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# An Endonuclease Excising 8-Oxo-2'-deoxyguanosine in Regenerating Rat Liver

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**Oxidative DNA damage is generated in every tissue in the body, and is mainly excised by glycosylases. However, endonucleases and exonucleases are suggested as being present in tissues, since oxidized nucleosides such as thymidine glycol and 8-Oxo-2'-deoxyguanosine are detected in urine. For this reason, we studied repair enzymes induced in regenerating rat liver, and detected an enzyme with endonuclease activity by phosphocellulose column chromatography. The enzyme cleaves phosphodiester bonds on the 5' side of 8-oxo-2'-deoxyguanosine nucleotides in DNA. The coexistence of this enzyme and phosphodiesterase II results in the release of 8-oxo-2'-deoxyguanosine monophosphate from calf thymus DNA enriched with 8-oxoguanine by $\gamma$-ray irradiation. This enzyme is found in regenerating rat liver, but not in normal rat liver. The enzyme may have a specific connection to DNA replication.**

**Key words :** oxidative DNA damage; repair; endonuclease; 8-oxo-2'-deoxyguanosine; regenerating rat liver

The accurate transfer of genetic information to daughter cells is very important for maintaining the characteristics of the parent cells. However, cells in aged animals contain a diversity of altered genes[1]. Significant decreases in the fidelities of DNA polymerases and proofreading activity with age are reported as causes for the occurrence of this genetic instability[2,3]. Oxidative damage to DNA bases is also proposed as another main cause of gene mutations[4,5].

More than 20 oxidized DNA bases are known[6], and reactive oxygen species (ROS), such as superoxide radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen, are believed to be responsible for this damage. 8-Oxo-2'-deoxyguanosine (8-oxodG) is one of the most frequent products of DNA base damage induced by oxidation. Mismatched nucleotides are incorporated at very high frequency into positions opposite and adjacent to 8-oxoguanine (8-oxoGua) residues during DNA replication[4,7], if the modified DNA remains unrepaired. Mispairing of 8-oxoGua gives rise to GC to AT transversions[8] because of the predominance of the energetically favored *syn* conformation of 8-oxoGua[9]. The amount of 8-oxodG remains steady at low levels in the tissues of 2- to 24-month-old rats, however, the amount increases progressively in rats older than 27 or 30 months[10,11]. The amount of oxidative DNA damage measured in tissues is thought to reflect the balance between the damage and repair rates. Base excision repair is generally responsible for the removal of non-bulky base adducts. In this process, DNA glycosylase removes the modified base leaving an apurinic or apyrimidinic (AP) site, which is subsequently removed by AP endonuclease[12]. A number of glycosylases for the removal of oxidative base damage have been identified[13], including thymine glycol DNA glycosylase (NTH1; endonuclease III)[14] and 8-oxodG DNA glycosylase (OGG1)[15]. These repair enzymes, furthermore, have been shown to possess both glycosylase and AP lyase activities. The resultant gaps are filled by a DNA polymerase. On the other hand, an endonuclease that gives rise to 8-oxodG-3', 5'-diphosphate as the putative product from DNA, which may subsequently be hydrolyzed to 8-oxodG by nucleotidase(s), has been found in mammals[16-18]. Both oxidized bases such as thymine glycol and 8-oxoGua

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and oxidized nucleosides such as thymidine glycol and 8-oxodG have been detected in the urine of rats and humans. It is recognized that the results of repair are embodied as modified bases and nucleosides in the urine. The measurement of urinary modified bases and nucleosides provides a non-invasive approach to monitoring *in vivo* damage and repair conditions. However, the concentration of oxidized bases in the urine is affected by nucleic acids in the diet[19–21]. It has therefore been proposed that urinary levels of 8-oxodG can be used as a non-invasive monitor of *in vivo* oxidative stress[22]. The origins of urinary 8-oxodG are considered to be 8-oxodGMP excised through the cooperative excision by an endonuclease and an exonuclease from DNA or the dephosphorylation of 8-oxodGTP by hMTH[23] in the nucleotide pool[24]. The subsequent digestion of 8-oxodGMP by a nucleotidase generates 8-oxodG, which can be transported across the cell membrane and excreted in the urine[25]. Therefore, investigations into the role of endonucleases are important to clarify the mechanism for the removal of oxidative DNA damage, but investigations into endonucleases that excise 8-oxodG remain few. The presence of transcription-repair coupling has been reported by several groups[26,27]. Since replicative misreadings lead directly to mutations, a repair system coupled with a replication system must be present in mammals. To clarify how oxidative DNA damage is removed before replication, we investigated 8-oxodG-releasing enzymes in regenerating rat liver in which extensive DNA replication need to be carried out.

## Materials and Methods

***Animals.***

Specific pathogen-free male Wistar strain rats were obtained at 4–6 months of age from the Animal Facility of the Tokyo Metropolitan Institute of Gerontology. Rats were fed *ad libitum* a commercial diet, CRF-1 (Oriental Yeast Co., Tokyo, Japan), and water. Partial hepatectomy was performed by the method of Higgins and Anderson[27]. The regenerating livers were removed from the rats 48 h after partial hepatectomy. All experimental procedures involving animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology.

***Chemicals.***

Phosphodiesterase II from bovine spleen and alkaline phosphatase from calf intestine were purchased from Worthington Biochemical Co. (Freehold, NJ). Exonuclease III from *Escherichia coli* (*E. coli*) was obtained from Toyobo Co. Ltd. (Osaka, Japan). Nuclease P1 and alkaline phosphatase type III from *E. coli* were from Sigma Chemical Co. (St. Louis, MO) and 8-oxodG was from Wako Pure Chemical Industries (Osaka, Japan).

***Quantitation of 8-oxodG in DNA.***

DNA (25 $\mu$g) was digested with nuclease P1 (2 $\mu$g) in 20 mM sodium acetate buffer (pH 4.8) at 37°C for 30 min under an argon atmosphere, and then with alkaline phosphatase (0.65 units) in 100 mM Tris-HCl buffer (pH 7.5) under an argon atmosphere. The resulting mixture was filtered through an Ultrafree-MC filter (Millipore Co., Bedford, MA), and the filtrate was applied to a high-performance liquid chromatography (HPLC) system with a Symmetry C18 column (4.6 × 75 mm; Waters Co., Milford, MA) and an ESA Coulochem II 5200 electrochemical detector (ESA, Bedford, MA) with a guard cell 5020 and an analytical cell 5011. The mobile phase consisted of citrate buffer (12.5 mM, pH 5.1) and methanol (94 : 6, v/v), and the flow rate was 0.8 ml/min. The measurement of 8-oxodG was performed by electrochemical detection (ECD) at an oxidation potential of + 350 mV. The 8-oxodG content is expressed as the molar ratio of 8-oxodG to $10^5$ 2'-deoxyguanosine (dG). The amount of dG was calculated from the absorption at 260 nm in the same measurement.

***Extraction of 8-oxodG-releasing enzyme.***

Regenerating rat liver was homogenized with a teflon-glass homogenizer in 4 volumes of 5 mM Tris-HCl buffer, pH 8.0, containing 0.34 M sucrose, 25 mM KCl, and 5 mM $MgCl_2$. The homogenate was centrifuged at 100,000 × g for 120 min; the supernatant was used as the crude enzyme extract.

***Phosphocellulose column chromatography.***

Crude enzyme extracts (4 ml each) were dialyzed against 10 mM Tris-maleate buffer, pH 6.5, containing 50 mM KCl and 1 mM 2-mercaptoethanol (buffer A) for 5 h. This dialyzed extract (3.5 ml) was applied to a phosphocellulose column (0.5 × 3 cm) equilibrated with buffer A. The column was washed with 2 ml of buffer A and developed with a 30 ml linear gradient of 50 mM to 1.0 M KCl in the same buffer. One milliliter fractions were collected.

***Assay of 8-oxodG-releasing activity.***

Fifty microliters of each eluate was mixed with 75 $\mu$l of 2.5 nmol Tris-HCl buffer, pH 8.3, containing 50 mg of calf thymus DNA exposed to $\gamma$-rays from a [$^{60}$Co] source, 1.0 nmol $MgCl_2$, and 0.5 nmol dithiothreitol. After incubation for 1 h at 37°C, 8-oxodG excising activities in the reaction mixtures were measured by four methods. In Method 1, samples were cooled in ice-cold water without any treatment. In Method 2,

the reaction mixtures were additionally incubated with 0.5 U of alkaline phosphatase for 10 min at 37 °C. In Method 3, the reaction mixtures were further hydrolyzed with 1.0 U of exonuclease III for 5 min at 37 °C, and then treated with 0.5 U of alkaline phosphatase for 10 min at 37 °C. In Method 4, the reaction mixtures were further treated with 0.05 U of phosphodiesterase II for 10 min at 37 °C, and then treated with 0.5 U of alkaline phosphatase for 10 min at 37 °C. In all cases, the reactions were stopped by cooling in ice-cold water. The samples were centrifuged using a 10,000 NMWL Filter Unit of an Ultrafree-MC (Millipore Co, Bedford, MA) to remove enzymes and high molecular weight DNA. The amounts of 8-oxodG in the filtrates were measured by HPLC/ECD as described above.

## Results

### *Oxidation of calf thymus DNA by γ-ray irradiation*

Aqueous solutions of calf thymus DNA (1 mg/ml) were irradiated with 100, 200, 400, or 600 Gy γ-rays from a [$^{60}$Co] source to prepare oxidatively damaged substrate DNA. The amounts of 8-oxodG in the DNA increased linearly up to a dose of 200 Gy and then reached a plateau at higher doses of irradiation (Fig. 1). The amount of 8-oxodG in DNA irradiated with 600 Gy γ-rays was about 690 8-oxodG / $10^5$ dG (0.7 %). We used this γ-ray-irradiated DNA as a substrate in this study.

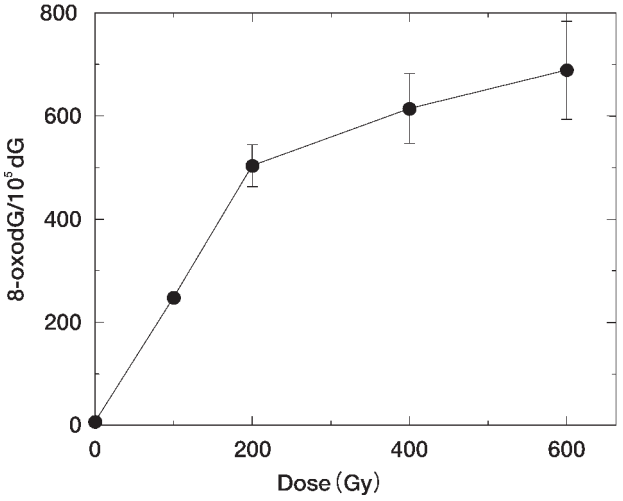


Fig. 1 Formation of 8-oxodG in calf thymus DNA irradiated with γ-rays. An aqueous solution of calf thymus DNA was irradiated with various doses of γ-rays. After irradiation, the amount of 8-oxodG was measured using an HPLC/ECD system. Points and bars represent means and SD of four independent irradiations.

### *Separation of 8-oxodG-releasing enzymes from regenerating rat liver*

DNA replication should occur at high frequency in regenerating liver, so multiple deoxyribonucleases should be present in regenerating liver. We attempted to detect 8-oxodG-releasing enzymes in regenerating rat liver. It is necessary for the activities of individual 8-oxodG deoxyribonucleases to be measured separately; therefore, we attempted to separate deoxyribonucleases by phosphocellulose column chromatography. In this study, deoxyribonuclease activities for 8-oxodG excision were measured by four methods. In Method 1, γ-ray-irradiated calf thymus DNA was treated with fractions eluted from phosphocellulose column chromatography. In this method, if an endonuclease, exonuclease, and phosphatase activity are present in the same fraction, free 8-oxodG should be excised from the substrate DNA. In the pass-through fractions, 8-oxodG-releasing activity (Fractions 3-5; Peak-1) was observed (Fig. 2A). When the substrate DNA was treated with alkaline phosphatase in addition to treatment with the eluate fractions (Method 2), three small peaks, Peak-1 (Fractions 3-5), Peak-2 (Fractions 9-13), and Peak-3 (Fractions 13-21), were observed (Fig. 2B). The enzymes contained in Peak-2 and Peak-3 may be nucleases that release 8-oxodG mono- or di-phosphates from DNA, and were not found in eluates (Method 1) not subjected to additional digestion. To detect an endonuclease that cleaves on the 3' side of 8-oxodG residues, the substrate DNA was treated first with the eluate fractions, and then with exonuclease III. If an enzyme that cleaves the 3' side of 8-oxodG residues is present in the fractions, 8-oxodG mononucleotides will be released from the sites excised by the endonuclease. Finally, 8-oxodG is produced by treatment with alkaline phosphatase (Method 3). An increase in Peak-1 was found by Method 3 as shown in Fig. 2C. The maximum amount of 8-oxodG released from the substrate DNA was observed following treatment with eluates (Fractions 9-13), phosphodiesterase II from bovine spleen as a 5' →3' exonuclease, and alkaline phosphatase (Method 4; Fig. 2D). This indicates the presence of an endonuclease that digests on the 5' side of 8-oxodG residues. The increase in Peak-2 that occurs by Method 4 was not observed in normal rat liver.

## Discussion

ROS are produced in the course of normal metabolism, such as during respiration and phagocytosis[28], and by exogenous factors such as radiation and ultraviolet light[29]. ROS cause a variety of damage, includ-

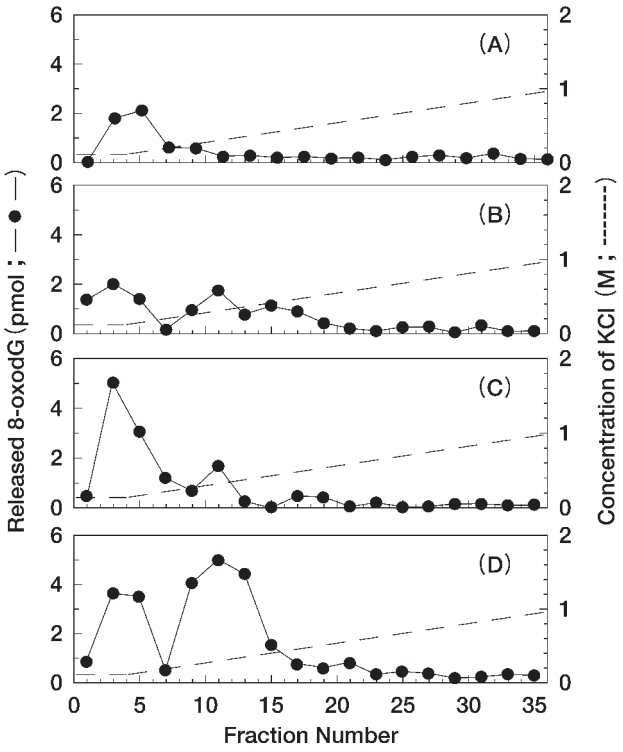


Fig. 2 Elution profiles of enzymes that release 8-oxodG (A) or 8-oxodG mono- or diphosphate (B) or cleave on the 3'- (C) or 5'- (D) side of 8-oxodG residues in fractions eluted from phosphocellulose column chromatography of extracts of regenerating rat liver homogenates. The eluted fractions were treated with the substrate DNA (A) and then with alkaline phosphatase (B), exonuclease III and alkaline phosphatase (C), or phosphodiesterase II and alkaline phosphatase (D). The 8-oxodG-releasing activity was estimated from the amount of free 8-oxodG released by treatment as described in Materials and Methods. The concentration of KCl in the elution buffer is shown by the broken line.

ing base and sugar modifications, covalent crosslinks with proteins and single- and double-strand breaks, to nuclear and mitochondrial DNA. Damage to bases and sugars is repaired by endonucleases and glycosylases, and the products are excreted into the urine in the form of nucleosides and bases.

Base excision repair is mainly responsible for the removal of non-bulky base modifications, involving specialized enzymes that recognize specific lesions. In this process, a DNA glycosylase removes the modified base leaving an apurinic-apyrimidinic (AP) site (AP-deoxyribose), which is subsequently removed by two AP endonucleases that incise on the 3' (AP lyase) and 5' (AP hydrolase) sides of the AP site[12]. However, some repair enzymes have been shown to possess both glycosylase and AP lyase activities. The resultant gap is then filled by a DNA polymerase.

The modified base 8-oxoGua and its deoxynucleoside derivative 8-oxodG have been the targets of intense investigation. The OGG1 protein removes 8-oxoGua from 8-oxoGua・C pairs produced in DNA by oxidative damage[15]. The MYH protein removes adenine from 8-oxoGua・A pairs produced by misreading during replication; the remaining 8-oxoGua is paired with C by DNA polymerase $\beta$, and then 8-oxoGua is finally released by the OGG1 protein from the 8-oxoGua・C pairs[30]. Furthermore, the MTH1 protein specifically dephosphorylates 8-oxodGTP in the nucleotide pool and produces the 8-oxodG monophosphate[31]. If oxidatively damaged nucleotides, such as 8-oxodGTP or 2-hydroxydeoxyadenine triphosphates, are not removed from the nucleotide pool, they are misincorporated by DNA polymerases[32]. In mammalian cell repair systems, OGG1, MYH, and MTH1 all play important roles in the release of 8-oxoGua. Although an endonuclease that excises 8-oxodG residues in DNA has been reported[18], the endonuclease has also been pointed out to have glycosylase and lyase activities. For example, OGG1 has been reported to have an AP endonuclease activity[33]. The presence of 8-oxodG in the urine suggests the presence of an endonuclease cleaving bonds near 8-oxodG in DNA as well as hMTH in the nucleotide pool. Therefore, we attempted to detect endonucleases against 8-oxodG in regenerating rat liver, in which repair systems must be activated.

Partial hepatectomy causes the remaining liver tissue to enter a proliferative state. It has been reported that the amounts of 8-oxodG in the liver decrease in the early stages of liver regeneration after partial hepatectomy, despite the enhancement in the susceptibility to oxidative stress of the DNA in regenerating liver[34]. The endonuclease activity, that cleaves phosphodiester bonds on both sides (5' and 3' sides) of nucleotides containing 8-oxoGua residues to generate a one-nucleotide gap[32] is very rare in regenerating rat liver as shown in Fig. 2B. Deoxynucleoside monophosphates are generally excised through the cooperation of endonucleases and exonucleases. Thus, to detect other endonucleases that cleave bonds adjacent to nucleotides containing 8-oxoGua residues, the substrate DNA treated with the eluate fraction was further digested with exonuclease III or phosphodiesterase II, and then treated with alkaline phosphatase to prepare nucleosides. The activities in Peak-1 treated with either of the two exonucleases increased by about 2-fold (Figs. 2C & 2D). Fractions 3-5 may contain an endonuclease that hydrolyzes on the 3' or 5'

side of 8-oxodG residues in DNA. However, this peak was also found by Methods 1 and 2 without exonuclease treatment (Figs. 2A & 2B). No attempt was made to purify the enzymes in Peak-1 further in this study, because this fraction is the pass-through fraction and often not sufficiently resolved. The activity in Peak-2 following treatment with fractions 9-13, phosphodiesterase II, and alkaline phosphatase (Fig. 2D) is about 7-fold higher than that observed after treatment with fractions 9-13 and alkaline phosphatase (Fig. 2B) or fractions 9-13, exonuclease III, and alkaline phosphatase (Fig. 2C). No endonuclease that releases 8-oxodG through the cooperative action of a 5'→3' exonuclease, such as phosphodiesterase II, and alkaline phosphatase has yet been reported. These results indicate that the activity of the 8-oxodG endonuclease reported previously is very low in regenerating liver, but that an 8-oxodG-releasing enzyme that cooperates with a 5'→3' exonuclease is present. This endonuclease activity has not been observed in normal liver. This 8-oxodG-releasing enzyme may induce the excision of 8-oxodG from DNA before replication to prevent mutations, while the 8-oxodG endonuclease reported previously may act during usual DNA repair. The detection of endonucleases cleaving phosphodiester bonds on the sides of nucleotides containing 8-oxoGua residues has been reported based on electrophoretic observations using synthetic oligonucleotides containing a single 8-oxoGua at a defined position as the substrate[18,35,36]. However, this method is unable to distinguish between an endonuclease activity and a combination of glycosylase and AP lyase activities. On the other hand, an endonuclease that cleaves bonds near 8-oxodG residues is confirmed in our method, since excised 8-oxodG is directly observed by the HPLC/ECD system. The 8-oxodG-releasing enzyme found in this study need to be purified and its characteristics clarified.

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# ラット再生肝における8-オキソ-2'-デオキシグアノシン除去エンドヌクレアーゼ

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DNA酸化傷害は身体中のあらゆる細胞で発生し，主にグリコシラーゼ類によって除去されている．しかし，チミジングリコールや8-オキソ-2'-デオキシグアノシンのような酸化ヌクレオシドも尿中に排泄されてきて検出されるので，エンドヌクレアーゼとエキソヌクレアーゼが組織中に存在することが示唆される．本研究は複製が活発に行われる再生肝を用いて，エンドヌクレアーゼ活性を持つ修復酵素をホスホセルロースカラムによって分離して検出することを試みた．その結果，再生肝中にDNA中の8-オキソ-2'-デオキシグアノシンヌクレオシドの5'側のホスホジエステル結合を切断する活性があることが判明した．本酵素とホスホジエステラーゼIIを共存させると，γ-線を照射して8-オキソグアニンを豊富に含む子ウシ胸腺DNAから，8-オキソ-2'-デオキシグアノシンが放出された．本酵素はラット再生肝では認められたが，通常のラット肝では認められなかった．本酵素は，DNA複製と特異的に関連しているのかもしれない．

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