in male mice [4]. In rat, co-administration of $NaNO_2$ with PG promoted forestomach carcinogenesis after initiation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [5]. It is reported that PG is not easily excreted and tends to accumulate in the body [6]. As measured by the Salmonella/microsome mutagenesis assay, PG caused an enhancement of the mutagenic activities of the carcinogens *N*-hydroxy-2-acetylaminofluorene and 4-nitroquinoline 1-oxide [7]. PG induced sister-chromatid exchanges and chromosomal aberrations in CHO-K1 cells [8]. These reports have suggested that accumulation of PG may contribute to carcinogenesis. However, the mechanism leading to carcinogenesis has not yet been clarified.

To investigate the ability of PG to cause oxidative DNA damage, the amount of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a characteristic oxidative DNA lesion, induced by PG was measured in a human leukemia cell line, HL-60, and its hydrogen peroxide (H_2O_2)-resistant clone HP100 by using an electrochemical detector coupled to high-performance liquid chromatography (HPLC-ECD). 8-OxodG is known to cause DNA misreplication resulting in mutation or cancer [9,10]. It has been reported that PG in the hepatocyte suspensions is converted to gallic acid (GA) [6]. To make sure that PG is converted to GA, we analyzed products generated from PG incubated with esterase by using an HPLC equipped with a photodiode array. Furthermore, to clarify the mechanism of carcinogenesis by PG, we examined the DNA damage caused by PG, esterase-treated PG and GA in the presence of metal ions, using ^{32}P-5'-end-labeled DNA fragments obtained from the human *p16* and *p53* tumor suppressor genes. In addition, we analyzed the formation of 8-oxodG in calf thymus DNA caused by GA in the presence of metal ions.

2. Materials and methods

2.1. Materials

Restriction enzymes and calf intestine phosphatase (CIP) were purchased from Boehringer Mannheim GmbH (Germany). T4 polynucleotide kinase was from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP was from New England Nuclear (Boston, MA). Diethylenetriamine-*N*,*N*,*N'*,*N''*,*N''*-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemical Co. (Kumamoto, Japan). Acrylamide, dimethylsulfoxide (DMSO), bisacrylamide, piperidine, PG, and GA were from Wako Pure Chemical Industries (Osaka, Japan). $CuCl_2$, ethanol, D-mannitol, sodium formate, and proteinase K were from Nacalai Tesque (Kyoto, Japan). Calf thymus DNA, bacterial alkaline phosphatase (BAP, from *Escherichia coli*), superoxide dismutase (SOD, 3000 U/mg from bovine erythrocytes), catalase (45,000 U/mg from bovine liver), esterase (250 U/mg from porcine liver), and RNase A were from Sigma Chemical Co. (St. Louis, MO). Nuclease P1 was from Yamasa Shoyu Co. (Chiba, Japan). Lysis buffer for DNA extraction was from Applied Biosystems (Foster City, CA).

2.2. Measurement of 8-oxodG in cultured cell

Human leukemia HL-60 cells and its H_2O_2-resistant clone HP100 were grown in RPMI 1640 supplemented with 6% fetal bovine serum at 37 °C under 5% CO_2 in a humidified atmosphere. Cells (10^6 cells/ml) were incubated with PG for 2 h at 37 °C and immediately washed three times with phosphate-buffered saline. DNA was extracted under anaerobic condition digested to component nucleosides with nuclease P_1 and BAP and analyzed by HPLC-ECD [11,12].

2.3. Analysis of PG and its metabolite by HPLC

The reaction mixture containing 100 μM PG and 0.625 U esterase in 50 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA was incubated at 37 °C. To analyze the products generated from PG incubated with esterase, HPLC was carried out on an LC-10A HPLC system (Shimadzu, Kyoto, Japan) using a Cosmosil column (Nacalai Tesque, 4.6 mm internal diameter × 150 mm), flow rate of 1 ml/min, linear gradient in 12.5 min of 0–50% acetonitrile. The HPLC eluate was routed directly into a photodiode array UV-Vis detector (SPD-M10A, Shimadzu) and the spectrum of the eluate was measured [13].

2.4. Preparation of ^{32}P-5'-end-labeled DNA fragments

Two fragments containing exon 1 or 2 of the human *p16* tumor suppressor gene [14] were obtained as described previously [15]. The 5'-end-labeled 490 bp

fragment (*Eco*RI* 5841–*Eco*RI* 6330) containing exon 1 was further digested with *Mro*I to obtain the singly labeled 328 bp fragment (*Eco*RI* 5841–*Mro*I 6168) and the 158 bp fragment (*Mro*I 6173–*Eco*RI* 6330). The 5′-end-labeled 460 bp fragment (*Eco*RI* 9481–*Eco*RI* 9940) containing exon 2 was also further digested with *Bss*H II to obtain the singly labeled 309 bp fragment (*Eco*RI* 9481–*Bss*H II 9789), and the 147 bp fragment (*Bss*H II 9794–*Eco*RI* 9940).

DNA fragments were also obtained from the human *p53* tumor suppressor gene [16]. The ^{32}P-5′-end-labeled 650 bp (*Hin*dIII* 13972–*Eco*RI* 14621) and 460 bp (*Hin*dIII* 13038–*Eco*RI* 13507) fragments were obtained as described previously [17]. The 650 bp fragment was digested with *Apa*I to obtain the singly labeled 211 bp (*Hin*dIII* 13972–*Apa*I 14182) and 443 bp (*Apa*I 14179–*Eco*RI* 14621) DNA fragments. The 460 bp fragment was digested with *Sty*I to obtain the singly labeled 118 bp (*Hin*dIII* 13038–*Sty*I 13155) and 348 bp (*Sty*I 13160–*Eco*RI* 13507) fragments. For reference, nucleotide numbering starts with the Bam HI site [18]. An asterisk indicates ^{32}P-labeling.

2.5. Detection of DNA damage caused by GA in the presence of metal ions

The standard reaction mixture contained GA, ^{32}P-5′-end-labeled DNA fragments, 20 μM/base calf thymus DNA and 20 μM metal ions in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37 °C for 1 h, the DNA fragments were heated at 90 °C in 1 M piperidine for 20 min.

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure [19] using a DNA-sequencing system (LKB 2010 Macrophor, Pharmacia Biotech, Uppsala, Sweden). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech).

2.6. Analysis of formation of 8-oxodG in calf thymus DNA by GA in the presence of metal ions

The amounts of 8-oxodG were measured by modified methods of Kasai et al. [20]. Calf thymus DNA (100 μM/base) and 20 μM metal ($CuCl_2$, Fe(III)EDTA or Fe(III)ADP) were incubated with GA, in 200 μl of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA for 1 h at 37 °C. After ethanol precipitation, DNA fragments were digested to individual nucleosides with nuclease P1 and CIP, and analyzed with an HPLC-ECD, as described previously [11].

2.7. UV-Vis spectra measurement during autooxidation of GA and PG

UV-Vis spectra of GA and PG were measured with a UV-Vis spectrometer (UV-2500PC, Shimadzu, Kyoto). The reaction mixture contained 2 mM GA or PG and 200 μM $CuCl_2$ or Fe(III)EDTA in 10 mM phosphate buffer (pH 7.8). The spectra of the mixtures were measured repeatedly at 37 °C for the indicated duration.

2.8. Ab initio molecular orbital calculation of GA and PG

Energies of highest occupied molecular orbital (HOMO) of GA and PG were estimated from ab initio molecular orbital (MO) calculation at Hartree–Fock 6-31G* level. The calculations were performed using Spartan 02′ for Windows (Wavefunction Inc., CA) as previously reported [21].

3. Results

3.1. Formation of 8-oxodG in human cultured cells by PG

Fig. 1 compared 8-oxodG formation in HL-60 cells and HP100 cells, which are an H_2O_2-resistant clone of HL-60 cells. The content of 8-oxodG of DNA in HL-60 cells treated with 200 and 500 μM PG was significantly increased in comparison with no treated cells, whereas PG did not significantly increase the amount of 8-oxodG in HP100 cells. Catalase activity of HP100 cells is 18 times higher than that of parent HL-60 cells [22]. These findings suggested that the generation of H_2O_2 plays a critical role in PG-induced DNA damage.

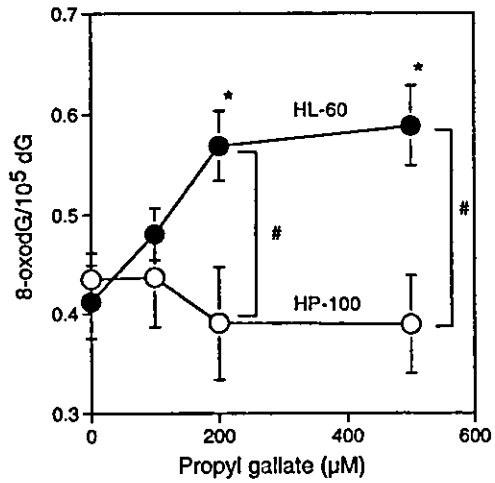

Fig. 1. Comparison of 8-oxodG formation in HL-60 and HP100 cells treated with PG. HL-60 (●) and HP100 (○) cells (10^6 cells/ml) were incubated with PG for 2 h at 37 °C and the DNA was extracted immediately. DNA was digested to nucleosides enzymatically and 8-oxodG content was analyzed by HPLC-ECD, as described in Section 2. Results are expressed as mean ± S.E. of values obtained from six independent experiments. Symbols indicate significant differences compared with control (*: $P < 0.05$) and significant differences between HL-60 and HP100 at the same dose of PG (#: $P < 0.05$) by *t*-test.

3.2. Identification of a product generated from PG treated with esterase

The product generated from PG treated with esterase was analyzed with an HPLC equipped with a photodiode array. The product eluting at 3.0 min showed a maximum absorption at 272 nm (Fig. 2). This product was identified to be GA based on its HPLC elution profile and UV spectral properties. The amounts of GA generated by PG plus esterase were measured with passage of time. When PG was incubated with esterase for 1 and 3 h, 40 and 70% of PG were converted into GA, respectively.

3.3. Damage to ^{32}P-labeled DNA fragments by PG, PG with esterase, and GA in the presence of metal ions

PG induced no or little DNA damage in the presence of metal ions (Fig. 3A). However, when PG

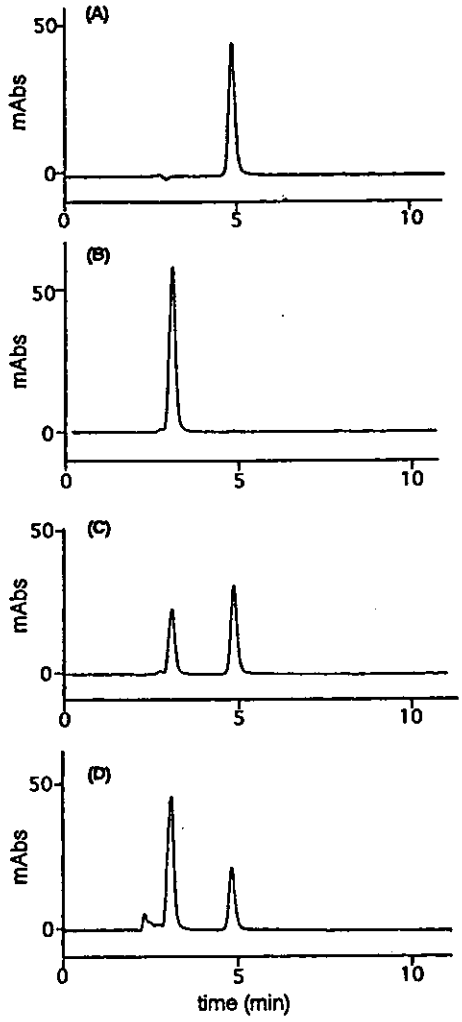

Fig. 2. HPLC chromatograms of PG, GA, and PG treated with esterase. (A) Chromatogram of authentic PG. The mixture containing 100 μM PG in 50 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA was analyzed with an HPLC as described in Section 2. (B) Chromatogram of authentic GA. The mixture containing 100 μM GA in 50 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA was analyzed with an HPLC. (C) Chromatogram of PG incubated with esterase for 1 h. The mixture containing 100 μM PG in 50 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA was incubated with 0.625 U esterase for 1 h at 37 °C and analyzed with an HPLC. (D) Chromatogram of PG incubated with esterase for 3 h. The mixture containing 100 μM PG in 50 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA was incubated with 0.625 U esterase for 3 h at 37 °C and analyzed with an HPLC.

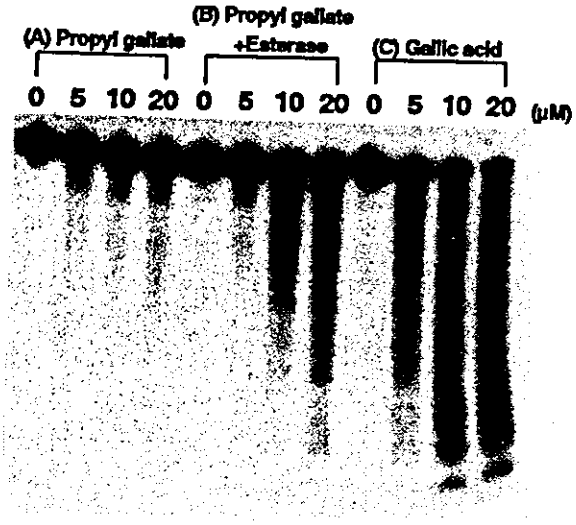

Fig. 3. Damage to ^{32}P-labeled DNA fragments by PG, esterase-treated PG, and GA in the presence of Cu(II). (A) The reaction mixtures containing ^{32}P-5′-end-labeled DNA fragments, 20 μM/base calf thymus DNA, indicated concentrations of PG and 20 μM $CuCl_2$ were incubated for 1 h at 37 °C. (B) The reaction mixtures containing indicated concentrations of PG and 0.625 U esterase in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 3 h at 37 °C. After preincubation, ^{32}P-5′-end labeled DNA fragments, 20 μM/base calf thymus DNA and 20 μM $CuCl_2$ were added to the mixtures, followed by the incubation for 1 h at 37 °C. (C) The reaction mixtures containing ^{32}P-5′-end labeled DNA fragments, 20 μM/base calf thymus DNA, indicated concentrations of GA and 20 μM $CuCl_2$ were incubated for 1 h at 37 °C. Subsequently, DNA fragments were treated with 1 M piperidine for 20 min at 90 °C, then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was visualized by exposing an X-ray film to the gel.

was incubated with esterase, DNA damage was observed in the presence of Cu(II) (Fig. 3B). GA also induced DNA damage in a dose-dependent manner in the presence of Cu(II) (Fig. 3C). Piperidine treatment enhanced DNA cleavage by GA, suggesting that GA plus Cu(II) caused not only direct breakage of the deoxyribose phosphate backbone but also base modification (data not shown). Similar results was obtained with GA in the presence of Fe(III)EDTA. Direct breakage and piperidine-sensitive base modification caused by GA plus Cu(II) were stronger than those by GA plus Fe(III)EDTA (data not shown).

3.4. Effects of scavengers and metal chelators on DNA damage induced by GA in the presence of metal ions

In the presence of Fe(III)EDTA, DNA damage induced by GA was inhibited by free hydroxyl radical (•OH) scavengers such as ethanol, mannitol, sodium formate, DMSO and methional (Fig. 4A). Catalase and deferoxamine mesylate, an iron chelating agent, also inhibited the DNA damage (Fig. 4A). SOD did not inhibit the DNA damage (Fig. 4A). These results indicated that •OH, H_2O_2, and iron participated in the Fe(III)EDTA-mediated DNA damage.

In the presence of Cu(II), •OH scavengers showed little or no inhibitory effect on DNA damage by GA (Fig. 4B). However, the DNA damage was inhibited by methional (Fig. 4B), which can scavenge not only •OH but also species with weaker reactivity than •OH [23]. Catalase and bathocuproine, a Cu(I) chelator, inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I) (Fig. 4B). From these results, we speculated that reactive oxygen species such as DNA-copper–hydroperoxo complex participated in the Cu(II)-mediated DNA damage caused by GA.

3.5. Site specificity of DNA cleavage by GA in the presence of metal ions

Fig. 5 shows the patterns of DNA damage induced by GA in the presence of Fe(III)EDTA or Cu(II). The relative intensity of DNA damage was obtained by scanning autoradiogram with a laser densitometer. In the presence of Fe(III)EDTA, GA caused DNA damage at every nucleotide in DNA fragments treated with piperidine (Fig. 5A). In the presence of Cu(II), DNA damage caused by GA occurred frequently at thymine and cytosine with piperidine treatment (Fig. 5B).

3.6. Formation of 8-oxodG in calf thymus DNA by GA in the presence of metal ions

We measured the 8-oxodG content in calf thymus DNA incubated with GA. As shown in Fig. 6, GA increased formation of 8-oxodG in the presence of metal ions. At GA concentrations above 50 μM, Cu(II) led to the formation of 8-oxodG more efficiently than

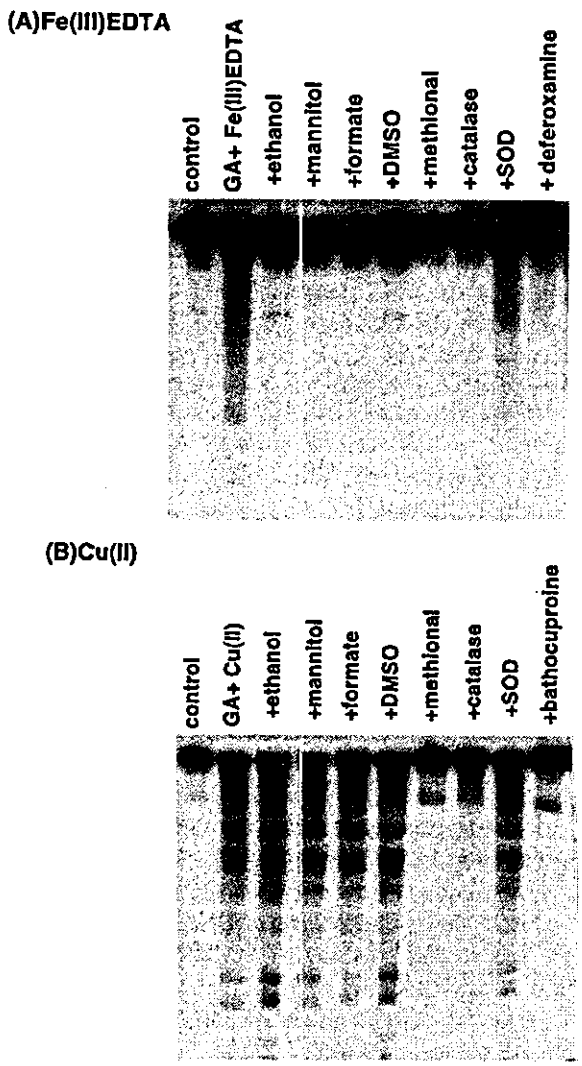

Fig. 4. Effects of scavengers and metal chelators on DNA damage induced by GA in the presence of Cu(II) and Fe(III)EDTA. Reaction mixtures contained the ^{32}P-5′-end-labeled DNA fragments, 20 μM/base calf thymus DNA, 200 μM (A) or 20 μM (B) GA and 20 μM Fe(III)EDTA (A) or $CuCl_2$ (B) in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were incubated for 1 h at 37 °C. DNA fragments were treated with 1 M piperidine for 20 min at 90 °C, then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was visualized by exposing an X-ray film to the gel. The concentration of scavengers and metal chelators was as follows, 0.8 M ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.8 M DMSO, 0.1 M methional, 30 U catalase, 30 U SOD, 20 μM bathocuproine, and 1 mM deferoxamine.

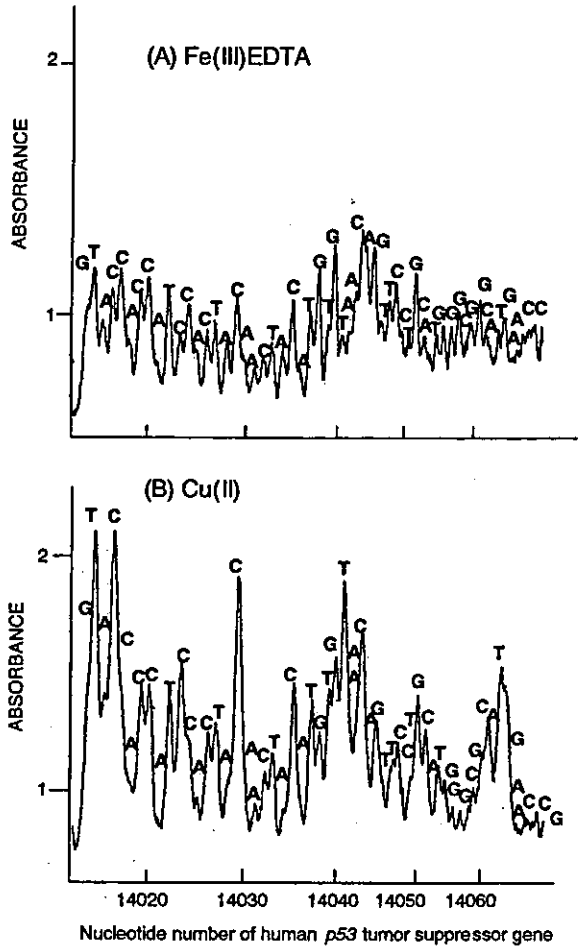

Fig. 5. Site specificity of DNA damage induced by GA in the presence of Cu(II) and Fe(III)EDTA. Reaction mixtures contained the ^{32}P-5′-end-labeled 211 bp DNA fragment (*Hin*dIII* 13972–*Apa*I 14182), 20 μM/base of calf thymus DNA, 200 μM (A) or 20 μM (B) GA and 20 μM Fe(III)EDTA (A) or $CuCl_2$ (B) in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were incubated for 1 h at 37 °C. Following piperidine treatment, the DNA fragments were analyzed as described in Section 2.

Fe(III)EDTA. However, GA led to the formation of 8-oxodG in the presence of Fe(III)EDTA as much as that in the presence of Cu(II) at concentrations below 50 μM. The level of 8-oxodG also increased depending on the concentrations of GA in the presence of Fe(III)ADP. PG did not increase the level of 8-oxodG in the presence of metal ions (data not shown).

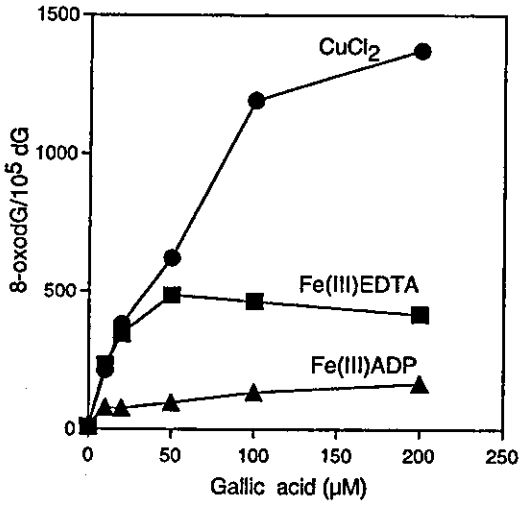

Fig. 6. Formation of 8-oxodG by GA in the presence of metal ions. Reaction mixtures contained 100 μM/base of calf thymus DNA, GA and 20 μM metal ion ($CuCl_2$, Fe(III)EDTA or Fe(III)ADP) was incubated at 37 °C for 1 h. After ethanol precipitation, the DNA was subjected to enzyme digestion and analyzed by HPLC-ECD as described in Section 2.

3.7. UV-Vis spectroscopic study on the autooxidation of GA and PG

The absorption spectra of GA and PG were red-shifted immediately by the addition of $CuCl_2$, possibly due to a complex formation with copper ion. Their absorption bands at ca. 420 nm gradually increased in the presence of Cu(II) (Fig. 7). It has been reported that oxidation of pyrogallol derivatives produce *o*-benzoquinone derivatives, which show characteristic absorption spectrum at ca. 420 nm [24,25]. Therefore, it is reasonably considered that GA and PG, pyrogallol derivatives, produce their corresponding *o*-benzoquinone derivatives via Cu(II)-mediated autooxidation, although a possibility of the formation of the stable one-electron oxidative products cannot be neglected. The spectra changes showed that the oxidation rate of GA is much faster than that of PG. Similar spectra changes were observed in the presence of Fe(III)EDTA (data not shown). Autooxidation of GA mediated by Fe(III)EDTA was faster than that by Cu(II).

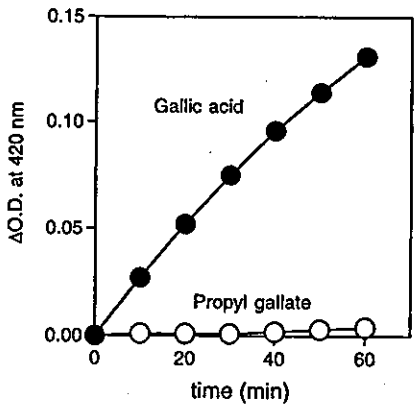

Fig. 7. Increase of absorbance of GA and PG during their autooxidation. Reaction mixture contained 2 mM GA or PG and 200 μM $CuCl_2$ in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The absorption spectra were measured every 10 min for 60 min with a UV-Vis spectrometer at 37 °C. The increments of absorbance at 420 nm (ΔO.D.) were plotted against the reaction time.

3.8. Calculated energies of the HOMO of GA and PG

The ab initio MO calculation has indicated that the HOMO of these compounds are localized on their phenyl rings. Since pK_a of GA is below 3.4 [26], GA becomes anion form in this experimental condition (pH 7.8). The HOMO energy of anion form of GA (4.71 eV) is smaller than that of PG (8.53 eV). This calculation study suggested that anion form of GA, a hydrolysis product of PG, easily undergo the oxidation rather than the parent PG.

4. Discussion

In this study, we demonstrated that PG significantly increased 8-oxodG formation in HL-60 cells, but did not increased it in HP100 cells. The catalase activity of HP100 cells was 18 times higher than that of HL-60 cells [22]. Therefore, it is suggested that generation of H_2O_2 plays an important role in PG-induced 8-oxodG formation in human cultured cells. The DNA base damage 8-oxodG formation, is a prominent indicator of oxidative stress and has been well-characterized as a premutagenic lesion in mammalian cells. Numerous studies have indicated that the formation of 8-oxodG causes misreplication of DNA that may lead to mutation or cancer [9,10]. It has been reported that guanine to thymine transversion is the most common mispairing-type mutation produced by 8-oxodG in site

specificity assays [9,27]. It is reasonably considered that oxidative DNA damage participates in carcinogenesis induced by PG.

Although PG increased 8-oxodG formation in HL-60 cells, PG itself did not increased the level of 8-oxodG in isolated calf thymus DNA in the presence of metal ions. To clarify the mechanism of cellular DNA damage induced by PG, we investigated formation of 8-oxodG induced by GA in calf thymus DNA. PG is hydrolyzed enzymatically to GA by cellular carboxylesterase. We demonstrated that GA increased the amounts of 8-oxodG in the presence of Cu(II), Fe(III)EDTA, and Fe(III)ADP. From these results, it is considered that GA, produced from PG by esterase, may be involved in oxidative DNA damage in human cultured cell.

Furthermore, we investigated site-specific DNA damage by GA, using ^{32}P-labeled DNA fragments obtained from the human *p53* and *p16* tumor suppressor genes. GA induced DNA damage in the presence of Fe(III) complex or Cu(II). GA caused cleavage uniformly at every nucleotide in the presence of Fe(III)EDTA. A similar pattern was observed in GA induced-DNA damage in the presence of Fe(III)ADP. It is reported that •OH causes DNA cleavage without site specificity [28–31]. In order to confirm what kinds of reactive oxygen species cause oxidative DNA damage, the effects of various scavengers on the Fe(III)EDTA-mediated DNA damage by GA were examined. The inhibitory effects of •OH scavengers, catalase and the iron chelating agent suggested that •OH, H_2O_2, and iron play important roles in the DNA damage. Therefore, we conclude that the Fe(III) complex-mediated DNA damage caused by GA is mainly due to •OH generated via the Fenton reaction.

GA plus Cu(II) induced piperidine-labile site at thymine and cytosine. In addition, using an HPLC-ECD, we also observed the increase of 8-oxodG, which is piperidine-inert site. Thus, GA with Cu(II) should cause oxidative damage at guanine. DNA damage caused by GA plus Cu(II) was inhibited by catalase and bathocuproine, a Cu(I) chelator. Although •OH scavengers showed no or little inhibitory effect on the DNA damage, methional, which can scavenge not only •OH but also species with weaker reactivity than •OH [23], inhibited the DNA damage. From these results, we considered that reactive species such as Cu(I)–hydroperoxo complex partic-

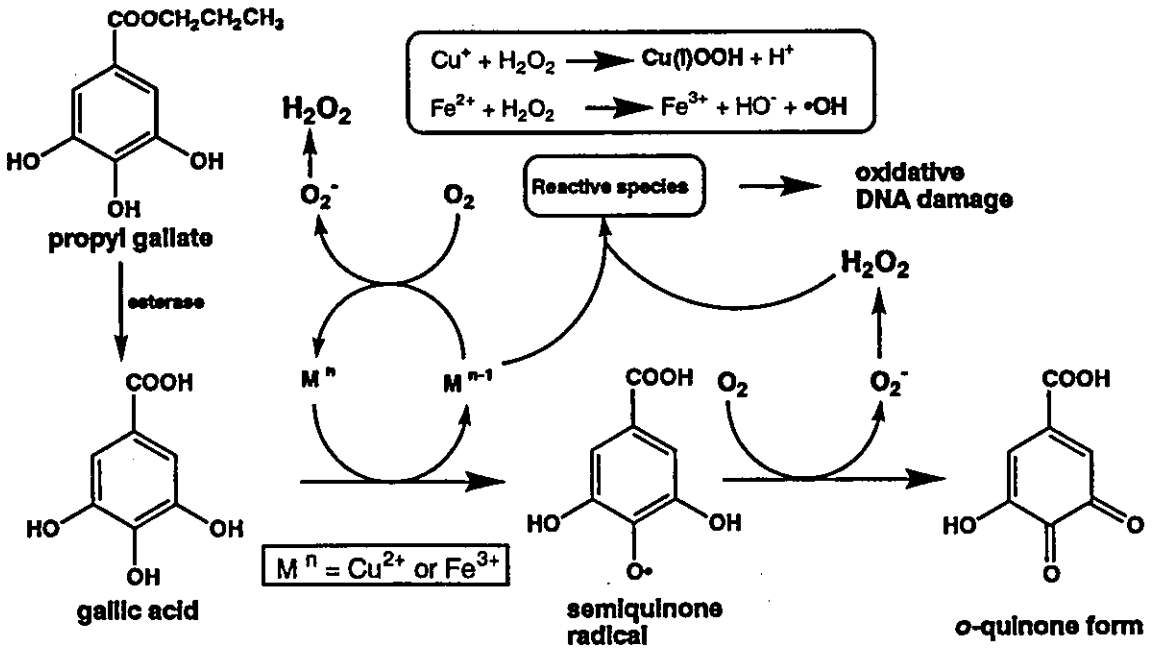

Fig. 8. Possible mechanism of metal-mediated DNA damage induced by GA, a metabolite of PG.

ipate in the Cu(II)-mediated DNA damage by GA. Copper, which occurs in the mammalian cell nucleus, is believed to play a central role in the formation of reactive oxygen species and produce DNA damage leading to carcinogenesis [32].

On the basis of these results, the possible mechanisms of metal-mediated DNA damage induced by GA, a metabolite of PG, are proposed in Fig. 8. Metal-mediated autooxidation of GA generate to the semiquinone radical. In the presence of metal ion (M^n), H_2O_2 was generated by $O_2^{\bullet -}$ dismutation with concomitant reduction of M^n to M^{n-1}. In the presence of Cu(II), GA induced DNA damage by the interaction of Cu(I) and H_2O_2 to form a Cu(I)–hydroperoxo complex such as Cu(I)OOH. Fe(III)EDTA-mediated DNA damage resulting from exposure to GA is caused by $^{\bullet}OH$ generated from the Fenton reaction. $^{\bullet}OH$ is extremely short-lived and travels a very short distance in water [33–35]. This could be one of the reasons that Cu(II)-mediated DNA damage caused by GA was stronger than Fe(III)EDTA-mediated damage, although autooxidation of GA mediated by Fe(III)EDTA was faster than that by Cu(II).

It has been reported that PG can act as carcinogen in several tumor in experimental animals [4,5]. Jacobi et al. [36] reported that PG induced single strand breaks in the presence of Cu(II). However, our study revealed that although PG induced no or little DNA damage in the presence of metal ions, GA efficiently induced DNA damage in the presence of metal ions such as iron and copper under the experimental condition. Absorption spectroscopic studies demonstrated that the autooxidation of GA was markedly faster than that of PG. This observation was supported by MO calculation suggesting that oxidation potential of GA is smaller than that of PG. The experimental and the calculation results lead us to the idea that GA, hydrolysis product of PG, can be easily oxidized than PG, resulting in the enhancement of redox activity to produce reactive oxygen species. Finally, we conclude that GA plays an important role in the express of PG carcinogenicity.

Acknowledgements

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Photo-irradiated Titanium Dioxide Catalyzes Site Specific DNA Damage via Generation of Hydrogen Peroxide

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Titanium dioxide (TiO_2) is a potential photosensitizer for photodynamic therapy. In this study, the mechanism of DNA damage catalyzed by photo-irradiated TiO_2 was examined using [^{32}P]-5′-end-labeled DNA fragments obtained from human genes. Photo-irradiated TiO_2 (anatase and rutile) caused DNA cleavage frequently at the guanine residue in the presence of Cu(II) after *E. coli* formamidopyrimidine-DNA glycosylase treatment, and the thymine residue was also cleaved after piperidine treatment. Catalase, SOD and bathocuproine, a chelator of Cu(I), inhibited the DNA damage, suggesting the involvement of hydrogen peroxide, superoxide and Cu(I). The photocatalytic generation of Cu(I) from Cu(II) was decreased by the addition of SOD. These findings suggest that the inhibitory effect of SOD on DNA damage is due to the inhibition of the reduction of Cu(II) by superoxide. We also measured the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine, an indicator of oxidative DNA damage, and showed that anatase is more active than rutile. On the other hand, high concentration of anatase caused DNA damage in the absence of Cu(II). Typical free hydroxyl radical scavengers, such as ethanol, mannitol, sodium formate and DMSO, inhibited the copper-independent DNA photodamage by anatase. In conclusion, photo-irradiated TiO_2 particles catalyze the copper-mediated site-specific DNA damage via the formation of hydrogen peroxide rather than that of a free hydroxyl radical. This DNA-damaging mechanism may participate in the photo-toxicity of TiO_2.

Keywords: Titanium dioxide; Oxidative DNA damage; Superoxide; Hydrogen peroxide; Copper; Free hydroxyl radicals

Abbreviations: TiO_2, titanium dioxide; ROS, reactive oxygen species; $O_2^{\cdot-}$, superoxide anion radical; H_2O_2, hydrogen peroxide; $^{\cdot}OH$, free hydroxyl radical; PDT, photodynamic therapy; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; dGuo, 2′-deoxyguanosine; HPLC-ECD, high-performance liquid chromatography equipped with an electrochemical detector; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; Fpg, *E.coli* formamidopyrimidine-DNA glycosylase

INTRODUCTION

Titanium dioxide (TiO_2) is a well-known photocatalyst.[1] The crystalline forms of TiO_2, anatase and rutile, are semiconductors with band gap energies of 3.26 and 3.06 eV, respectively. TiO_2 absorbs UVA light, catalyzing the generation of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), free hydroxyl radical ($^{\cdot}OH$), and singlet oxygen, in aqueous media.[1–3] Photo-irradiated TiO_2 demonstrates bactericidal effects and is widely used for photocatalytic sterilization.[1,4–6] Recently, the application of TiO_2 as a photosensitizer of photodynamic therapy (PDT) was proposed.[1,7–10] PDT is a relatively new treatment for certain types of cancer, including endobronchial and esophageal cancers.[11] TiO_2 particles can be incorporated into cells[7,12] and kill cancer cells during UVA irradiation.[1,7–10,12] The inhibitory effect of tumor growth by photo-irradiated TiO_2 was also reported in an animal experiment using mice.[1,10] The mechanism of cytotoxicity by photocatalysis of TiO_2 is accompanied by cell membrane damage.[13] In addition, TiO_2 induces photodamage to DNA in human cells,[14] mouse lymphoma cells,[15] and phage.[16] However, the mechanism underlying

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DNA damage photocatalyzed by TiO_2 is not well understood.

In this study, the mechanism and the site specificity of DNA damage by photo-irradiated TiO_2 (anatase and rutitle) were examined using a ^{32}P-5′-end-labeled DNA fragment obtained from the human *p53* and *p16* tumor suppressor genes and the c-Ha-*ras*-1 protooncogene. The formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), an oxidation product of 2′-deoxyguanosine (dGuo), was also measured using an electrochemical detector coupled to high-performance liquid chromatography (HPCL-ECD).

MATERIALS AND METHODS

Materials

TiO_2 particles (anatase and rutile) with an average size of 50–300 nm in diameter were purchased from Kanto Chemical Co. (Tokyo, Japan). The particles were ultra-sonically dispersed in water. Restriction enzymes (*Ava*I and *Pst*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Restriction enzymes (*Apa*I, *BssH*II, *EcoR*I, *Mro*I and *Xba*I) and calf intestine phosphatase were from Boehringer Mannheim GmbH (Mannheim, Germany). [γ-^{32}P]-ATP was from New England Nuclear (Boston, MA). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). SOD (3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver) were from Sigma Chemical Co. (St Louis, MO). Methional (3-methylthiopropionaldehyde) was from Tokyo Kaksei (Tokyo, Japan). DMSO was from Aldrich Chemical Co. (Milwaukee, WI). Copper(II) chloride dihydrate was from Nacalai Tesque, Inc. (Kyoto, Japan). *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) was from Trevigen Co. (Gaithersburg, MD).

Preparation of ^{32}P-5′-end-labeled DNA Fragments

DNA fragments were obtained from the human *p53*[17] and *p16*[18] tumor suppressor genes and the c-Ha-*ras*-1 protooncogene.[19] The DNA fragment of the *p53* tumor suppressor gene was prepared from pUC18 plasmid, ligated fragments containing exons of *p53* gene. A singly ^{32}P-5′-end-labeled double-stranded 443-bp fragment (*Apa*I 14179-*EcoR*I*14621) and a 211-bp fragment (*Hind*III* 13972-*Apa*I 14182) were prepared according to the method described previously.[20] Exon-containing DNA fragments were also obtained from the human *p16* tumor suppressor gene; these fragments were subcloned into the Pgem-T Easy Vector (Promega Corp. Madison, WI). A singly labeled 324 bp DNA fragment (*EcoR*I* 9466-*BssH*II 9789) and a 158-bp fragment (*Mro*I 6173-*Eco*RI* 6330) were prepared as described previously.[21] The DNA fragment of the c-Ha-*ras*-1 protooncogene was prepared from plasmid pbcNI, which carries a 6.6 kb *Bam*HI chromosomal DNA segment containing the c-Ha-*ras*-1 gene. A singly labeled 337 bp fragment (*Pst*I 2345-*Ava*I* 2681) and a 261-bp fragment (*Ava*I* 1645-*Xba*I 1905) were obtained according to a method described previously.[22] Nucleotide numbering starts with the *Bam*HI site.[19] The asterisk indicates the ^{32}P labeling.

Detection of Damage to Isolated DNA by Photo-irradiated TiO_2

The standard reaction mixture in a microtube (1.5 ml Eppendorf) contained the ^{32}P-DNA fragment (<1 μM) and 20 μM calf thymus DNA, indicated amounts of TiO_2, and 5 μM DTPA in a 10 mM sodium phosphate buffer (pH 7.8). DTPA was used to remove the contaminated metal ions. To clarify the effect of metal ions on DNA photodamage, a 20 μM metal ion, such as $CuCl_2$ was used. The mixtures were exposed to 10 J/cm^2 UVA light using 10-W UV lamp (λ_{max} = 365 nm, 1.4 mW/cm^2) (UVP Inc., CA). Subsequently, the DNA was treated with 1 M piperidine for 20 min at 90°C or 10 units of Fpg in the reaction buffer (10 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10 mM EDTA and 0.1 mg/ml BSA) for 2 h at 37°C. The DNA fragments were subjected to electrophoresis on an 8 M urea/8% polyacrylamide gel. The autoradiogram was obtained by exposing an X-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure[23] using a DNA-sequencing system (LKB 2010 Macrophor, Pharmacia Biotech, Uppsala, Sweden). A relative amount of DNA fragments was measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech).

Measurement of 8-OxodGuo Formation in Calf Thymus DNA by Photo-irradiated TiO_2

Formation of 8-oxodGuo was measured by a modification of a reported method.[24] The reaction mixture in a tube (1.5 ml Eppendorf) contained indicated concentration of TiO_2 (anatase or rutile), 20 μM $CuCl_2$; 100 μM/base calf thymus DNA and 5 μM DTPA in 100 μl of 4 mM sodium phosphate buffer (pH 7.8). The mixtures were exposed to 10 J/cm^2 UVA light using 10-W UV lamp (λ_{max} = 365 nm, 1.4 mW/cm^2). After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase,

and analyzed with an HPLC-ECD, as described previously.[25]

UV–visible Spectra Measurements on Cu(II) Reduction Photocatalyzed by TiO_2

UV–visible spectra for the reduction of Cu(II) to Cu(I) by photo-irradiated TiO_2 were measured with a UV–visible spectrometer (UV-2500PC, Shimadzu, Kyoto, Japan) using bathocuproine as a Cu(I)-chelator. The standard reaction mixture contained 8 μg/ml TiO_2, 20 μM $CuCl_2$ and 10 μM bathocuproine in 1 ml of 10 mM sodium phosphate buffer (pH 7.8). The mixtures were exposed to 2 J/cm^2 UVA light using 10-W UV lamp (λ_{max} = 365 nm, 1.4 mW/cm^2). After irradiation, TiO_2 particles were removed by centrifugation and the absorption maximum at 480 nm of the Cu(I)-bathocuproine complex[26] was measured.

RESULTS

DNA Damage by Photo-irradiated TiO_2

Photo-irradiated TiO_2 particles (anatase and rutile) caused DNA damage in the presence of Cu(II) (Fig. 1). Mn(II), Fe(III), Co(II) and Ni(II) did not mediate DNA damage (data not shown). Even without piperidine treatment, oligonucleotides were slightly formed by photo-irradiated TiO_2 (data not shown), indicating the breakage of the deoxyribose phosphate backbone. The extent of DNA damage was increased by piperidine treatment, suggesting that base modifications were also induced by photo-irradiated TiO_2 in the presence of Cu(II). Without irradiation, TiO_2 showed no damage to DNA (Fig. 1). DNA damage induced by anatase was stronger than that by rutile.

Effects of Scavengers and Bathocuproine on DNA Damage by Photo-irradiated TiO_2

To investigate the identity of the reactive species involved in DNA damage, we evaluated the inhibitory effects of scavengers of ROS and bathocuproine, a chelator of Cu(I), on DNA damage (Fig. 2).

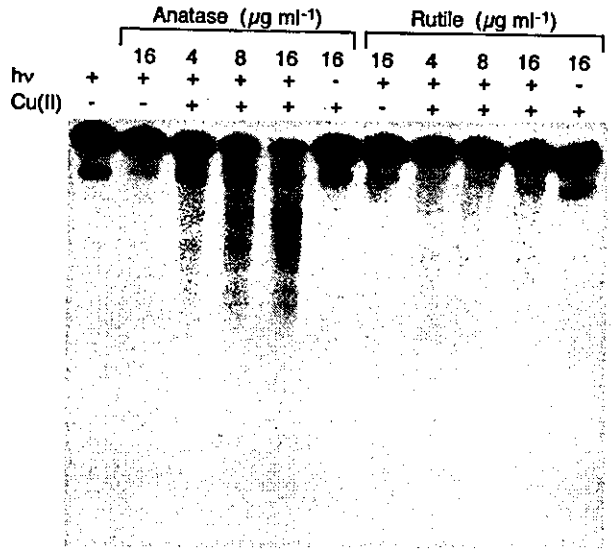

FIGURE 1 Autoradiogram of ^{32}P-labeled DNA fragment irradiated with UVA light in the presence of TiO_2. The reaction mixtures contained the ^{32}P-5′-end-labeled 158 bp DNA fragment, 20 μM/base calf thymus DNA, the indicated concentrations of TiO_2, 20 μM $CuCl_2$, and 5 μM DTPA in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were irradiated with UVA light (λ_{max} = 365 nm, 10 J/cm^2). Then, the DNA fragments were treated with 1 M piperidine for 20 min at 90°C and electrophoresed on an 8% polyacrylamide/8M urea gel.

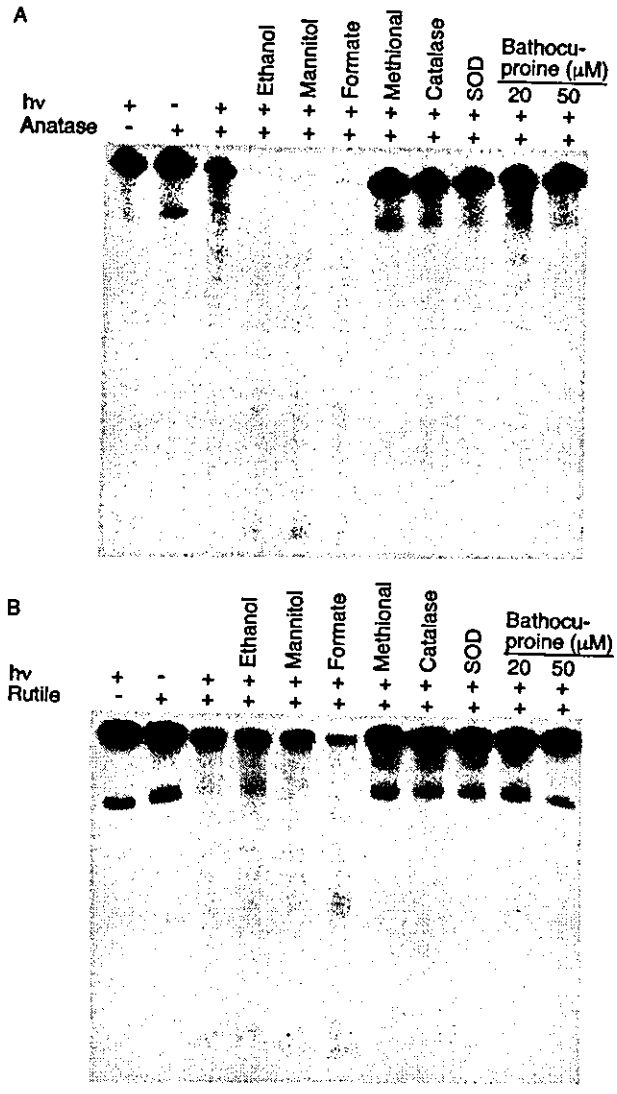

FIGURE 2 Effects of scavengers on DNA damage induced by photo-irradiated TiO_2 in the presence of Cu(II). The reaction mixtures contained the ^{32}P-5′-end-labeled 261 bp (A) or 443 bp (B) DNA fragment, 20 μM/base calf thymus DNA, 20 μM $CuCl_2$, 5 μM DTPA and 8 μg/ml anatase (A) or 8 μg/ml rutile (B) in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were irradiated with UVA light (λ_{max} = 365 nm, 10 J/cm^2) and treated as described in the legend to Fig. 1. The concentrations of scavengers and bathocuproine were as follows: 5v% ethanol, 0.1 M mannitol, 0.1 M sodium formate, 0.1 M methional, 30 units of SOD and 50 units of catalase.

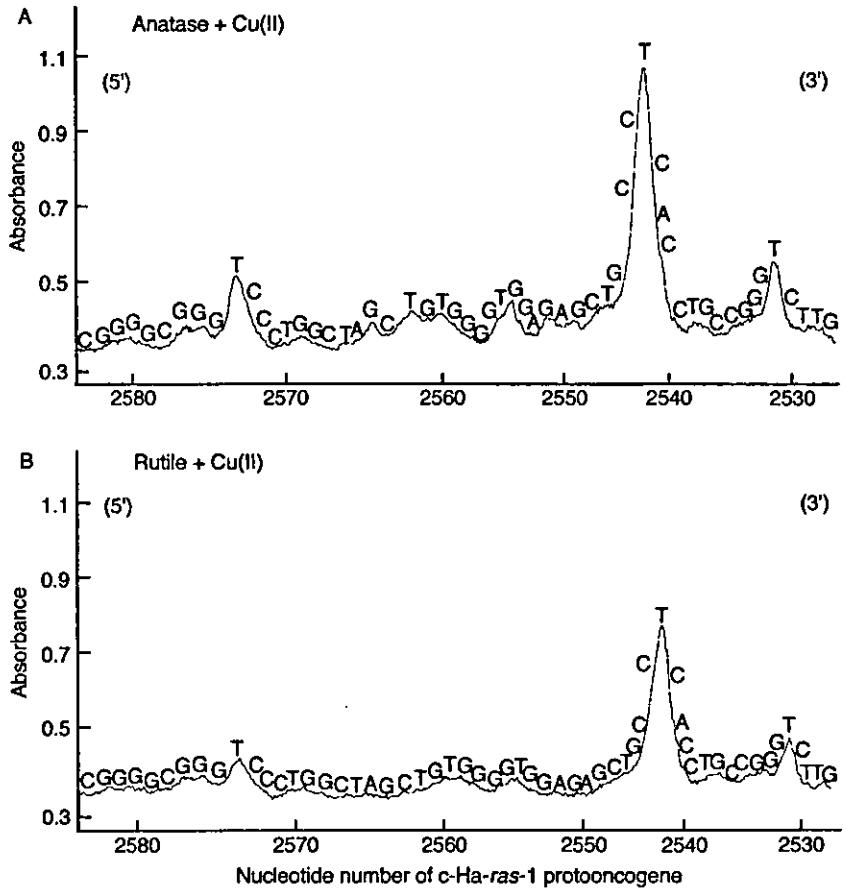

FIGURE 3 Site specificity of DNA damage induced by photo-irradiated TiO_2 in the presence of Cu(II). The reaction mixtures contained the ^{32}P-5'-end-labeled 337 bp DNA fragment (c-Ha-*ras*-1 protooncogene), 20 μM/base calf thymus DNA, 20 μM $CuCl_2$, 5 μM DTPA and 8 μg/ml anatase (A) or 8 μg/ml rutile (B) in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). Mixtures were irradiated with UVA light (λ_{max} = 365 nm, 10 J/cm^2). The DNA fragments were then treated with piperidine. Subsequently, the DNA was analyzed and the relative amounts of oligonucleotides were measured by the methods described in the "Materials and methods section". The horizontal axis shows the nucleotide number of the human c-Ha-*ras*-1 protooncogene.

DNA damage induced by photo-irradiated anatase plus Cu(II) was significantly inhibited by catalase, SOD and bathocuproine (Fig. 2A). Similar scavenging effects were observed in the case of rutile plus Cu(II) (Fig. 2B). These results suggest the involvement of H_2O_2, $O_2^{\cdot-}$, and Cu(I). Methional also inhibited DNA damage. Typical $^{\cdot}OH$ scavengers, such as ethanol, mannitol and sodium formate, could not inhibit DNA damage. Addition of ethanol, mannitol and sodium formate enhanced DNA photodamage by anatase plus Cu(II) (Fig. 2A).

Site Specificity of DNA Damage by Photo-irradiated TiO_2

The patterns of DNA damage induced by photo-irradiated anatase was quite similar to that induced by rutile (Fig. 3A and B). Photo-irradiated TiO_2 particles formed piperidine-labile products at the underlined bases of 5'-TC (Figs. 3 and 4A) and 5'-TG (Fig. 4A) in the presence of Cu(II). With Fpg treatment, the DNA cleavage occurred frequently at the underlined guanine residue of 5'-TG, another guanine and cytosine (Fig. 4B). Fpg mainly catalyzes the excision of piperidine-resistant 8-oxodGuo, an oxidative product of dGuo.[27] Fpg also mediates the cleavages of the oxidative cytosine, such as 5-hydroxycytosine.[28]

Formation of 8-OxodGuo in Calf Thymus DNA by Photo-irradiated TiO_2

Photo-irradiated anatase and rutile induced 8-oxodGuo formation in the presence of Cu(II) (Fig. 5). The formation of 8-oxodGuo by photo-irradiated anatase was increased in a dose-dependent manner, whereas that by rutile plateaued when more than 4 μg/ml TiO_2 was used. A comparison of 8-oxodGuo formation by anatase and rutile suggested that the DNA-damaging ability of anatase is stronger than that of rutile.

Reduction of Cu(II) by Photo-irradiated TiO_2

After photo-irradiation of the mixture including TiO_2, Cu(II) and bathocuproine, a typical absorption spectrum of Cu(I)-bathocuproine complex[26] with

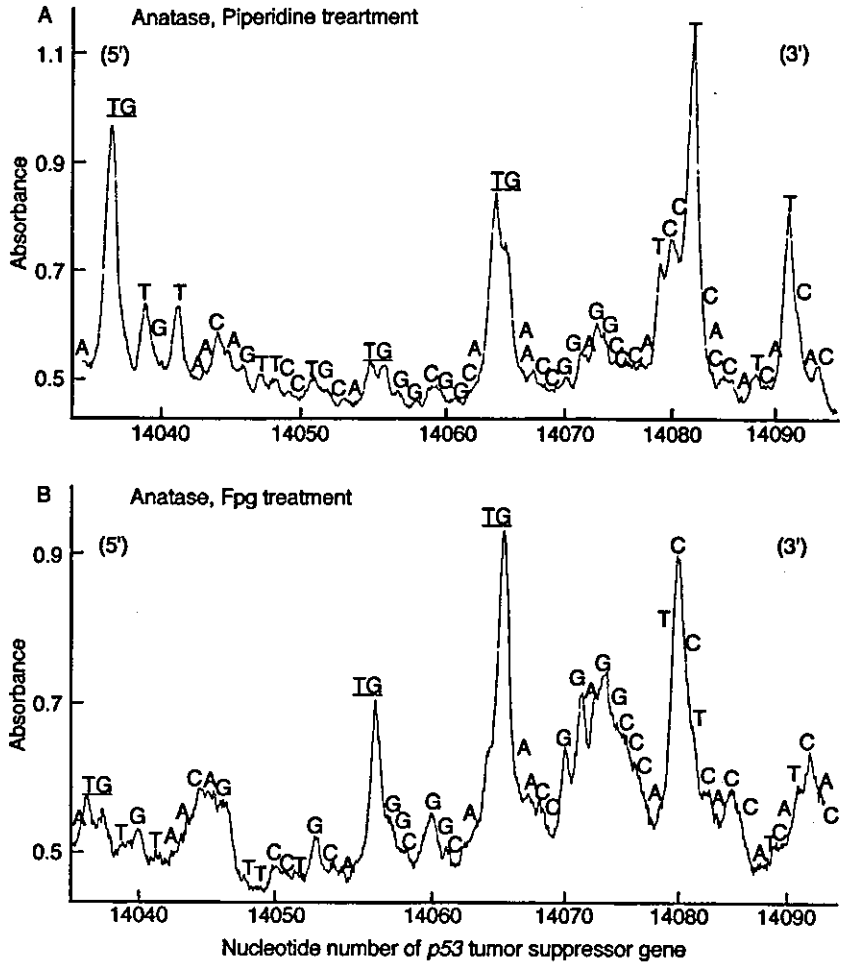

FIGURE 4 Site specificity of DNA damage induced by photo-irradiated anatase. The reaction mixtures contained the ^{32}P-5'-end-labeled 211 bp DNA fragment (*p53* tumor suppressor gene), 20 μM/base calf thymus DNA, 5 μM DTPA, 20 μM $CuCl_2$ and 8 μg/ml anatase in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). Mixtures were irradiated with UVA light (λ_{max} = 365 nm, 10 J/cm^2). Subsequently, the DNA fragments were treated with piperidine (A) or Fpg (B). The DNA was analyzed and the relative amounts of oligonucleotides were measured by the methods described in the Materials and methods section. The horizontal axis shows the nucleotide numbers of the *p53* tumor suppressor gene.

the maximum at 480 nm was observed and increased depending on the concentration of TiO_2 (Fig. 6), indicating the reduction of Cu(II) to Cu(I) by the photocatalysis of TiO_2. The formation of the Cu(I)-bathocuproine complex was decreased by SOD, suggesting the Cu(II) reduction by $O_2^{\cdot-}$. SOD did not completely inhibit Cu(I) generation because Cu(II) can be easily reduced in the presence of bathocuproine. The formation of the Cu(I)-bathocuproine complex was accelerated under argon (data not shown), indicating that Cu(II) can be directly reduced by the electron formed in the conductive band of TiO_2 in the absence of molecular oxygen.

DNA Photodamage by a High Concentration of Anatase in the Absence of Cu(II)

A high concentration of anatase caused DNA damage in the absence of Cu(II). No metal-independent DNA photodamage was detected when rutile was used, but as the DNA targets employed were relatively short and therefore, cannot detect rare damage this dose not imply that rutile is incapable of inflicting metal-independent photodamage on DNA. DNA photodamage induced by a high concentration of anatase was inhibited by ·OH scavengers and methional (Fig. 7), suggesting the involvement of ·OH. A high concentration of anatase induced piperidine-labile sites at every nucleobase in the absence of Cu(II) (Fig. 8). This cleavage pattern is quite different from the Cu(II)-dependent DNA photodamage by anatase.

DISCUSSION

The present study has demonstrated that photo-irradiated TiO_2 particles catalyze DNA damage in the presence of Cu(II). DNA damage induced by anatase was stronger than that by rutile. The DNA damage was enhanced by piperidine treatment, suggesting that photo-irradiated TiO_2 caused not only DNA strand breakage but also base

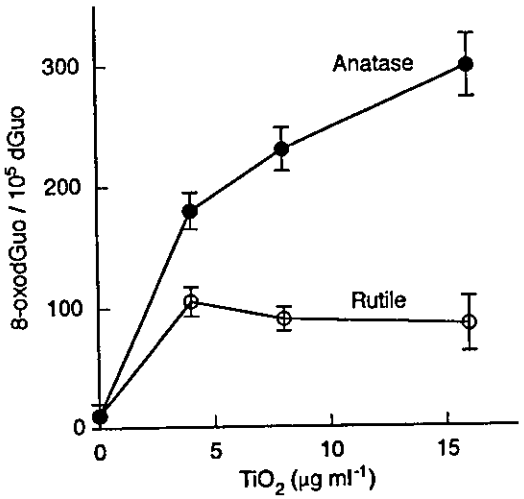

FIGURE 5 Formation of 8-oxodGuo induced by photo-irradiated TiO_2 in the presence of Cu(II). The reaction mixtures contained 100 μM/base calf thymus DNA, TiO_2, 20 μM $CuCl_2$ and 5 μM DTPA in 100 μl of 4 mM sodium phosphate buffer (pH 7.8). After photo-irradiation (λ_{max} = 365 nm, 10 J/cm^2), DNA fragment was enzymatically digested into nucleosides, and 8-oxodGuo formation was measured with an HPLC-ECD as described in the Materials and methods section.

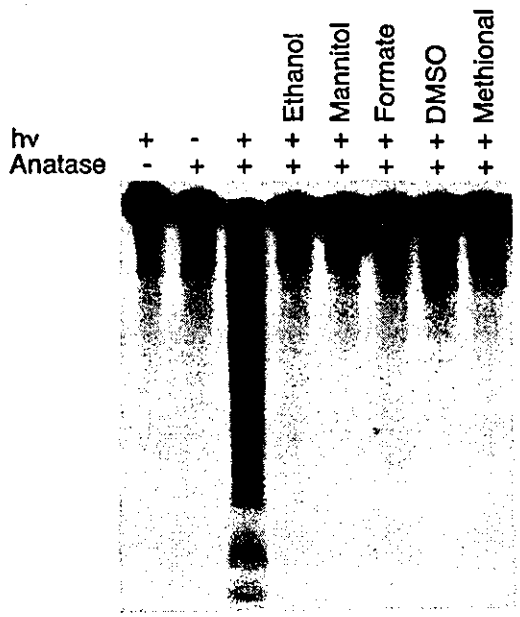

FIGURE 7 Effects of hydroxyl radical scavengers on DNA damage induced by photo-irradiated anatase. The reaction mixtures contained the ^{32}P-5′-end-labeled 324 bp DNA fragment 20 μM/base calf thymus DNA, 5 μM DTPA and 80 μg/ml anatase in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were irradiated with UVA light (λ_{max} = 365 nm, 10 J/cm^2) and treated as described in the legend of Fig. 1. The concentrations of scavengers were as follows: 5 v% ethanol, 0.1 M mannitol, 0.1 M sodium formate, 5 v% DMSO and 0.1 M methional.

modification. Photo-irradiated TiO_2 formed piperidine-labile lesions at the underlined bases of 5′-TG and 5′-TC. Furthermore, TiO_2 caused DNA photocleavage at the underlined guanine of 5′-TG and the cytosine residues in a DNA fragment treated with Fpg, which catalyzes the excision of piperidine-resistant 8-oxodGuo.[27] Fpg also mediated the cleavages of the oxidative products of cytosine, such as 5-hydroxycytosine.[28] The present study suggests that photo-irradiated TiO_2 induces 8-oxodGuo formation adjacent to piperidine-labile thymine lesions. Although the present method based on Maxam–Gilbert procedure dose not clearly show double-base damage on the same DNA molecule, the data from the DNA cleavage pattern stochastically suggest the involvement of a double-base lesion. It has been appropriately postulated that double-base lesions can be generated from one radical hit that leads through a secondary reaction to a tandem base modification at pyrimidine and the adjacent residues.[29–31] Indeed, tandem mutations in human cells can be induced by H_2O_2 plus Cu(II) via vicinal or cross-linked base damage.[32]

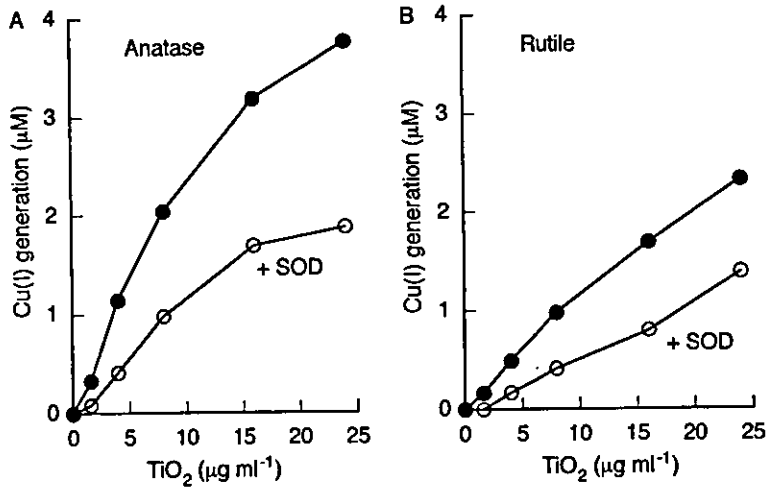

FIGURE 6 Reduction of Cu(II) by photo-irradiated TiO_2. The reaction mixtures contained 20 μM $CuCl_2$, TiO_2 and 10 μM bathocuproine in 1 ml of 10 mM sodium phosphate buffer (pH 7.8). After photo-irradiation (λ_{max} = 365 nm, 2 J/cm^2), the concentration of formed Cu(I)-bathocuproine complex was determined by measurement of absorbance at 480 nm.

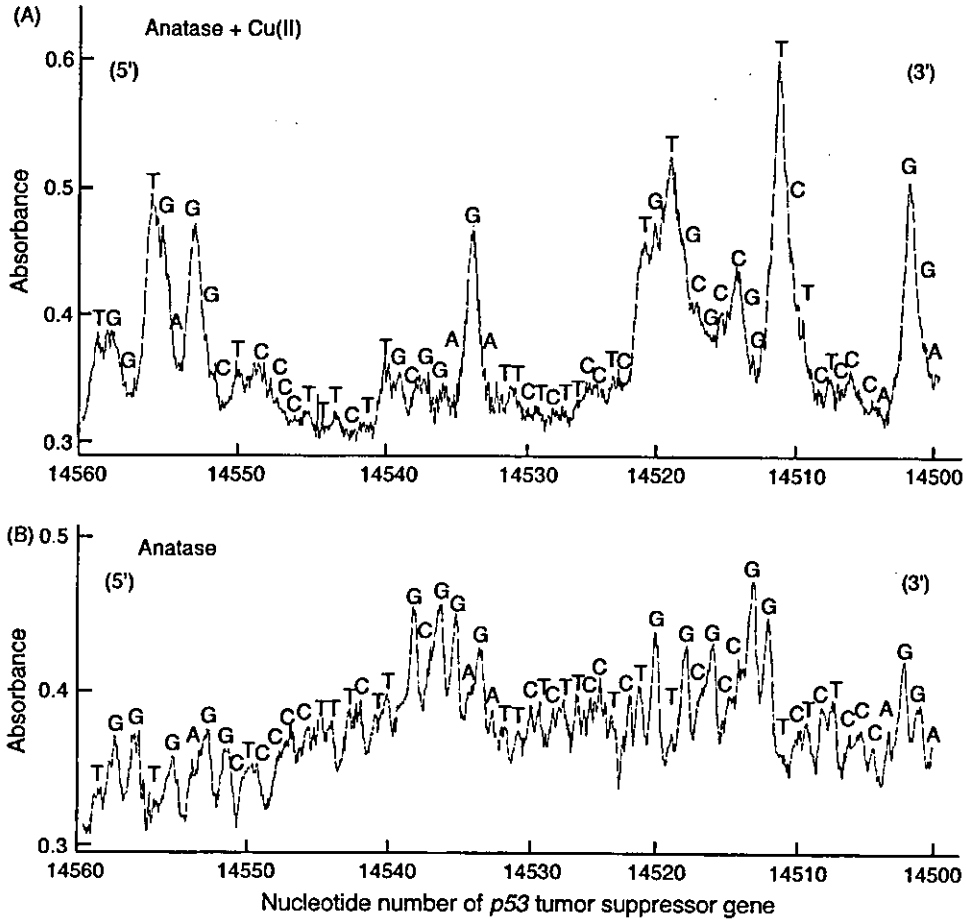

FIGURE 8 Site specificity of DNA damage induced by photo-irradiated anatase. The reaction mixtures contained the ^{32}P-5'-end-labeled 443 bp DNA fragment (*p53* tumor suppressor gene), 20 μM/base calf thymus DNA, 5 μM DTPA and 8 μg/ml anatase with 20 μM $CuCl_2$ (A) or 80 μg/ml anatase without $CuCl_2$ (B) in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). Mixtures were irradiated with UVA light (λ_{max} = 365 nm, 10 J/cm^2). Subsequently, the DNA fragments were treated with piperidine. The DNA was analyzed and the relative amounts of oligonucleotides were measured by the methods described in the Materials and methods section. The horizontal axis shows the nucleotide numbers of the *p53* tumor suppressor gene.

Since cluster damage in living cells is poorly repaired,[33] such clustered damage, including double-base lesions, appears to play an important role in the phototoxicity of TiO_2.

The effects of ROS scavengers and bathocuproine on DNA damage suggest the participation of H_2O_2 and Cu(I). Typical 'OH scavengers showed no or little inhibitory effects on DNA damage, although the possibility of DNA damage by *in situ*-produced 'OH cannot be ignored. The inhibitory effect of methional on DNA damage can be explained by the assumption that sulfur compounds scavenge less reactive species than 'OH.[34] It has also been reported that 'OH is not the main reactive species involved in DNA damage by H_2O_2 and Cu(I).[22,31] DNA-associated Cu(I) ions may generate other oxidants, including a copper-peroxo intermediate, such as Cu(I)-OOH, which is generated from the reaction of H_2O_2 and Cu(I).[35,36] The generation of these reactive species should be involved in the formation of piperidine-labile products and 8-oxodGuo. On the other hand, a high concentration of anatase could induce DNA photodamage in the absence of Cu(II). The effects of typical 'OH scavengers on DNA damage suggest the involvement of 'OH. The DNA damage induced by photo-irradiated anatase without Cu(II) was observed at every nucleotides with little site specificity, supporting the contribution of 'OH to DNA damage.[35]

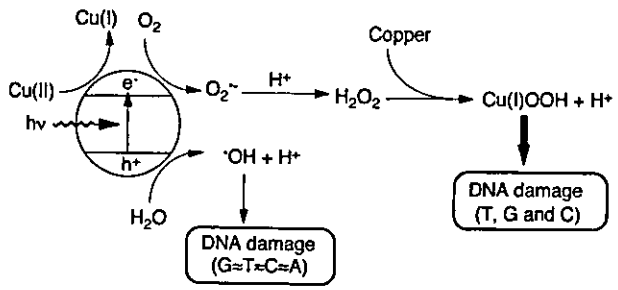

FIGURE 9 Proposed mechanism of DNA damage induced by photo-irradiated TiO_2.

A possible mechanism of DNA damage induced by photo-irradiated TiO_2 is shown in Fig. 9. The crystalline forms of TiO_2, anatase and rutile, are semiconductors with band gap energies of 3.26 and

3.06 eV, corresponding to light of 385 and 400 nm, respectively. When a TiO_2 semiconductor absorbs light with energy greater than its band gap, electrons in the valence band are excited to the conduction band, creating electron-hole pairs and causing various chemical reactions.[1] The electron (e^-) is a reducing agent, whereas the hole (h^+) is a powerful oxidizing agent. In aqueous environments, the electron reduces oxygen to give $O_2^{\cdot-}$, and the hole oxidizes a water molecule to yield $^{\cdot}OH$. Formed $O_2^{\cdot-}$ can be dismutated into H_2O_2. The experimental results of the formation of the Cu(I)-bathocuproine complex suggest that oxygen reduction precedes the Cu(II) reduction in the photocatalytic reaction of TiO_2 under aerobic condition, since the concentration of dissolved oxygen is much higher than that of Cu(II). The Cu(I) generation can be mediated by $O_2^{\cdot-}$. H_2O_2 reacts with Cu(I) to generate other oxidants, including a copper-peroxo intermediate, resulting in the oxidation of nucleobases. Copper, which is an essential component of chromatin,[37,38] is found to bind DNA with high affinity.[39,40] Therefore, copper may play an important role in ROS generation *in vivo*, although mammals have evolved means of minimizing the levels of free copper ions and most copper ions bind to protein carriers and transporters.[41] $^{\cdot}OH$ formed by the reaction of water with a hole in the valence band of TiO_2 also slightly participates in DNA damage by anatase. Because $^{\cdot}OH$ is strong oxidant, $^{\cdot}OH$ can damage every nucleobase.[35] This study suggested that H_2O_2 mainly participates in the phototoxicity of TiO_2 and that the contribution of $^{\cdot}OH$ is small. Quite appropriately, Fujishima *et al.* reported the involvement of peroxide generated from $O_2^{\cdot-}$ in the cytotoxicity of illuminated TiO_2.[1] These findings were also supported by the relatively small quantum yield of $^{\cdot}OH$ generation[42] in TiO_2 photocatalysis.

TiO_2 is a potential photosensitizer for PDT.[1,7–10] TiO_2 particles can be incorporated into cancer cells and demonstrate cytotoxicity under photo-irradiation.[1,7–10,12] Photo-irradiated TiO_2 catalyzes a number of functional changes in cells including altered permeability of cellular membranes to potassium and calcium ions, release of RNA and proteins and cytotoxicity.[13] It has also been reported that DNA can be a target molecule of the photocatalysis of TiO_2 *in vivo*.[14–16] The present study has shown that, under photo-irradiation, TiO_2 particles mainly caused copper-dependent DNA damage through H_2O_2 generation *in vitro*. Other metal ions may play an important role in the phototoxicity of TiO_2 *in vivo*. Although TiO_2 is not likely to be incorporated in a cell nucleus, H_2O_2 generated via a photocatalytic reaction can be easily diffused and incorporated in a cell nucleus, leading to DNA damage. Several studies have demonstrated that DNA can be an alternative potential target of PDT.[43,44] Therefore, the metal-mediated DNA damage through the photocatalysis of TiO_2 may participate in cytotoxicity by photo-irradiated TiO_2.

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Genistein and Daidzein Induce Cell Proliferation and Their Metabolites Cause Oxidative DNA Damage in Relation to Isoflavone-Induced Cancer of Estrogen-Sensitive Organs†

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ABSTRACT: The soy isoflavones, genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone), are representative phytoestrogens that function as chemopreventive agents against cancers, cardiovascular disease, and osteoporosis. However, recent studies indicated that genistein and/or daidzein induced cancers of reproductive organs in rodents, such as the uterus and vulva. To clarify the molecular mechanisms underlying the induction of carcinogenesis by soy isoflavones, we examined the ability of genistein, daidzein, and their metabolites, 5,7,3′,4′-tetrahydroxyisoflavone (orobol), 7,3′,4′-trihydroxyisoflavone (7,3′,4′-OH-IF), and 6,7,4′-trihydroxyisoflavone (6,7,4′-OH-IF), to cause DNA damage and cell proliferation. An E-screen assay revealed that genistein and daidzein enhanced proliferation of estrogen-sensitive breast cancer MCF-7 cells, while their metabolites had little or no effect. A surface plasmon resonance sensor showed that binding of isoflavone-liganded estrogen receptors (ER) to estrogen response elements (ERE) was largely consistent with cell proliferative activity of isoflavones. Orobol and 7,3′,4′-OH-IF significantly increased 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation in human mammary epithelial MCF-10A cells, while genistein, daidzein, and 6,7,4′-OH-IF did not. Experiments using isolated DNA revealed a metal-dependent mechanism of oxidative DNA damage induced by orobol and 7,3′,4′-OH-IF. DNA damage was enhanced by the addition of endogenous reductant NADH, formed via the redox cycle. These findings suggest that oxidative DNA damage by isoflavone metabolites plays a role in tumor initiation and that cell proliferation by isoflavones via ER−ERE binding induces tumor promotion and/or progression, resulting in cancer of estrogen-sensitive organs.

Epidemiological and experimental studies have shown that soy products can reduce the risk of cancer (*1*−*5*) and provide other benefits including lowering cholesterol (*6*, *7*) and blood pressure (*8*) and preventing cardiovascular diseases (*1*, *6*) and osteoporosis (*9*). The soy isoflavones, genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone), are representative phytoestrogens (*10*) and act as chemopreventive agents against cancers, cardiovascular disease, and osteoporosis. Due to these health benefits, the consumption of soy food and the use of isoflavone supplements have been increasing (*11*). However, recent studies revealed that genistein and/or daidzein induced cancers of reproductive organs in rodents, such as the uterus (*12*) and vulva (*13*). In addition, genistein was reported to have tumor-enhancing effects on breast (*14*) and colon cancer (*15*). Dietary soy increased the rate of epithelial proliferation in histologically normal human breasts in premenopausal women (*16*). A stimulatory influence of soy on breast secretion and hyperplastic epithelial cells was also observed in pre- and postmenopausal women (*17*). These reports led us to consider that soy isoflavones may have a carcinogenic effect on female reproductive organs.

Epidemiological studies and animal experiments suggest that estrogens have carcinogenic actions in humans (*18*, *19*). Recent meta analyses have revealed that users of postmenopausal estrogen as hormone replacement therapy have an increased risk of breast and endometrial cancer (*20*). According to the hypothesis of estrogen-induced carcinogenesis (*21*, *22*), catechol estrogens, which are metabolites of estrogen, play a role in tumor initiation through oxidative DNA damage, whereas estrogen itself induces tumor promotion and/or progression by enhancing cell proliferation. Therefore, there arises the possibility that genistein, daidzein, and their metabolites may participate in tumor initiation and promotion by causing DNA damage and cell proliferation, thereby leading to carcinogenesis. Like endogenous estrogens, genistein and daidzein may have the capacity to produce not only beneficial actions but also adverse effects including carcinogenesis.

To investigate whether soy isoflavones affect tumor initiation and promotion, we investigated DNA damage and cell proliferative activity induced by genistein, daidzein, and their metabolites. The chemical structures of the isoflavones and their metabolites tested are shown in Figure 1. These metabolites have been detected as products of oxidative

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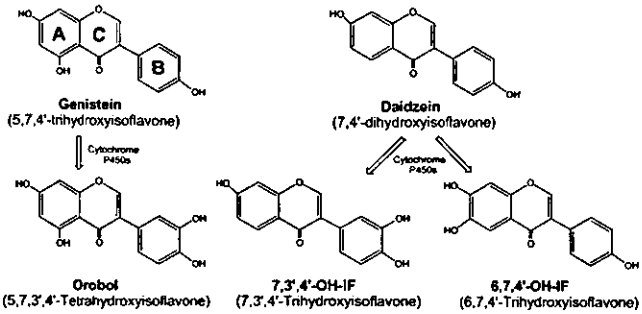

FIGURE 1: Chemical structures of isoflavones (genistein, daidzein) and their metabolites used in this study.

metabolism of genistein and daidzein, in vitro and in vivo (*23*, *24*). We examined the effects of these substances on cell proliferation of estrogen-dependent MCF-7 cells, using an E-screen assay. Furthermore, to study interactions between isoflavone-liganded estrogen receptors and estrogen response elements, we measured binding affinity using a surface plasmon resonance (SPR) sensor. Formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG),[1] an indicator of oxidative damage, in human mammary epithelial cells treated with genistein, daidzein, and their metabolites, was measured using an electrochemical detector coupled to HPLC (HPLC-ECD). To elucidate the mechanism of DNA damage, we measured 8-oxodG formation in calf thymus DNA and examined DNA damage using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* and *p16* tumor suppressor genes and the c-Ha-*ras*-1 protooncogene.

MATERIALS AND METHODS

Materials. Restriction enzymes (*Sma*I, *Eco*RI, *Bss*HII, *Apa*I, and *Sty*I) and proteinase K were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Restriction enzymes (*Hin*dIII, *Ava*I, and *Xba*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was obtained from New England Nuclear (Boston, MA). Genistein was purchased from Wako Chemical Co. (Osaka, Japan). We isolated orobol (5,7,3′,4′-tetrahydroxyisoflavone), one of the metabolites of genistein, from *Streptomyces* according to a method described previously (*25*). 7,3′,4′-Trihydroxyisoflavone (7,3′,4′-OH-IF) and 6,7,4′-trihydroxyisoflavone (6,7,4′-OH-IF), which are metabolites of daidzein, were obtained from Extrasynthèse (Genay, France). β-Nicotinamide adenine dinucleotide disodium salt (reduced form) (NADH) was purchased from Kohjin Co. (Tokyo, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were obtained from Dojin Chemicals Co. (Kumamoto, Japan). Fetal bovine serum (FBS), horse serum (HS), epidermal growth factor (EGF), Dulbecco's modified Eagle medium (DMEM), and Ham's F12 medium were purchased from Gibco (Grand Island, NY). Daidzein, superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes), catalase (45000 units/mg from bovine liver), L-buthionine (*S*,*R*)-sulfoximine (BSO), bacterial alkaline phosphatase, RNase A, phenol red free DMEM, insulin, hydrocortisone, and charcoal (activated) were purchased from Sigma Chemical Co. (St. Louis, MO). Formamidopyrimidine–DNA glycosylase (Fpg, 20000 units/mg from *Escherichia coli*) was from Trevigen Inc. (Gaithersburg, MD). Lysis buffer for DNA extraction (model 340A) was purchased from Applied Biosystems (Foster City, CA). 17β-Estradiol (E_2) was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Dimethyl sulfoxide (DMSO) and kanamycin sulfate were from Wako Chemical Co. (Osaka, Japan). L-Glutamine was from ICN Biomedicals Inc. (Aurora, OH). Dextran T70 was from Pharmacia Biotech (Uppsala, Sweden). The BIAcore sensor chips SA (modified with streptavidin) were obtained from Biacore Inc. (Uppsala, Sweden). Tween 20 was from Nacalai Tesque (Kyoto, Japan). Human recombinant estrogen receptor α (ERα) and estrogen receptor β (ERβ) were obtained from Panvera (Madison, WI).

Cell Culture. Human estrogen-sensitive breast cancer MCF-7 cells (ATCC No. HTB 22) and nontumorigenic mammary epithelial MCF-10A cells (ATCC No. CRL 10317) were obtained from American Type Culture Collection (Dainippon Pharmaceutical Co., Osaka, Japan). For routine maintenance, cells were grown in seeding medium (MCF-7 cells, DMEM supplemented with 100 ng/mL kanamycin and 5% FBS; MCF-10A cells, DMEM/F12 supplemented with 20 ng/mL EGF, 0.01 mg/mL insulin, 500 ng/mL hydrocortisone, 100 ng/mL kanamycin, and 5% HS) at 37 °C in a humidified atmosphere of 5% CO_2. Sex steroids in serum were removed by charcoal–dextran treatment for experimental medium by the method reported previously (*21*). Experimental medium was phenol red free medium supplemented with 5% charcoal–dextran–serum, 100 ng/mL kanamycin, and 4 mM L-glutamine.

Bioassay for Measuring Estrogenic Activity (E-Screen Assay). The E-screen assay was performed by a modified method of Soto et al. (*26*). Briefly, MCF-7 cells were trypsinized and plated into 12-well plates at an initial concentration of 3×10^4 cells per well with seeding medium. After the cells were allowed to attach for 24 h, the seeding medium was replaced with experimental medium. A range of concentrations (10^{-10}–10^{-5} M) of the test compounds was added. 17β-Estradiol (E_2) and isoflavones were dissolved in DMSO before being tested. The final solvent concentration in culture medium did not exceed 0.1%, as this concentration did not affect cell yields (*26*). The control condition also contained 0.1% DMSO. Cells were incubated for 6 days after treatment with the test compounds and were then trypsinized and harvested. Harvested cells were counted using a Coulter counter (Beckman Coulter, Tokyo, Japan).

Preparation of the Sensor Chip and Immobilization of ERE. The single-stranded biotinylated oligonucleotide (35mer, HPLC grade), containing the sequence of human *pS2* ERE (*27*), and the complementary unbiotinylated oligonucleotide (35mer, HPLC grade) were obtained from TaKaRa Biotechnology Co., Ltd. (Shiga, Japan). The sequence is 5′-XGTCCAAAGTCAGGTCACGGTGGCCTGATCAAAGTT-3′ (X indicates biotin-labeled). Oligonucleotides were biotinylated for immobilization to the streptavidin-treated sensor chip. The BIAcore-biosensor system (Biacore X,

[1] Abbreviations: Orobol, 5,7,3′,4′-tetrahydroxyisoflavone; 7,3′,4′-OH-IF, 7,3′,4′-trihydroxyisoflavone; 6,7,4′-OH-IF, 6,7,4′-trihydroxyisoflavone; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; HPLC-ECD, electrochemical detector coupled to HPLC; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; SOD, superoxide dismutase; DMEM, Dulbecco's modified Eagle medium; F12, Ham's F12 medium; FBS, fetal bovine serum; HS, horse serum; ·OH, hydroxyl radical; H_2O_2, hydrogen peroxide; BSO, buthionine sulfoximine.