

FIGURE 1: Phosphorylation of deoxyguanosine diphosphates by a Jurkat cell-free extract. One nanomole of labeled dGDP or 8-oxo-dGDP was incubated at 37 °C with 70 ng of crude protein extract (fraction I) in a reaction mixture (10 μL) containing 50 mM Tris-HCl, pH 7.5, 2 mM ATP, 25 mM $MgCl_2$, and 10 mM KCl. At the times indicated, 0.5−1-μL aliquots of the mixture were withdrawn and the reaction was terminated by addition of 1 μL of 50 mM EDTA. The samples were developed by TLC, and the amounts of deoxyguanosine triphosphates produced were measured by an image analyzer. (A) Profiles of TLC analyses. (B) Time courses of conversion of deoxyguanosine diphosphates to the corresponding triphosphates: ●, 8-oxo-dGDP; ○, dGDP.

gradient (0−100%) of methanol at flow rate of 0.5 mL/min. Eluates were monitored and quantified by UV absorption.

Other Procedures. 8-Oxo-dGTPase activity was measured as described by Mo et al. (1992). Protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard (Bradford, 1976).

RESULTS

Generation of 8-Oxo-dGTP in the Nucleotide Pool

Phosphorylation of 8-Oxo-dGDP by Nucleoside Diphosphate Kinase. Cellular extracts contain nucleoside diphosphate kinase, an enzyme activity which phosphorylates various nucleoside diphosphates to the corresponding nucleoside triphosphates. Thus, in addition to direct oxidation of dGTP, 8-oxo-dGTP may be generated by phosphorylation of 8-oxo-dGDP. To examine this possibility, radioactive 8-oxo-dGDP was incubated with a Jurkat cell extract in the presence of ATP. As shown in Figure 1A radioactive material migrating at the same position as marker 8-oxo-dGTP was present in the reaction mixture. By HPLC analysis, the labeled compound proved to be 8-oxo-dGTP (data not shown). A time-course analysis revealed that the rate of phosphorylation of 8-oxo-dGDP was one-third that of dGDP (Figure 1B).

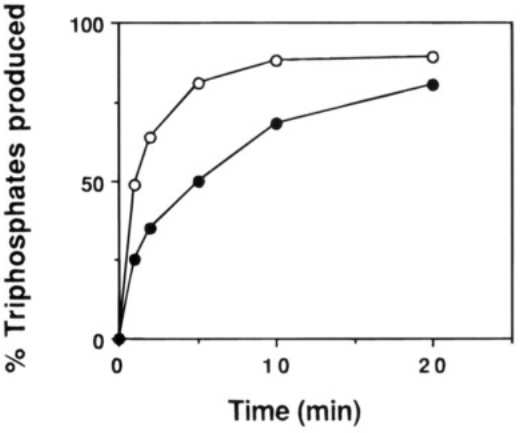

FIGURE 2: Phosphorylation of guanosine diphosphates by a Jurkat cell-free extract. One nanomole of labeled 8-oxo-GDP or GDP was incubated at 37 °C with 70 ng of crude protein extract (fraction I) in a reaction mixture (10 μL) containing 50 mM Tris-HCl, pH 7.5, 2 mM ATP, 25 mM $MgCl_2$, and 10 mM KCl. At the times indicated, 0.5−1-μL aliquots of the mixture were withdrawn and the reaction was terminated by addition of EDTA. ●, 8-oxo-GDP; ○, GDP.

Metabolic Fate of 8-OxoGDP. Deoxyribonucleoside diphosphates are generated by reduction of ribonucleoside diphosphates, and the enzyme catalyzing this reaction, ribonucleoside diphosphate reductase, has no preference for the base moiety (Kornberg & Baker, 1992). Thus, 8-oxo-dGDP may be produced by the enzymic reduction of 8-oxoGDP. However, it was difficult to show this with a crude enzyme preparation, since the Jurkat cell extract contains a powerful nucleoside diphosphate kinase activity. When 8-oxoGDP was incubated with the extract in the presence of ATP, it was rapidly converted to 8-oxoGTP (Figure 2). The rate of phosphorylation of 8-oxoGDP was about one-half that of GDP.

Cleavage of 8-OxoGTP by 8-Oxo-dGTPase. The Jurkat cell extract contains a high level of enzyme activity to degrade 8-oxo-dGTP to 8-oxo-dGMP (Mo et al., 1992). This enzyme appears to act on the ribonucleotide counterpart, 8-oxoGTP, but with less efficiency. Figure 3 shows time courses of cleavage of 8-oxo-dGTP, 8-oxoGTP, and GTP to the corresponding nucleoside monophosphates by the Jurkat cell extract. The rate of hydrolysis of 8-oxoGTP was far less than that of 8-oxo-dGTP, but it was significantly higher than the rate for GTP. A purified preparation of mouse 8-oxo-dGTPase gave a similar result with regard to degradation of the three types of nucleotides (data not shown).

Inability of Human Cell Extract To Phosphorylate 8-Oxo-dGMP

Inability of Cell-Free Extract to Phosphorylate 8-Oxo-dGMP. When dGMP was incubated with a crude cell-free extract in the presence of ATP, the two major radioactive materials generated subsequently proved to be dGDP and dGTP (Figure 4A). When 8-oxo-dGMP was incubated under the same conditions, we detected no phosphorylated compounds, such as 8-oxo-dGDP and 8-oxo-dGTP (Figure 4B); rather, a fast-moving radioactive material was observed in the case of a prolonged incubation. This material was identified as inorganic orthophosphate.

Substrate Specificity of Human Guanylate Kinase. Mammalian cells contain guanylate kinase that phosphorylates

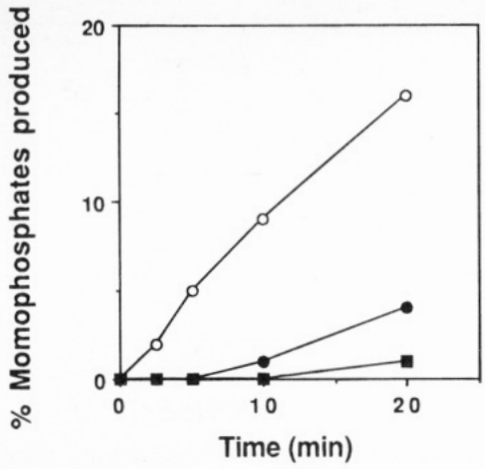

FIGURE 3: Hydrolysis of oxidized guanosine triphosphates by a Jurkat cell-free extract. Two hundred fifty picomoles of labeled guanosine triphosphates was incubated with 0.4 μg of Jurkat cell-free extract protein (fraction I) in a reaction mixture (12.5 μL) containing 20 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 40 mM NaCl, 80 μg of bovine serum albumin per milliliter, 8 mM DTT, and 10% glycerol. The reaction was run at 30 °C and terminated at the times indicated by adding 2.5 μL of 50 mM EDTA. ●, 8-oxo-GTP; ○, 8-oxo-dGTP; ■, GTP.

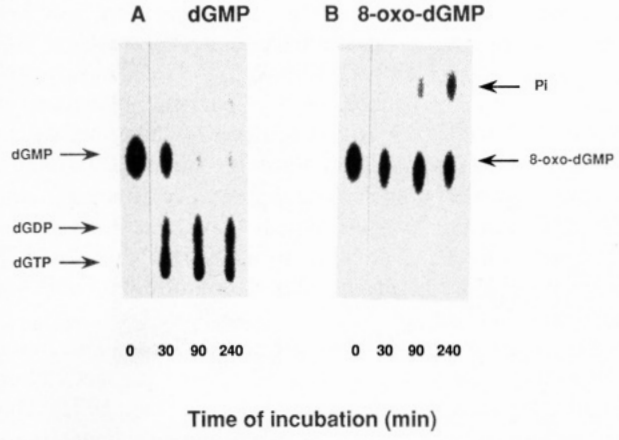

FIGURE 4: Fate of 8-oxo-dGMP on incubation with a Jurkat cell-free extract (fraction I) in the presence of ATP. Eight nanomoles of labeled 8-oxo-dGMP and dGMP was incubated with 2.8 μg of crude extract protein (fraction I) in a reaction mixture (10 μL) containing 0.1 M Tris-HCl, pH 8.0, 0.25 M KCl, 20 mM $MgCl_2$, and 5 mM ATP. When the reaction mixture was incubated for over 30 min, one drop of mineral oil was overlaid on the reaction mixture to avoid evaporation. After incubation at 37 °C for the times indicated, an aliquot (0.5 μL) of the reaction mixture was withdrawn and spotted onto a PEI-cellulose F plate. After development in 1 M LiCl, the radioactivity was measured by an image analyzer. Panel A, dGMP; panel B, 8-oxo-dGMP.

GMP and dGMP to the corresponding nucleoside diphosphates, with almost equal efficiencies (Miech & Parks, 1965). Our findings imply that the guanylate kinase enzyme cannot act on 8-oxo-dGMP. To gain support for this notion, the enzyme was partially purified from Jurkat cells and the substrate specificity was examined. The cell-free extract was subjected to ammonium sulfate precipitation, and the dialyzed fraction was applied to a DEAE-Bio-Gel A column. As shown in Figure 5, the major guanylate kinase activity eluted so as to be completely separable from 8-oxo-dGTPase. Analysis of this 8-oxo-dGTPase-free guanylate kinase preparation was made, and the results are given in Figure 6. dGMP was rapidly converted to dGDP, but there was no evidence of phosphorylation of 8-oxo-dGMP under the same conditions. Quantitative analyses revealed the rate of 8-oxo-dGMP conversion to 8-oxo-dGDP to be less than 1% of that for dGMP.

Characterization of 8-Oxo-dGMPase

Dephosphorylation of 8-Oxo-dGMP. 8-Oxo-dGMP thus produced would have to be dephosphorylated to pass through the cell membrane for excretion. As inorganic phosphate was slowly generated from 8-oxo-dGMP on incubation with a cell-free extract, 8-oxo-deoxyguanosine was probably produced by the reaction. To gain support for this notion, the reaction mixture was applied to a μBondapak C18 column followed by HPLC. A newly generated UV-absorbing material was eluted at the same position as the marker 8-oxo-deoxyguanosine. Thus, human cells do contain an enzyme activity which degrades 8-oxo-dGMP to the corresponding nucleoside.

To characterize the enzyme, we partially purified the 8-oxo-dGMPase activity from Jurkat cells. When the ammonium sulfate fraction derived from the Jurkat cell extract was applied to a Bio-Gel A chromatography column, 8-oxo-dGMPase activity was eluted as a distinct peak between the peaks for guanylate kinase and 8-oxo-dGTPase activities (see Figure 5). The Bio-Gel A fraction was further purified with HiTrap−heparin, Q sepharose, and Mono Q column chromatography (Table 1). When examined using SDS−polyacrylamide gel electrophoresis, the peak enzyme fraction from the Mono Q column was seen to contain as many as 20 proteins (data not shown).

Properties of 8-Oxo-dGMPase. To characterize the enzyme, an active fraction from Mono Q column chromatography (fraction VI) was used in the following experiments. The optimum pH of the enzyme activity was examined in Tris-HCl buffer between pH 9.0 and 7.0 and in MES−NaOH buffer between pH 5.5 and 6.5. Although the optimal pH range was broad, the activity was highest in the range between pH 5.5 and 6.5. The enzyme reaction is absolutely dependent on Mg^{2+}, and the maximal activity was attained at 4−12 mM. Mn^{2+} can substitute for Mg^{2+}, but the activity attained with 4 mM $MnCl_2$ was about 10% of that obtained with 4 mM $MgCl_2$; Ca^{2+} gave no such activity. When 8-oxo-dGMP was incubated with the enzyme (fraction VI), inorganic phosphate and 8-oxo-deoxyguanosine were generated, in a similar fashion (Figure 7). In a gel filtration experiment, calibrated with standard proteins, the molecular mass of the native enzyme was determined to be 40.5 kDa (data not shown).

Inorganic phosphate, one of the reaction products, significantly inhibited the reaction (Table 2). The enzyme activity was inhibited by high concentrations of NaCl and also by relatively low concentrations of ATP.

Substrate Specificity of 8-Oxo-dGMPase. The activities toward various nucleoside monophosphates (at 20 μM) were examined using fraction VI, and the results are shown in Figure 8. 8-Oxo-dGMP was cleaved at a rate approximately twice that for dGMP. TMP and dAMP were also cleaved, but at lower rates. Neither dCMP nor GMP was hydrolyzed by the enzyme.

From Lineweaver−Burk plots, kinetics parameters for various substrates were determined. The K_m value for hydrolysis of 8-oxo-dGMP was 0.25 mM, about 10-fold lower than that for dGMP (3 mM), whereas the V_{max} values

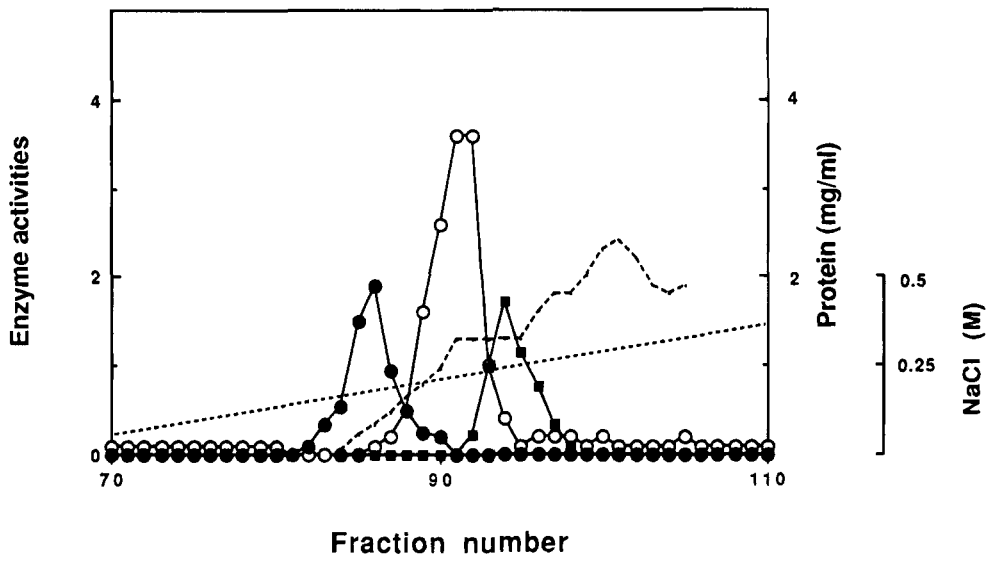

FIGURE 5: Elution profiles of guanylate kinase, 8-oxo-dGMPase, and 8-oxo-dGTPase from a DEAE-Bio-Gel A column. Fraction II was applied to a DEAE-Bio-Gel A column, and proteins were eluted with a linear gradient (0−0.5 M) of NaCl: ●, guanylate kinase activity (10 × unit/μL); ○, 8-oxo-dGMPase activity (unit/μL); ■, 8-oxo-dGTPase activity (0.1 × unit/μL); - - -, protein concentration (mg/mL); • • •, NaCl concentration (M).

Table 1: Purification of 8-Oxo-dGMPase from Jurkat Cells[a]

fraction	total act. ($\times 10^{-3}$ units)	total protein (mg)	sp act. (units/μg)	purification (x-fold)	yield (%)
I. crude extract		868			
II. ammonium sulfate	2040	410	4.9	1.0	100
III. DEAE-Bio-Gel A	1104	35	31	6.3	54
IV. HiTrap heparin	744	2.5	300	61	36
V. Q-Sepharose	240	0.36	670	137	12
VI. Mono Q	79	0.20	400	82	4

[a] Enzyme activity of fraction I was not determined because an inhibitor was present.

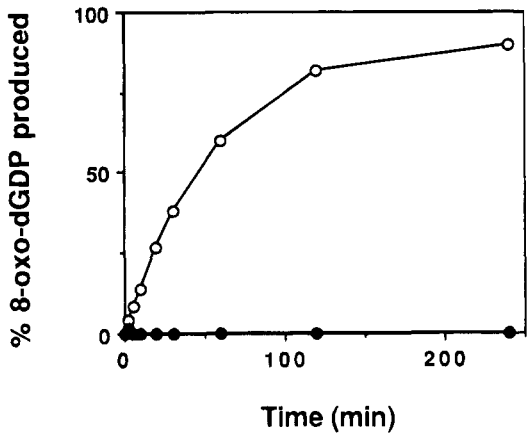

FIGURE 6: Phosphorylation of dGMP and 8-oxo-dGMP by human guanylate kinase. Eight nanomoles of nucleotides was incubated with a partially purified preparation of guanylate kinase (0.2 unit) in a reaction mixture (10 μL) containing 0.1 M Tris-HCl, pH 8.0, 0.25 M KCl, 20 mM $MgCl_2$, and 5 mM ATP. At the times indicated, an aliquot (0.5 μL) of the reaction mixture was withdrawn and analyzed by TLC. When the reaction mixture was incubated for over 10 min, one drop of mineral oil was overlaid on the reaction mixture. After incubation, the aqueous phase was recovered by extraction with 100 μL of a mixture of $CHCl_3$ and distilled water (1:1) and then analyzed by TLC. ●, 8-oxo-dGMP; ○, dGMP.

observed with the two substrates were similar. The K_m values for TMP and dAMP were 2 and 5 mM, respectively. When 40 μM uridine 2′-monophosphate was added to the reaction mixture, dephosphorylation of 8-oxo-dGMP was considerably inhibited. This compound acts as a competitive inhibitor with a K_i value of 20 μM (data not shown).

DISCUSSION

An intriguing question is how cells manage to prevent regeneration of 8-oxo-dGTP from 8-oxo-dGMP, which was produced by the antimutagenic 8-oxo-dGTPase activity. We have shown in the present study that human guanylate kinase is unable to phosphorylate 8-oxo-dGMP. Synthetic analogs for nucleotides are generally converted by cellular salvage pathways to deoxyribonucleoside triphosphates and can compete with the natural nucleotides for base pairing with the template. For instance, 5-bromouracil-containing nucleotide is readily incorporated into DNA to replace the thymine-containing one, and a small but significant incorporation of 8-azaguanine is also detected. Miech and Parks (1965) reported that porcine guanylate kinase phosphorylates 8-azaguanosine monophosphate at a rate about 10% of that for GMP. Therefore, the observation that phosphorylation of 8-oxo-dGMP is strictly prevented may reflect the biological significance for exclusion of this mutagenic substrate from the DNA precursor pool.

The salvage pathway is a mechanism for reutilization of materials generated from degradation of cellular macromolecules, including DNA. Reichard and collaborators (Nicander & Reichard, 1985; Bianchi et al., 1986; Höglund et al., 1991) proposed the "substrate cycle model" that links the salvage pathway and reactions for degradation of nucleotides; that is, the size of the cellular nucleotide pool is regulated by the continual dephosphorylation and rephosphorylation of nucleotides synthesized *de novo*. In the present work, we found that 8-oxo-dGTP is more efficiently dephosphorylated than other nucleoside triphosphates, and

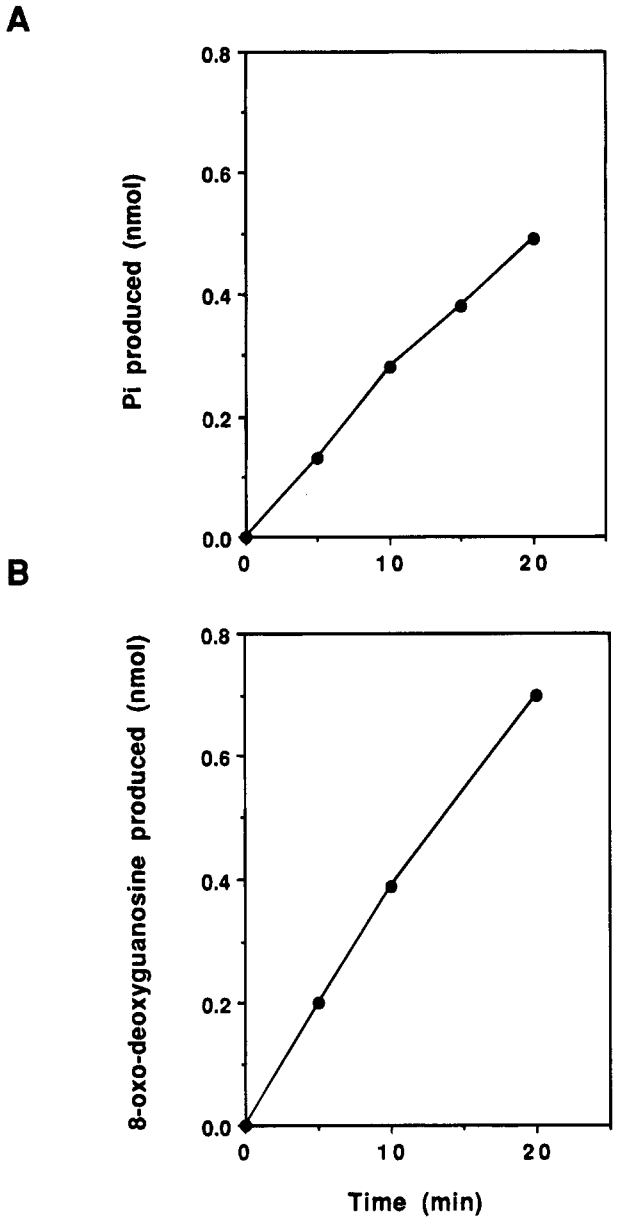

FIGURE 7: Dephosphorylation of 8-oxo-dGMP. Five nanomoles of 8-oxo-dGMP was incubated with 6.6 units of 8-oxo-dGMPase (fraction VI) in a reaction mixture (10 μL) containing 20 mM MES−NaOH, pH 6.5, 4 mM $MgCl_2$, 80 μg of bovine serum albumin per milliliter, 8 mM DTT, 2% glycerol, and an extract. The reaction was carried out at 37 °C for the time indicated. Aliquots (2 μL) of the mixture were withdrawn, and the reaction was terminated by addition of EDTA. A portion of the reaction mixture was used for analysis by TLC to quantify amounts of inorganic phosphate, and the remaining material was applied to a Waters μBondapak C18 column (3.9 × 300 mm) to quantify amounts of 8-oxodeoxyguanosine. Samples on the column were eluted with 10 mL of water with a linear gradient (0−100%) of methanol at a flow rate of 0.5 mL/min. Elutions were monitored and quantified by UV absorption. As a marker, 8-oxodeoxyguanosine was generated from 8-oxo-dGMP by digestion with bacterial alkaline phosphatase. Panel A, amount of inorganic phosphate produced in 2 μL of reaction mixture; panel B, amount of 8-oxodeoxyguanosine produced in 2 μL of reaction mixture.

the resultant 8-oxo-dGMP can be differentiated from deoxyribonucleoside monophosphates with respect to reutilization. Continual dephosphorylation and prevention of rephosphorylation may contribute to elimination of inappropriate nucleotide substrates misgenerated in the cell.

Table 2: Effects of Various Compounds on 8-Oxo-dGMPase Activity[a]

addition	concn (mM)	remaining act. (%)
none		100
ATP	2.5	30
	5.0	10
H_3PO_4	4	5
NaCl	50	60
	100	40
	200	20

[a] 0.6−5.4 units of 8-oxo-dGMPase (fraction VI) was used for each assay.

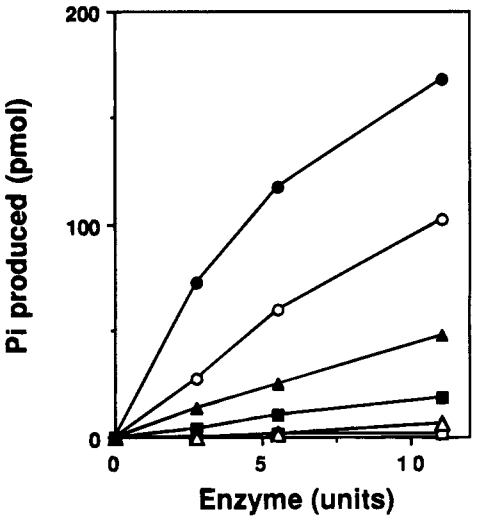

FIGURE 8: Substrate specificity of human 8-oxo-dGMPase. Two hundred fifty picomoles of various nucleotides was incubated with 2.8−11 units of 8-oxo-dGMPase (fraction VI) in a reaction mixture (12.5 μL) containing 20 mM MES−NaOH, pH 6.5, 4 mM $MgCl_2$, 80 μg of bovine serum albumin per milliliter, 8 mM DTT, and 2% glycerol. The reaction was carried out at 37 °C for 20 min and terminated by adding 2.5 μL of 50 mM EDTA. An aliquot (0.5−1 μL) of the reaction mixture was spotted onto a PEI-cellulose F plate and developed with 1 M LiCl (0.7 M for the TMPase assay). Amounts of inorganic phosphate produced were measured by an image analyzer: ●, 8-oxo-dGMP; ○, dGMP; ■, dAMP; ▲, TMP; □, GMP; △, dCMP.

The 8-oxo-dGTPase enzyme that hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP has been isolated from various organisms (Maki & Sekiguchi, 1992; Sakumi et al., 1993; Kamath & Yanofsky, 1993; Bullions et al., 1994). This type of unique cleavage pattern is also observed in the case of elimination of dUTP from the cellular nucleotide pool (Tye et al., 1977). dUTP is not a mutagenic nucleotide; rather, it is a normal precursor for thymidylate biosynthesis. Exclusion of uracil from DNA may be a prerequisite to preventing spontaneous mutations caused by deamination of cytosine residues in DNA (Hayakawa & Sekiguchi, 1978). All other enzymes convert nucleoside triphosphates to diphosphates, and this type of cleavage is restricted to such "sanitizing" enzymes.

A significant amount of 8-oxo-deoxyguanosine is found in mammalian urine. Shigenaga and colleagues (Shigenaga et al., 1989) proposed that urinary 8-oxo-deoxyguanosine may be a biological marker for oxidative stress. In the present work, we obtained evidence that human cells contain an enzyme to degrade 8-oxo-dGMP further to 8-oxo-deoxyguanosine. Nucleosides are readily transported through the cell membrane, and extracellular nucleosides can be excreted into urine. Dephosphorylation of 8-oxo-dGMP may

be an essential step for excretion of 8-oxoguanine-containing materials.

5′(3′)-Nucleotidase has been purified from human placenta and characterized (Höglund & Reichard, 1990), and a similar enzyme activity was found in rat tissues (Fritzson, 1991). The 8-oxo-dGMPase preparation obtained in the present study shows enzymatic properties similar to those of the nucleotidase. The human 5′(3′)-nucleotidase has a native molecular mass of 44−45 kDa and dephosphorylates certain 2′-, 3′-, and 5′-nucleotides with absolute dependence on Mg^{2+}. Uridine 2′-monophosphate proved to be one of the best substrates for the nucleotidase; when uridine 2′-monophosphate was added to a reaction mixture containing 8-oxo-dGMPase, dephosphorylation of 8-oxo-dGMP was dramatically reduced. Thus, the human 5′(3′)-nucleotidase and the 8-oxo-dGMPase may be analogous, or one and the same. Studies are ongoing to characterize these enzymes.

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