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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, mannnitol, 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 ($\lambda_{max} = 365$ 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.

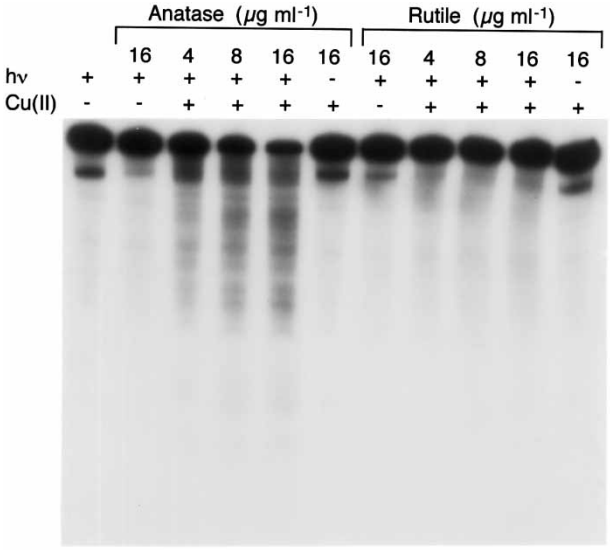

FIGURE 1 Autoradiogram of ^{32}P-labeled DNA fragment irradiated with UVA light in the presence of TiO_2. The reaction mixtures contained the ^{32}P-5′-end-labeled 158 bp DNA fragment, 20 μM/base calf thymus DNA, the indicated concentrations of TiO_2, 20 μM $CuCl_2$, and 5 μM DTPA in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were irradiated with UVA light ($\lambda_{max} = 365$ 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.

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).

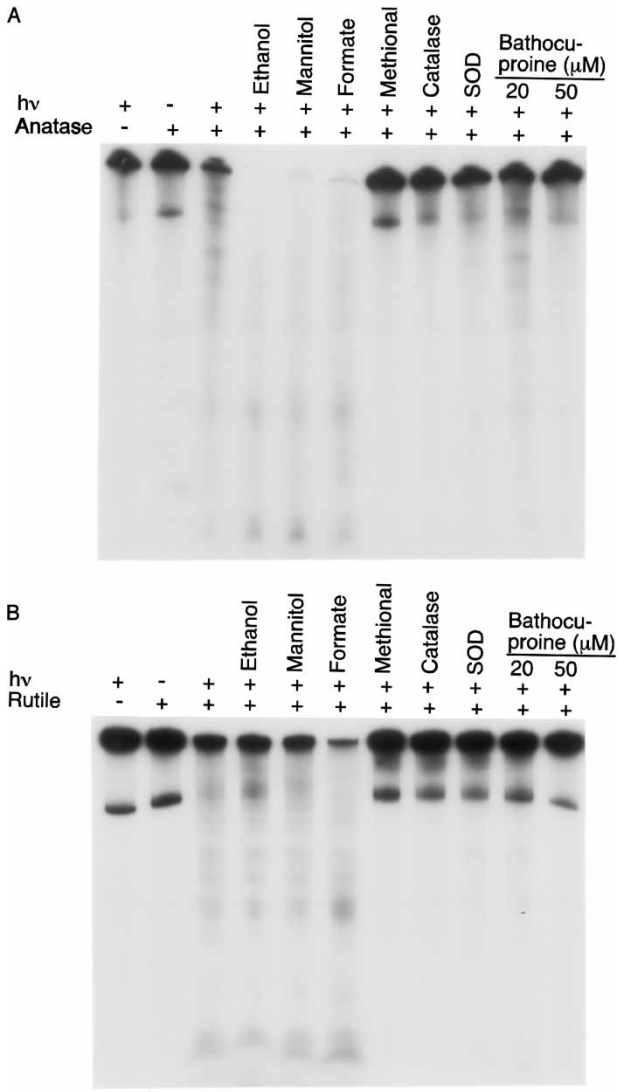

FIGURE 2 Effects of scavengers on DNA damage induced by photo-irradiated TiO_2 in the presence of Cu(II). The reaction mixtures contained the ^{32}P-5′-end-labeled 261 bp (A) or 443 bp (B) DNA fragment, 20 μM/base calf thymus DNA, 20 μM $CuCl_2$, 5 μM DTPA and 8 μg/ml anatase (A) or 8 μg/ml rutile (B) in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were irradiated with UVA light ($\lambda_{max} = 365$ 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.

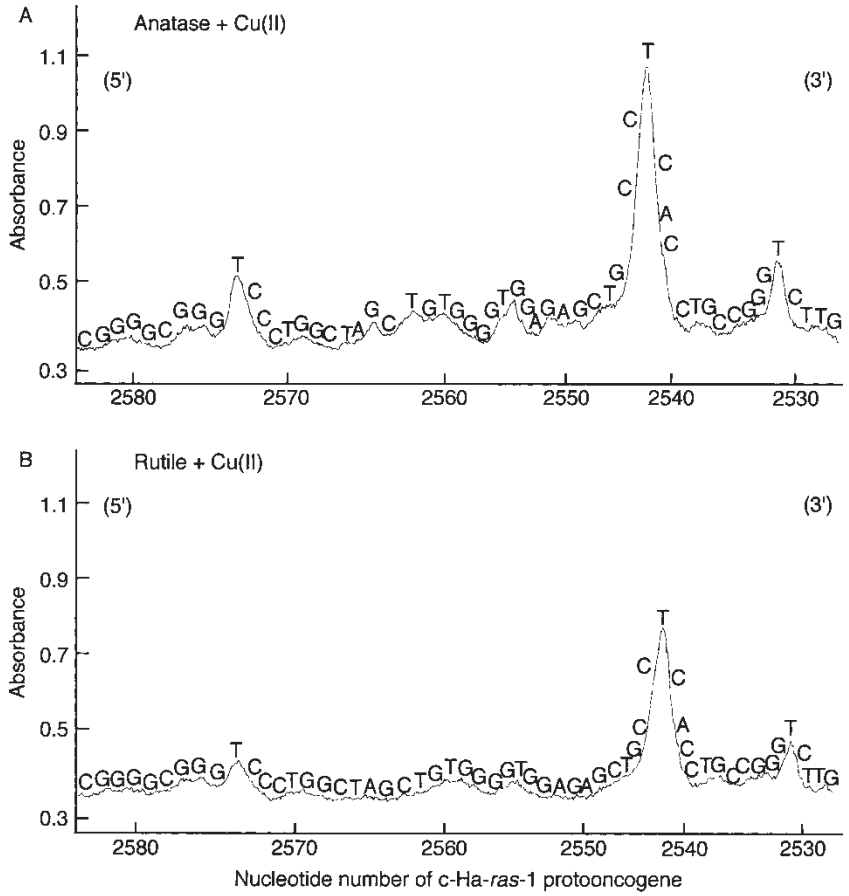

FIGURE 3 Site specificity of DNA damage induced by photo-irradiated TiO_2 in the presence of Cu(II). The reaction mixtures contained the ^{32}P-5′-end-labeled 337 bp DNA fragment (c-Ha-*ras*-1 protooncogene), 20 μM/base calf thymus DNA, 20 μM $CuCl_2$, 5 μM DTPA and 8 μg/ml anatase (A) or 8 μg/ml rutile (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

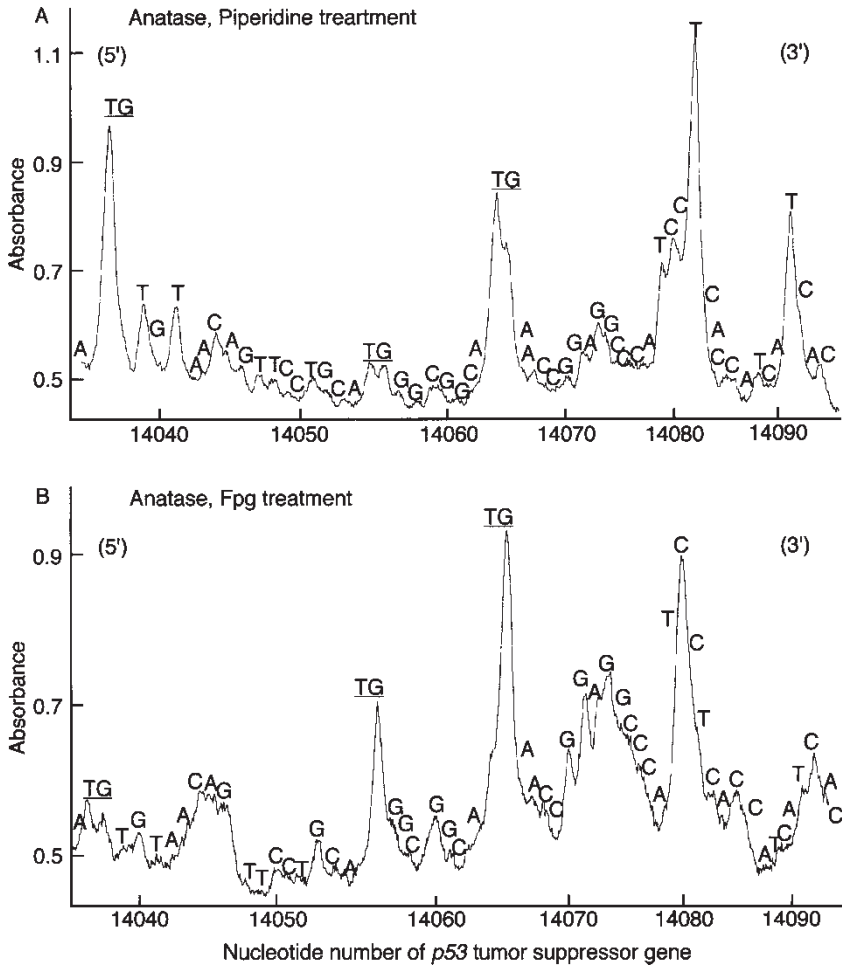

FIGURE 4 Site specificity of DNA damage induced by photo-irradiated anatase. The reaction mixtures contained the ^{32}P-5′-end-labeled 211 bp DNA fragment (*p53* tumor suppressor gene), 20 μM/base calf thymus DNA, 5 μM DTPA, 20 μM $CuCl_2$ and 8 μg/ml anatase in 100 μl of 10 mM sodium phosphate buffer (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 $^{\cdot}$OH scavengers and methional (Fig. 7), suggesting the involvement of $^{\cdot}$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

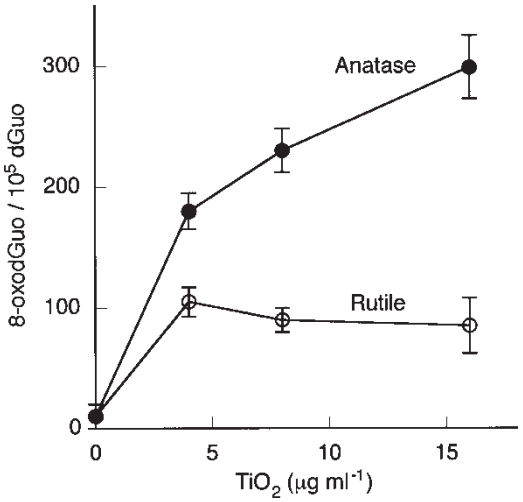

FIGURE 5 Formation of 8-oxodGuo induced by photo-irradiated TiO_2 in the presence of Cu(II). The reaction mixtures contained 100 μM/base calf thymus DNA, TiO_2, 20 μM $CuCl_2$ and 5 μM DTPA in 100 μl of 4 mM sodium phosphate buffer (pH 7.8). After photo-irradiation (λ_{max} = 365 nm, 10 J/cm^2), DNA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

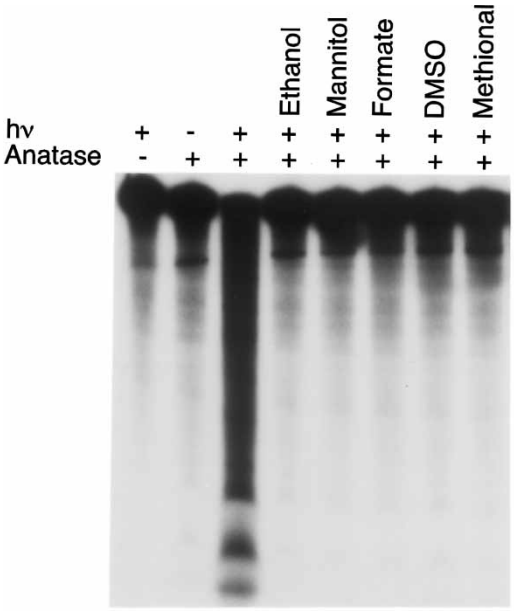

FIGURE 7 Effects of hydroxyl radical scavengers on DNA damage induced by photo-irradiated anatase. The reaction mixtures contained the ^{32}P-5′-end-labeled 324 bp DNA fragment 20 μM/base calf thymus DNA, 5 μM DTPA and 80 μg/ml anatase in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). The reaction 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]

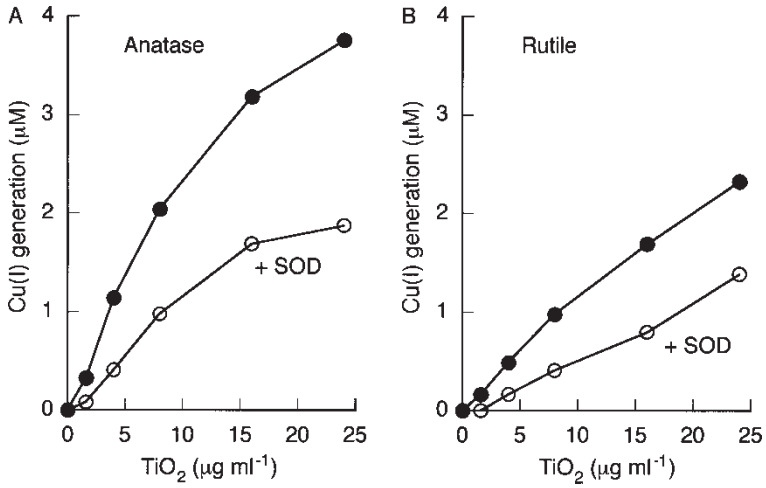

FIGURE 6 Reduction of Cu(II) by photo-irradiated TiO_2. The reaction mixtures contained 20 μM $CuCl_2$, TiO_2 and 10 μM bathocuproine in 1ml of 10 mM sodium phosphate buffer (pH 7.8). After photo-irradiation (λ_{max} = 365 nm, 2 J/cm^2), the concentration of formed Cu(I)-bathocuproine complex was determined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

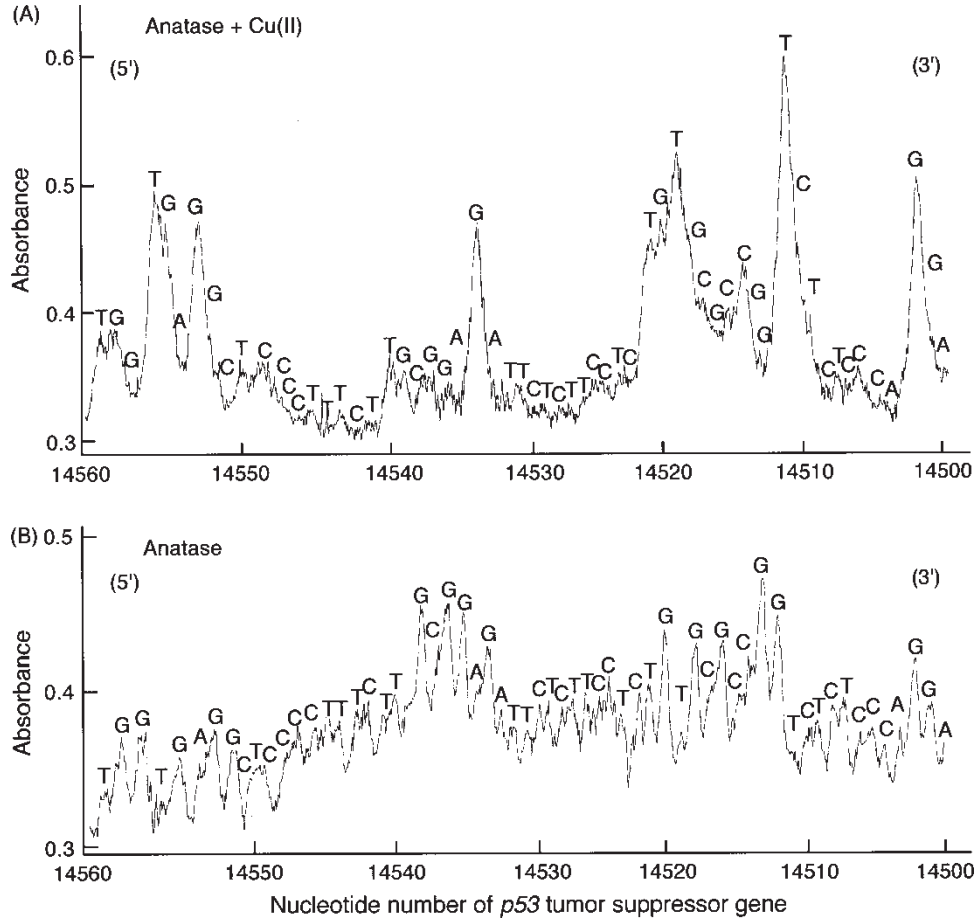

FIGURE 8 Site specificity of DNA damage induced by photo-irradiated anatase. The reaction mixtures contained the ^{32}P-5′-end-labeled 443 bp DNA fragment (*p53* tumor suppressor gene), 20 μM/base calf thymus DNA, 5 μM DTPA and 8 μg/ml anatase with 20 μM $CuCl_2$ (A) or 80 μg/ml anatase without $CuCl_2$ (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]

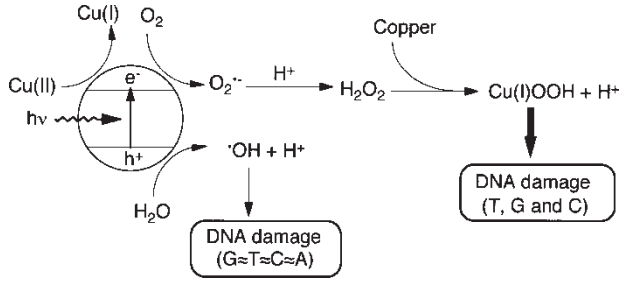

FIGURE 9 Proposed mechanism of DNA damage induced by photo-irradiated TiO_2.

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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