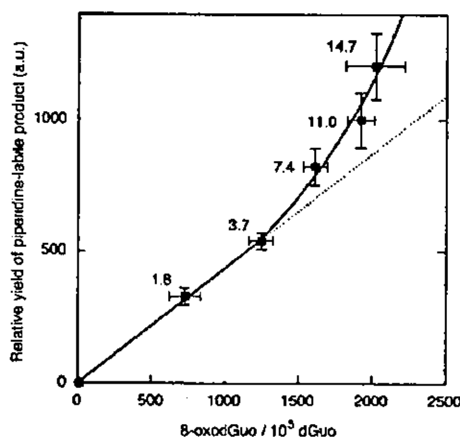

Figure 4. Plots of the relative yield of piperidine-labile product by photoexcited XAN *vs* that of 8-*oxo*-dGuo. The numerical values indicate the irradiated photon fluence (10^{18} cm^{-2}). Amounts of 8-*oxo*-dGuo formed by 10 μ*M* XAN with indicated irradiation dose were measured as described in the legend to Fig. 3. The relative yield of the piperidine-labile product was estimated from the absorbance of the bands in the fragmentation patterns of X-ray film, which was obtained according to the procedure described in the legend to Fig. 1 using the ^{32}P-labeled 98 base pair fragment and 10 μ*M* XAN. The relative yield was determined by normalizing the value at 11.0 × 10^{18} cm^{-2} (6 J·cm^{-2}) photon fluence as 1000 (arbitrary unit). Results represent means ± SD of four independent experiments.

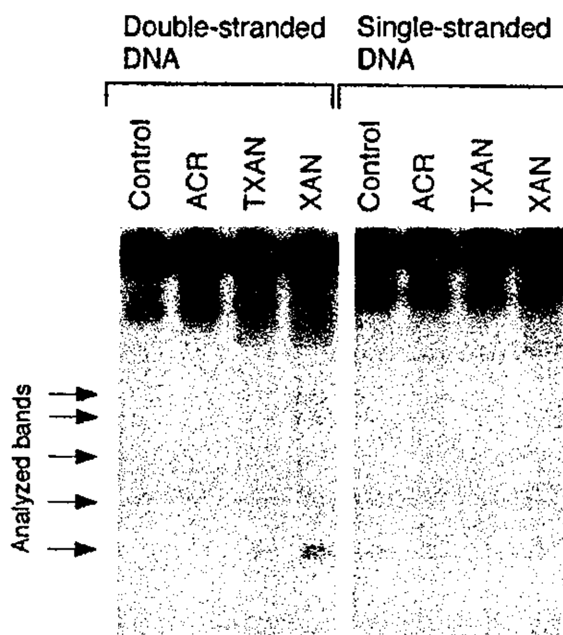

Figure 5. Comparison of UVA-induced DNA damage in the presence of XAN analogues. The reaction mixture contained the ^{32}P-labeled 443 base pair fragment, 20 μ*M*/base of calf thymus DNA, 10 μ*M* XAN analogues, 5 μ*M* DTPA and 2.5% (vol/vol) ethanol in 100 μL of 10 m*M* sodium phosphate buffer (pH 7.8). For the experiment with denatured single-stranded DNA, the ^{32}P-labeled and calf thymus DNA fragments were treated by heating at 90°C for 5 min, followed by quick chilling on ice. The reaction mixtures were exposed to 6 J·cm^{-2} UVA light (λ_{max} = 365 nm, 1.2 mW·cm^{-2}). Then, the DNA fragments were treated with piperidine and analyzed by the method described in Materials and Methods.

Comparison of the DNA-damaging ability of photoexcited XAN analogues

Although photoexcited TXAN and ACR induced DNA damage in a similar manner to photoexcited XAN, the extent of DNA photodamage increased in the following order: XAN > TXAN > ACR (Fig. 5). This order is the same as that of the amounts of 8-*oxo*-dGuo formation by these photosensitizers. Relative extents of DNA damage were estimated from the absorbance of the main five bands in the fragmentation patterns. Obtained values in the case of double-stranded DNA were normalized at the value of XAN, determined to be 1000 (arbitrary unit), and those of TXAN and ACR were 184 and 17, respectively. DNA denaturation decreased DNA photodamage by each photosensitizer.

Calculated HOMO energies and molar absorption coefficients of XAN analogues

Calculated HOMO energies of XAN, TXAN and ACR were 8.57, 8.07 and 7.75 eV, respectively. These values are larger than that of 5′-G of GG in double-stranded DNA (6.73 eV) (17), suggesting that photoexcited XAN analogues can oxidize 5′-G of GG through electron transfer from an energetic viewpoint.

Absorption spectra of XAN analogues were measured in 10 m*M* sodium phosphate buffer (pH 7.8) containing 2.5% (vol/vol) ethanol. Molar absorption coefficients at 365 nm of XAN, TXAN and ACR were 1240, 2800 and 5200 M^{-1}·cm^{-1}, respectively.

DISCUSSION

The present study has demonstrated that photoexcited XAN analogues as well as riboflavin, a Type I photosensitizer (12), generate piperidine-labile products specifically at 5′-G of GG sequence and both guanines of 5′-AGGA sequence in double-stranded DNA. Effects of scavengers of reactive oxygen and D_2O on DNA damage suggested that the contribution of reactive oxygen (Type II mechanism) to the DNA photodamage is negligibly small. Therefore, these results can be reasonably explained by assuming that nucleobase oxidation is induced by photoexcited XAN analogues mainly through electron transfer (Type I mechanism) (Fig. 6).

Guanine is most easily oxidized among the four DNA bases because the oxidation potential of guanine is lower than that of the other DNA bases (36–38). MO calculations have revealed that 5′-G in GG sequence in double-stranded DNA significantly lowers the HOMO energy (17,18). Therefore, the cation radical on the 5′-G in GG sequence arises from either the initial electron abstraction of this guanine by photoexcited XAN analogues or through charge migration from a relatively distant one-electron oxidized nucleobase (8,36,37,39–41). Relevantly, Schuster *et al.* clarified the hopping mechanism of long-distance charge transfer in duplex DNA (42,43). Decrease of the extent of DNA damage by photoexcited XAN analogues in single-stranded DNA also supported the DNA photodamage through electron transfer. The formed guanine cation radicals may react with water molecules to form the C-8 OH adduct radical (8,36). This radical may be converted by a reducing process into 2,6-diamino-4-hydroxy-5-formamidopyrimidine residue, a piperidine-labile product (8,36). On the other hand, competitive oxidation, which may be achieved by molecular oxygen, gives rise to 8-*oxo*-dGuo (8,36,44). The formation of 8-*oxo*-dGuo causes DNA misreplication that may lead to mutations such as G–C → T–A transversion (45,46). Although

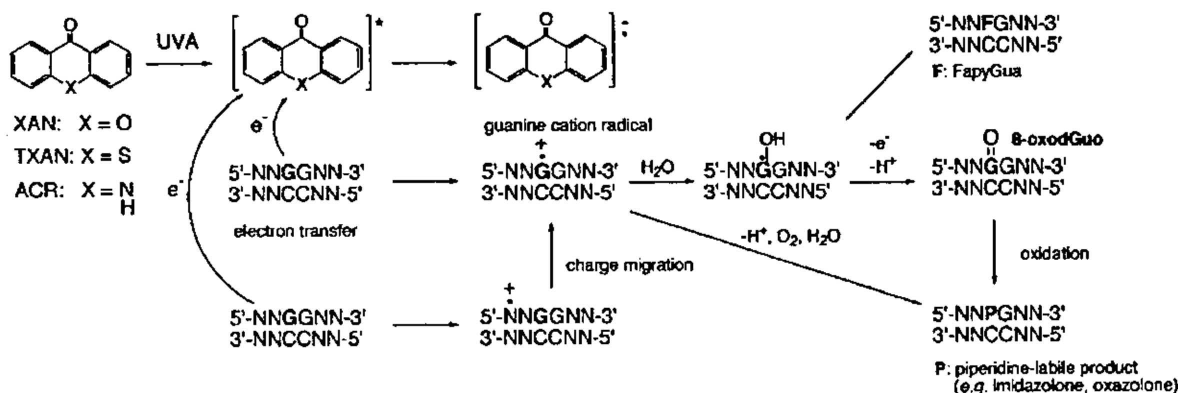

Figure 6. Possible mechanism of base oxidation at 5' site of GG sequence in double-stranded DNA induced by UVA-irradiated XAN analogues.

the 8-*oxo*-dGuo site is not efficiently cleaved under piperidine treatment (47), 8-*oxo*-dGuo can be converted into piperidine-labile products (*e.g.* imidazolone, oxazolone) through further oxidation (36,48,49). Imidazolone and oxazolone might be also produced through deprotonation of guanine cation radical followed by reaction with molecular oxygen (8,36,50,51). The present study regarding photon fluence dependence of DNA photodamage suggests that 8-*oxo*-dGuo oxidation into piperidine-labile products can occur in an irradiation dose-dependent manner. It has been reported that imidazolone and oxazolone are major oxidation products of guanine by the Type I mechanism (50,51). Imidazolone forms a stable base pair with G comparable with the Watson–Crick G–C base pair (48,49) and may cause G–C → C–G transversion (52,53). These transversions can partly explain the mutation induced by UVA as previously reported (5).

The extents of DNA damage increased depending on the HOMO energies of XAN, TXAN and ACR. Figure 7 shows the plots of the quantum yields of DNA damage against the gaps of HOMO energies (ΔE) between the photosensitizers and 5'-G of GG. The quantum yield of 8-*oxo*-dGuo formation ($\Phi_{8\text{-}oxo\text{-dGuo}}$) was estimated from the results in Fig. 3 using the photon fluence (1.10×10^{19} cm^{-2}, calculated under this experimental condition) and molar absorption coefficients at 365 nm of XAN analogues. Similarly, the relative quantum yield of the piperidine-labile product (Φ_P) was estimated from the results in Fig. 5. The logarithm plots indicate that the DNA-damaging abilities of these photosensitizers almost increased exponentially with ΔE (Fig. 7A). The plots have shown that Φ_P is almost proportional to $\Phi_{8\text{-}oxo\text{-dGuo}}$. The electron transfer reaction should also be affected by an interaction between DNA and photosensitizer. The absorption and fluorescence spectra of XAN analogues were not significantly changed by addition of DNA (data not shown), suggesting that noncovalent bonding to DNA can be negligible. Therefore, the quantum yield of the electron transfer reaction may be determined by the collision frequency between photoexcited XAN analogues and DNA in addition to ΔE. The collision frequency can be determined by the diffusion control rate coefficient (k_{dif}), concentration of nucleobase ([base]), triplet quantum yield (Φ_{isc}) and lifetime of excited triplet state (τ_0') of photosensitizer. Because k_{dif} and [base] can be taken as constants, the collision frequency varies depending on $\Phi_{isc} \times \tau_0'$. The values of Φ_{isc} and τ_0' of XAN analogues were previously reported (54). The values of $\Phi_{8\text{-}oxo\text{-dGuo}}$ and Φ_P were divided by $\Phi_{isc} \times \tau_0'$ and plotted against ΔE (Fig. 7B). These plots also showed an increase of extent of DNA damage exponentially, almost depending on ΔE. Strictly, the DNA-damaging ability of the photosensitizer should be determined by not only ΔE but also other factors containing the free energy of the electron transfer reaction and an interaction between DNA and photosensitizer. However, these results suggest that the

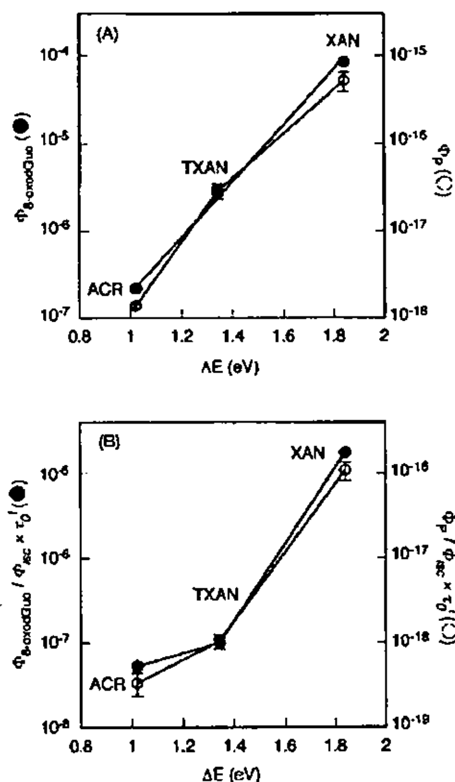

Figure 7. Relationship between the DNA-damaging abilities of XAN analogues and their HOMO energies. The quantum yield of 8-*oxo*-dGuo formation ($\Phi_{8\text{-}oxo\text{-dGuo}}$, ●) and the relative quantum yield of the piperidine-labile product (Φ_P, ○) by UVA irradiation (λ_{max} = 365 nm, 6 J·cm^{-2}) in the presence of 10 μM XAN analogues (A) and those values divided by $f = (\Phi_{isc} \times \tau_0')$ (B) are plotted against ΔE.

DNA-damaging abilities of these XAN analogues practically depend on their HOMO energies mainly.

In summary, this study demonstrated that photoexcited XAN analogues, exogenous photosensitizers, mediate poly-G–specific DNA oxidation through electron transfer. DNA oxidation through photoinduced electron transfer may play an important role in photocarcinogenesis mediated by photosensitizers. This study has also shown that the DNA-damaging abilities of XAN analogues increase exponentially with their HOMO energies. It is concluded that the DNA-damaging ability of derivatives of XAN analogues can be roughly estimated from their HOMO energies.

Acknowledgement—This work was supported by Grants-in-Aid for Scientific Research granted by the Ministry of Education, Science, Sports and Culture of Japan.

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Sequence-specific DNA damage induced by ultraviolet A-irradiated folic acid via its photolysis product

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Received 6 September 2002, and in revised form 18 November 2002

Abstract

DNA damage mediated by photosensitizers participates in solar carcinogenesis. Fluorescence measurement and high-performance liquid chromatography analysis demonstrated that photoirradiated folic acid, one of the photosensitizers in cells, generates pterine-6-carboxylic acid (PCA). Experiments using ^{32}P-labeled DNA fragments obtained from a human gene showed that ultraviolet A-irradiated folic acid or PCA caused DNA cleavage specifically at consecutive G residues in double-stranded DNA after *Escherichia coli* formamidopyrimidine–DNA glycosylase or piperidine treatment. The amount of 8-oxo-7,8-dihydro-2′-deoxyguanosine formed through this DNA photoreaction in double-stranded DNA exceeded that in single-stranded DNA. Kinetic studies suggested that DNA damage is caused mainly by photoexcited PCA generated from folic acid rather than by folic acid itself. In conclusion, photoirradiated folic acid generates PCA, which induces DNA photooxidation specifically at consecutive G residues through electron transfer. Excess intake of folic acid supplements may increase a risk of skin cancer by solar ultraviolet light.
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Keywords: DNA damage; Solar UV carcinogenesis; Folic acid; Pterine-6-carboxylic acid; Electron transfer

Solar UV radiation is a well-known carcinogen to humans [1]. UVA (320–400 nm) accounts for approximately 95% of solar UV radiation and is capable of causing mutation and skin cancer [1–4]. In contrast to UVB (280–320 nm), UVA indirectly induces DNA damage through activation of photosensitizers, because UVA is hardly absorbed by the DNA molecule. Therefore, solar carcinogenesis should involve UVA-mediated photoactivation of photosensitizers [5–12]. There are abundant porphyrin, flavin, and other cellular molecules that could act as endogenous photosensitizers. Exogenous photosensitizers from food and drugs are also considered to participate in solar carcinogenesis [12]. We previously reported that various photosensitizers mediated the DNA damage induced by UVA through Type I (electron transfer) [7–12] and Type II (singlet oxygen) [12] mechanisms.

Folic acid is one of the B vitamins essential for human nutrition. Supplements of folic acid are useful for prevention of cancer [13,14], neural tube defects [15,16], and other disorders (e.g., homocysteine increase [17]). It has been reported that folic acid fortification results in a significant increase in plasma folate concentration [18]. Folic acid can be easily decomposed by sunlight, UV light, oxidation, reduction, and acid [19–21] and undergoes photodegradation in human skin [19]. Therefore, photoproducts of folic acid may also act as photosensitizers in human skin cells.

In this study, the DNA-damaging ability of photoirradiated folic acid and its photolysis product was examined using ^{32}P-labeled DNA fragments obtained from the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene. The content of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo)[1] [22,23], an

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[1] *Abbreviations used:* 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; dGuo, 2′-deoxyguanosine, HPLC-ECD, HPLC equipped with an electrochemical detector; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; 4-oxo-TEMPO, 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl; PCA, pterine-6-carboxylic acid; Fpg, *E. coli* formamidopyrimidine–DNA glycosylase.

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doi:10.1016/S0003-9861(02)00722-1

oxidative product of 2′-deoxyguanosine (dGuo), formed by photoirradiated folic acid, was measured with an electrochemical detector coupled to HPLC (HPLC-ECD). Photochemical degradation of folic acid was investigated by fluorescence measurement and HPLC with photodiode array UV–visible detector.

Materials and methods

Chemicals

Restriction enzymes (*Ava*I, *Hin*dIII and *Pst*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Restriction enzymes (*Eco*RI and*Apa*I) and calf intestine phosphatase were from Boehringer Mannheim GmbH (Mannheim, Germany). Folic acid was from Wako Pure Chemical Industries (Osaka, Japan). [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) was from Dojin Chemicals Co. (Kumamoto, Japan). Calf thymus DNA, 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (4-oxo-TEMPO), and dGMP were from Sigma Chemical Co. (St. Louis, MO). Nuclease P_1 was from Yamasa Shoyu Co. (Chiba, Japan). Pterine-6-carboxylic acid (PCA) was from ICN Biomedicals (California). *E. coli* formamidopyrimidine-DNA glycosylase (Fpg) was from Trevigen Co. (Gaithersburg, MD).

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

The DNA fragment of the human *p53* tumor suppressor gene was prepared from pUC18 plasmid, with ligated fragments containing exons of the *p53* gene [24]. The 5′-end-labeled 650-bp (*Hin*dIII* 13972–*Eco*RI* 14621) fragment was obtained as described previously [25]. The fragment was further digested with *Apa*I to obtain the 5′-end-labeled 443-bp fragment (*Apa*I 14179–*Eco*RI* 14621). DNA fragments were also obtained from the human c-Ha-*ras*-1 protooncogene [26]. The fragment was prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the c-Ha-*ras*-1 gene. Singly labeled 98-bp fragment (*Ava*I 2247–*Pst*I* 2344) and 337-bp fragment (*Pst*I 2345–*Ava*I* 2681) were prepared as described previously [27].

Detection of DNA damage induced by UVA-irradiated folic acid or PCA

The standard reaction mixture in a microtube (1.5-ml Eppendorf) contained ^{32}P-labeled DNA fragment, 5 μM/base of calf thymus DNA, 5 μM DTPA, and folic acid or PCA in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). Denatured single-stranded DNA fragments were prepared by heating at 90 °C for 5 min followed by quick chilling before exposure to UVA light. The mixtures were exposed to UVA light using 10-W UV lamps (λ_{max} = 365 nm; UVP, Inc., CA, USA) placed at a distance of 20 cm. Subsequently, the DNA was treated with 1 M piperidine for 20 min at 90 °C or 6 units of Fpg protein in the reaction buffer (10 mM Hepes–KOH (pH 7.4), 100 mM KCl, 10 mM EDTA, and 0.1 mg/ml bovine serum albumin) 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 [28] using a DNA sequencing system (LKB2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

Measurement of 8-oxodGuo formation induced by UVA-irradiated folic acid or PCA

The reaction mixtures containing 100 μM/base calf thymus DNA, 5 μM DTPA, and folic acid or PCA in 4 mM sodium phosphate buffer (pH 7.8) were exposed to UVA light (λ_{max} = 365 nm). 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 [9].

Assay of photolysis of folic acid

Folic acid (50 μM) in 10 mM phosphate buffer (pH 7.8) was irradiated with UVA light (λ_{max} = 365 nm) and the fluorescence spectra were measured with a RF-5300PC spectrophotometer (Shimadzu, Kyoto, Japan) on 340-nm excitation. UVA-irradiated folic acid was also analyzed by HPLC consisting of a LC-6A pump (Shimadzu) equipped with a Wakopak ODS column (i.d. 4.6 mm × 150 mm; Wako). The mobile phase consisted of 89.8% (v/v) water, 10.0% (v/v) acetonitrile, and 0.2% (v/v) acetic acid. The analysis was carried out at a column temperature of 25 °C and a flow rate of 0.5 ml/min. The HPLC eluate was routed directly into a photodiode array UV–visible detector (SPD-M10A, Shimadzu) and the spectrum of the eluate was measured. The concentration of detected folic acid and photoproducts were estimated from absorbance at 296 nm.

Analysis of the reactivity of photoexcited PCA by EPR spin destruction method

The reactivity of photoexcited PCA with mononucleotides was examined by the EPR spin destruction

method [12,29,30]. The reaction mixtures containing 100 μM PCA and 5 μM 4-oxo-TEMPO, which is a stable nitroxide radical, in 100 μl of 10 mM sodium phosphate buffer (pH 7.8) were exposed to $3\,J\,cm^{-2}$ UVA light ($\lambda_{max} = 365\,nm$, $1.2\,mW\,cm^{-2}$) in the absence or presence of 2.5 mM dGMP. After the irradiation, EPR spectra were measured at 25 °C using a JES-TE100 (JEOL, Tokyo, Japan) spectrometer with 100-kHz field modulation. The spectra were recorded with a microwave power of 10 mW and a modulation amplitude of 0.1 mT.

Results

Damage to DNA fragments induced by UVA-irradiated folic acid or PCA

Fig. 1 shows the autoradiogram of ^{32}P-5′-end-labeled DNA fragments exposed to UVA light ($\lambda_{max} = 365\,nm$) in the presence of folic acid or PCA. UVA irradiation caused DNA damage in the presence of folic acid or PCA, whereas UVA irradiation alone did not cause DNA damage under the conditions used. The extent of DNA damage induced by UVA plus PCA was significantly greater than that induced by UVA plus folic acid. The DNA cleavage was observed only when the DNA fragment was treated with piperidine (data not shown), suggesting that base modification is induced by UVA-irradiated folic acid or PCA.

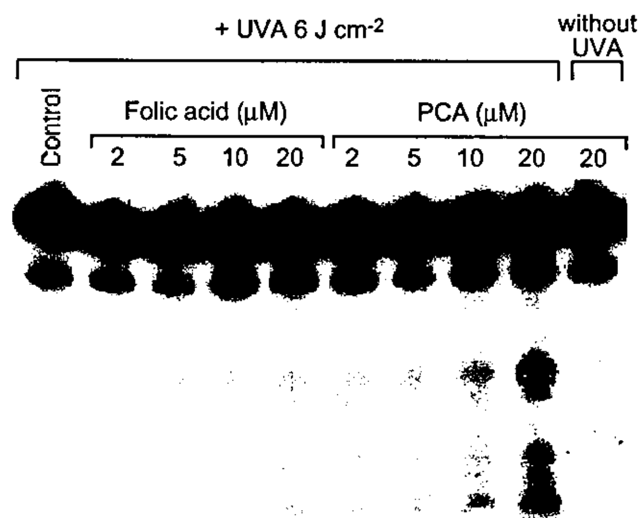

Fig. 1. Autoradiogram of DNA fragments exposed to UVA light in the presence of folic acid or PCA. The reaction mixture contained the ^{32}P-5′-end-labeled 98-bp DNA fragment, 5 μM/base of calf thymus DNA, 5 μM DTPA, and indicated concentration of folic acid or PCA in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were exposed to $6\,J\,cm^{-2}$ UVA light ($\lambda_{max} = 365\,nm$, $1.2\,mW\,cm^{-2}$). Then, the DNA fragments were treated with piperidine and analyzed by the method described under Materials and methods.

Sequence specificity of DNA damage by UVA-irradiated folic acid or PCA

Photoirradiated folic acid generated piperidine labile products specifically at the underlined G of 5′-<u>G</u>G-3′, 5′-G<u>G</u>G-3′, and 5′-G<u>G</u>GG-3′ sequences (Fig. 2B). When replacing folic acid with PCA, the result was almost the same (Fig. 2A). When denatured single-stranded DNA

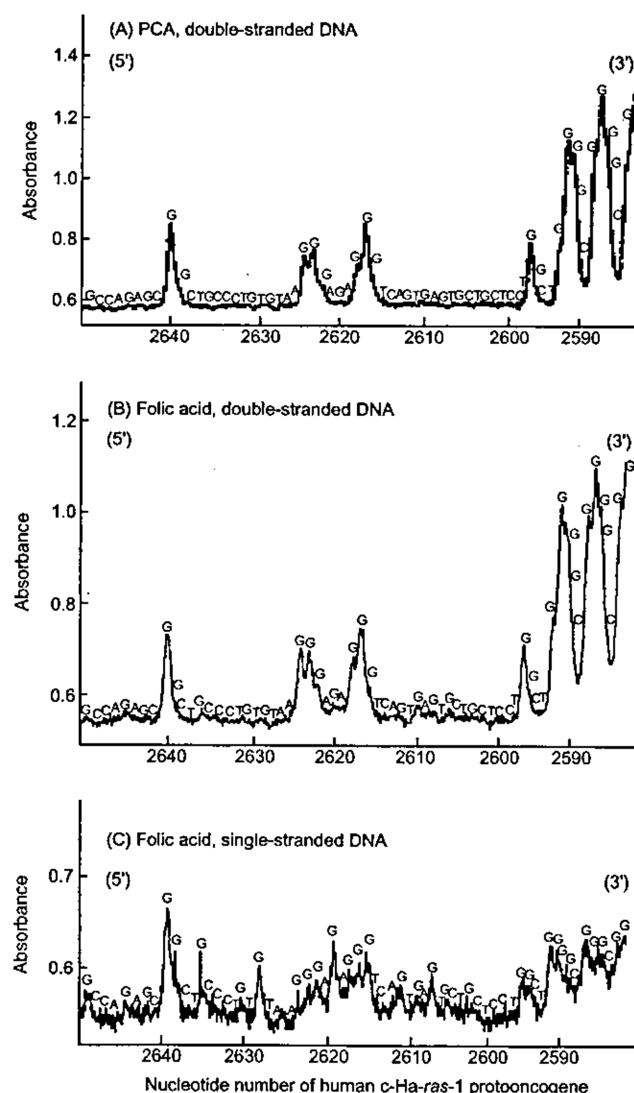

Fig. 2. Sequence specificity of DNA damage induced by UVA irradiation in the presence of PCA or folic acid. The reaction mixture contained the ^{32}P-5′-end-labeled 337-bp fragment (c-Ha-*ras*-1 protooncogene), 5 μM/base of calf thymus DNA, 5 μM DTPA, and 50 μM PCA (A) or 100 μM folic acid (B and C), in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). Where indicated, the ^{32}P-labeled and calf thymus DNA fragments were denatured by heating at 90 °C for 5 min, followed by quick chilling on ice before UVA irradiation (C). The reaction mixtures were exposed to $6\,J\,cm^{-2}$ UVA light ($\lambda_{max} = 365\,nm$, $1.2\,mW\,cm^{-2}$). Subsequently, the DNA fragments were treated with piperidine. The DNA was analyzed and the relative amounts of oligonucleotides were measured by the methods described under Materials and methods. Horizontal axis is the nucleotide numbers of the c-Ha-*ras*-1 protooncogene.

fragments were used, folic acid plus UVA caused DNA damage at the single guanines and consecutive guanine residues (Fig. 2C).

Fpg protein treatment and piperidine treatment demonstrated that photoexcited PCA caused preferential DNA cleavage at consecutive guanine residues in double-stranded DNA (Fig. 3). Fpg protein is known to recognize 8-oxodGuo and Fapy residues [31]. These results suggest that photoexcited PCA produces 8-oxodGuo and subsequently the piperidine-labile site at consecutive guanine residues in double-stranded DNA.

Formation of 8-oxodGuo induced by UVA-irradiated folic acid or PCA

Fig. 4 shows 8-oxodGuo formation induced by UVA irradiation in the presence of folic acid or PCA. The amount of 8-oxodGuo formation increased depending on concentration of folic acid or PCA. The amounts of 8-oxodGuo induced by photoirradiated folic acid and photoexcited PCA in double-stranded DNA exceeded those in single-stranded DNA. The formation of 8-oxodGuo induced by UVA plus PCA was markedly larger than that induced by UVA plus folic acid.

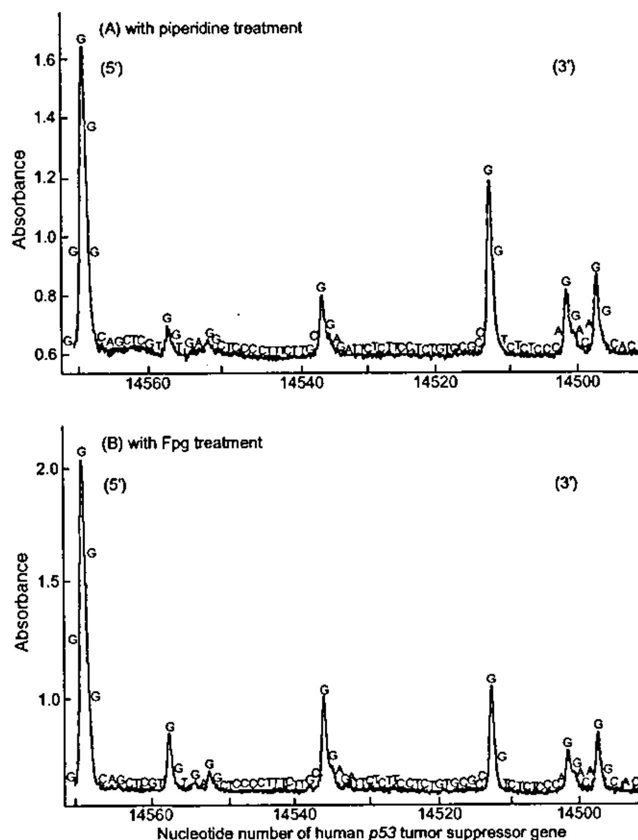

Fig. 3. Sequence specificity of DNA damage induced by UVA irradiation in the presence of PCA. The reaction mixture contained the ^{32}P-5′-end-labeled 443-bp fragment (*p53* tumor suppressor gene), 5 μM/base of calf thymus DNA, 5 μM DTPA, and 50 μM PCA in 100 μl of 10 mM sodium phosphate buffer (pH 7.8). The reaction mixtures were exposed to 6 J cm^{-2} UVA light (λ_{max} = 365 nm, 1.2 mW cm^{-2}). Subsequently, the DNA fragments were treated with piperidine (A) or Fpg protein (B). The DNA was analyzed and the relative amounts of oligonucleotides were measured by the methods described under Materials and methods. Horizontal axis is the nucleotide numbers of the *p53* tumor suppressor gene.

Photolysis of folic acid into PCA

Folic acid consists of the base pteridine attached to one molecule each of *p*-aminobenzoic acid and glutamic acid, and the pteridine moiety absorbs UVA light. Fluorescence intensity of the pteridine moiety of folic acid was very weak compared with that of PCA (Fig. 5), though pteridine is a strong fluorescent molecule. Relative fluorescence quantum yield (Φ_f) of folic acid with PCA is estimated from data shown in Fig. 5 to be $<2.8 \times 10^{-2}$. When folic acid was previously treated with photoirradiation, the fluorescence intensity increased depending on the extent of photoirradiation (Fig. 5), suggesting that UVA light converts folic acid into a stronger fluorescent molecule. The fluorescence spectrum of UVA-irradiated folic acid was quite similar to that of PCA.

The photolysis product of folic acid was analyzed by HPLC with a photodiode array UV-visible detector. In the chromatogram of folic acid, the peak height of folic

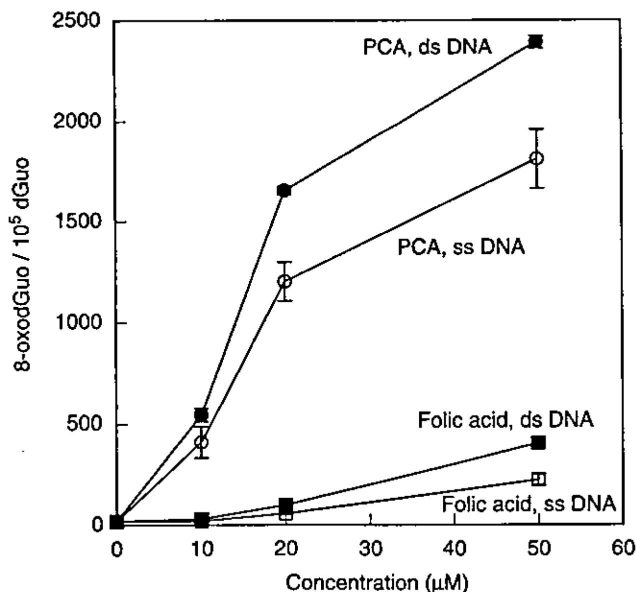

Fig. 4. Formation of 8-oxodGuo induced by UVA irradiation in the presence of folic acid or PCA. The reaction mixture containing 100 μM/base native (ds) or denatured (ss) calf thymus DNA, 5 μM DTPA, and the indicated concentration of folic acid or PCA in 100 μl of 4 mM sodium phosphate buffer (pH 7.8) was exposed to 6 J cm^{-2} UVA light (λ_{max} = 365 nm, 1.2 mW cm^{-2}). Where indicated, DNA fragment was denatured by heating for 5 min at 90 °C followed by chilling on ice before UVA irradiation. After the irradiation, DNA was treated and the amount of 8-oxodGuo was measured by the methods described under Materials and methods.

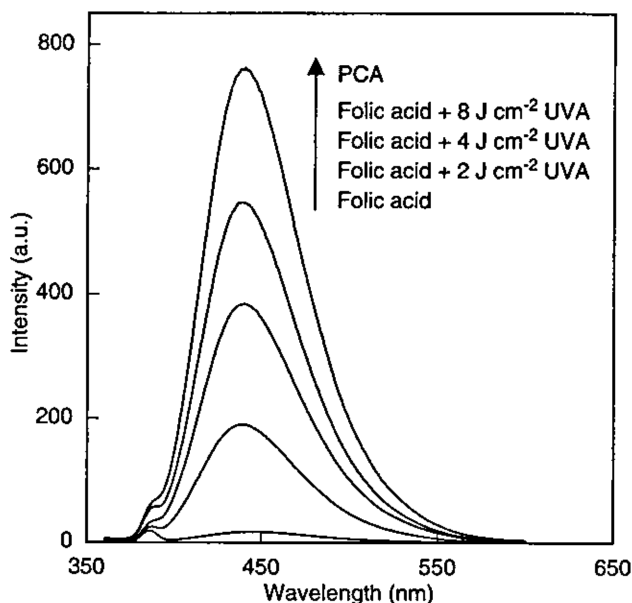

Fig. 5. Fluorescence spectra of PCA and folic acid previously treated with UVA irradiation. Folic acid (50 μM) in 10 mM sodium phosphate buffer (pH 7.8) was previously irradiated with UVA light (λ_{max} = 365 nm, 1.2 mW cm^{-2}) and diluted into 1 μM solution to be measured. The concentration of PCA was 1 μM. These spectra were measured in 10 mM sodium phosphate buffer (pH 7.8) on 340-nm excitation.

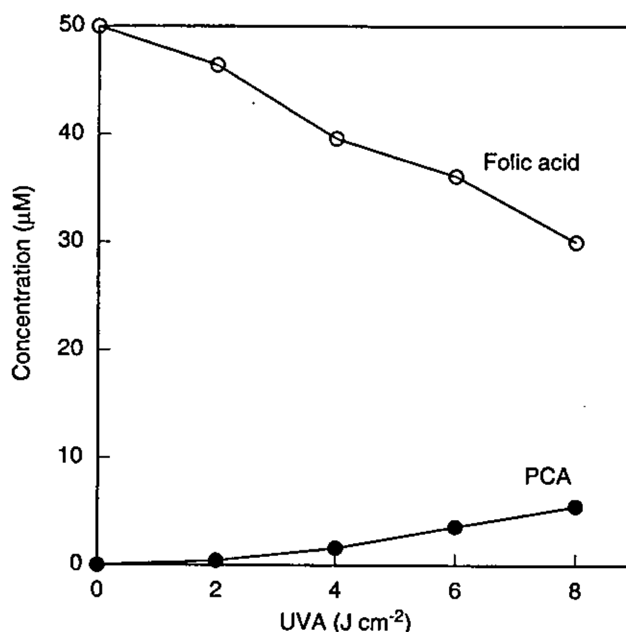

Fig. 6. Irradiation dose dependence of folic acid photolysis. Folic acid (50 μM) in 10 mM phosphate buffer (pH 7.8) was irradiated with UVA light (λ_{max} = 365 nm, 0.85 mW cm^{-2}) and analyzed by HPLC using a mobile phase consisting of 89.8% (v/v) water, 10.0% (v/v) acetonitrile, and 0.2% (v/v) acetic acid. The concentration of detected folic acid and PCA were estimated from absorbance at 296 nm.

acid was decreased by photoirradiation and the peak of a photolysis product was observed. The retention time (5.1 min) and the absorption spectrum of the photolysis product were quite similar to those of PCA (data not shown), indicating that the photoirradiated folic acid produces PCA. The concentration of folic acid decreased, whereas that of PCA generated from folic acid increased, depending on the photoirradiation dose (Fig. 6). The generation of PCA was not stoichiometric with the loss of folic acid, suggesting other routes of folic acid degradation and/or further decomposition of PCA.

Irradiation dose dependence of the 8-oxodGuo formation by UVA-irradiated folic acid or PCA

Formation of 8-oxodGuo by folic acid or PCA increased depending on the photoirradiation dose (Fig. 7). The 8-oxodGuo formation by PCA was markedly larger than that by folic acid. No or little DNA damage was induced by UVA in the presence of folic acid within 2 J cm^{-2}. The induction period within 2 J cm^{-2} suggests that folic acid itself could cause no or little DNA oxidation and photoproduct generated from photoirradiated folic acid participates in DNA oxidation.

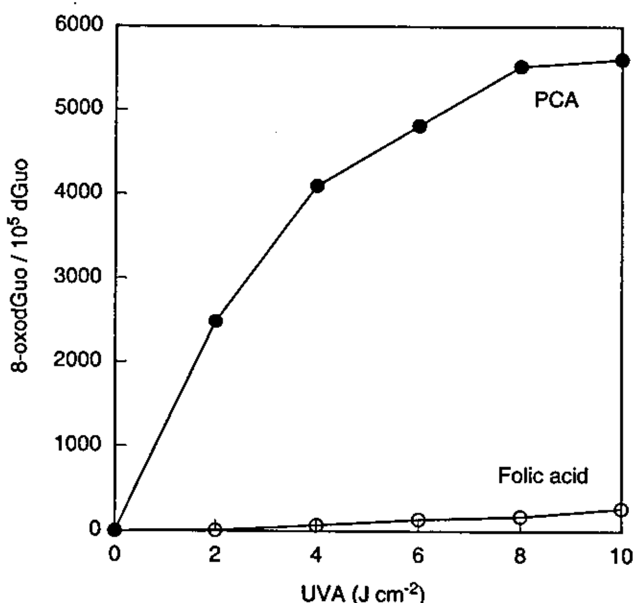

Fig. 7. Irradiation dose dependence of 8-oxodGuo formation induced by UVA irradiation in the presence of folic acid or PCA. The reaction mixture containing 100 μM/base calf thymus DNA, 5 μM DTPA, and 50 μM of folic acid or PCA in 100 μl of 4 mM sodium phosphate buffer (pH 7.8) was exposed to UVA light (λ_{max} = 365 nm, 0.85 mW cm^{-2}). After the irradiation, DNA was treated and the amount of 8-oxodGuo was measured by the methods described under Materials and methods.

Reactivity of photoexcited PCA with dGMP

The EPR signal of 4-oxo-TEMPO radical was clearly observed after photoirradiation in the presence of PCA (data not shown). The signal was completely diminished in the presence of dGMP. This result can be explained by assuming that 4-oxo-TEMPO radical was diminished

by the reaction with guanine cation radical generated through photoinduced electron transfer to photoexcited PCA.

Discussion

This study has demonstrated that photoirradiated folic acid induces DNA damage. Fluorescence spectrum and HPLC analysis have demonstrated that photoirradiated folic acid generates PCA depending on the irradiation dose. Similarly, Akhtar et al. [20] reported that PCA and *p*-aminobenzoyl-gulutamic acid are the photoproducts of UVA-irradiated folic acid. We have proposed the mechanism of PCA generation from photoexcited folic acid through an intramolecular electron transfer, following hydrolysis and oxidation. Pteridine is a strong fluorescent molecule, but the pteridine moiety of folic acid shows very weak fluorescence. This fluorescence property suggests that fluorescence of folic acid is quenched due to an intramolecular electron transfer from the *p*-aminobenzoyl-glutamic acid moiety to the photoexcited pteridine moiety. The intramolecular electron transfer rate constant (k_{et})[2] was roughly estimated to be $>10^9$–$10^{10}\,s^{-1}$ from the relative fluorescence quantum yield of folic acid (shown under Results: $\Phi_f < 2.8 \times 10^{-2}$) and the lifetime ($\tau_0$) of the photoexcited pteridine moiety almost ranges from 0.1 to 1 ns [32]. Therefore, photoirradiated folic acid should form radical ion pairs, which undergo hydrolysis and oxidation, leading to the formation of PCA.

Photoexcited PCA and photoirradiated folic acid induced DNA damage specifically at the underlined G in 5′-<u>G</u>G-3′, 5′-G<u>G</u>G-3′, and 5′-G<u>G</u>GG-3′ sequences in double-stranded DNA, and the extent of DNA damage by UVA plus PCA was much larger than that by UVA plus folic acid. Comparison of the irradiation dose dependence of the 8-oxodGuo formation by photoirradiated folic acid with that of PCA has suggested that DNA damage is due to photoactivation of PCA generated from folic acid rather than to folic acid itself. This can be explained by the rapid intramolecular electron transfer ($k_{et} > 10^9$–$10^{10}\,s^{-1}$) leading to the PCA formation preferentially occuring rather than the intermolecular reaction of DNA with the photoexcited state of folic acid (the maximum reaction rate constant $\approx 1.8 \times 10^5\,s^{-1}$).[3] These kinetic studies indicated that the photoexcited PCA mainly induces DNA damage.

Photoexcited PCA induced DNA cleavage especially at consecutive G sequences in double-stranded DNA after Fpg protein or piperidine treatment. Fpg protein is known to recognize 8-oxodGuo and Fapy residues [31]. The sequence specificity of DNA damage by photoexcited PCA was quite similar to that induced by Type I photosensitizers [7–12]. These results suggest that photoexcited PCA oxidizes the nucleobase through electron transfer (Type I mechanism), leading to the formation of 8-oxodGuo and/or related photoproducts (Fig. 8). The results of the EPR spin destruction method also support the Type I mechanism. Guanine is the most easily oxidized among the four DNA bases, because the oxidation potential of guanine is lower than that of the other DNA bases [33,34]. Molecular orbital calculations have revealed that stacking of two guanine bases in double-stranded DNA significantly lowers the ionization potential, and electron loss centers are localized on the consecutive G sequences [35,36]. The amount of 8-oxodGuo formation by photoexcited PCA in double-stranded DNA was larger than that in single-stranded

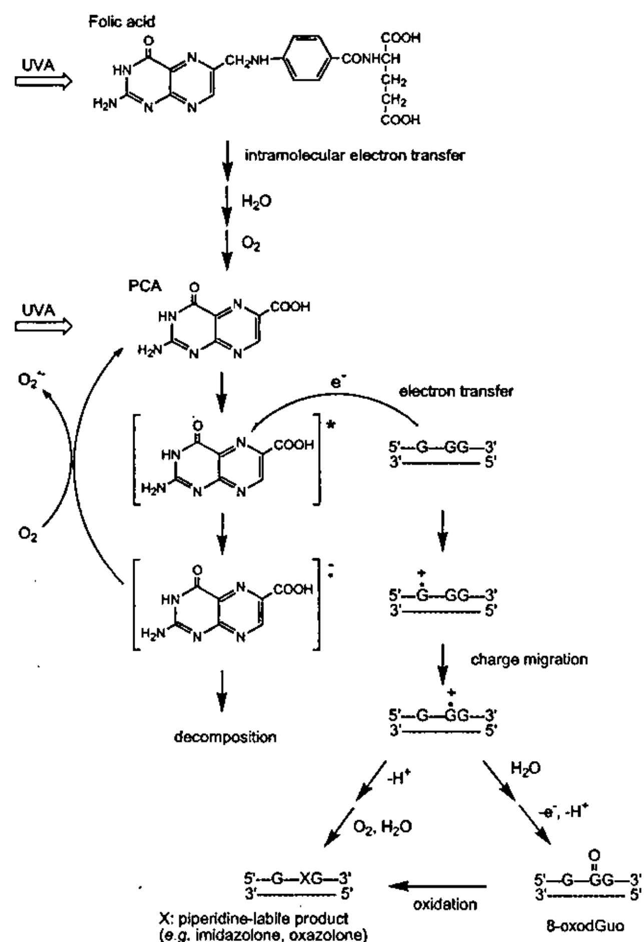

Fig. 8. Proposed mechanism of DNA damage induced by UVA-irradiated folic acid.

[2] The electron transfer rate constant (k_{et}) was estimated from $k_{et} = (1/\Phi_f - 1) \times 1/\tau_0$, where Φ_f and τ_0 are the relative fluorescence quantum yield and the fluorescence lifetime of the pteridine moiety of folic acid, respectively.

[3] The maximum reaction rate constant (k_{max}) between photoexcited folic acid and DNA was estimated from the equation of pseudo-first order diffusion control reaction rate constant (k_{dif}), $k_{max} \approx k_{dif} = 8RT[\mathrm{dGuo}]/3\eta$, where R, T, [dGuo], and η are gas constant, absolute temperature, concentration of dGuo (25 μM), and viscosity of water ($0.891 \times 10^{-3}\,kg\,m^{-1}\,s^{-1}$), respectively.

DNA, supporting the idea that DNA is damaged by this mechanism [7,8]. Thus, a guanine radical cation formed in DNA is finally localized on a consecutive guanine site through charge migration. The decomposition of a guanine radical cation might take two competitive pathways: deprotonation followed by reaction with O_2 leading to a piperidine-labile product (e.g., imidazolone, oxazolone) [6,33,36–38] or hydration leading to 8-oxodGuo [6,33,39,40], the latter being less efficiently cleaved by hot piperidine treatment [41,42]. Piperidine-labile products can be also formed from 8-oxodGuo through further oxidation [33,43–45]. The relative probability of hydration vs deprotonation of the radical cation is modulated by the local helical condition and dynamics variations of DNA [42]. The PCA anion radical generated through the electron transfer can be converted into PCA through oxidation by molecular oxygen and/or may undergo decomposition. Superoxide anion radical generation through O_2 reduction by the PCA anion radical was confirmed by the analysis of cytochrome *c* reduction (unpublished data).

The formation of 8-oxodGuo causes DNA misreplication that may lead to mutation such as G-C → T-A transversion [46,47]. The imidazolone residue formed in DNA may cause G-C → C-G transversion [48,49] due to the formation of a stable base pair with G comparable with the Watson–Crick G-C base pair [43,44]. These transversions can explain a part of the mutation induced by UVA radiation as previously reported [3,4].

In summary, the present study has demonstrated that photoirradiated folic acid induces poly-G specific DNA oxidation through photoinduced electron transfer. The reactive species to induce the DNA oxidation is mainly photoexcited PCA, which is generated via photolysis of folic acid. Since folic acid in human skin is easily decomposed by sunlight [19], PCA, an active photosensitizer, may be easily generated in human skin. This study suggests that decomposition of folic acid participates in solar-induced DNA damage, possibly resulting in the initiation process of skin cancers. Folic acid supplements are useful for prevention of cancer and other disorders [13–17], but the excess intake of folic acid might increase the risk of skin cancer by solar UV.

Acknowledgment

This work was supported by Grants-in-Aid for Scientific Research granted by the Ministry of Education, Science, Sports and Culture of Japan.

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Photochemistry and Photobiology, 2002, 76(5): 467–472

Photohydrolysis of Methotrexate Produces Pteridine, Which Induces Poly-G–specific DNA Damage Through Photoinduced Electron Transfer¶

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Received 26 April 2002; accepted 12 August 2002

ABSTRACT

Methotrexate (MTX), an antineoplastic agent, demonstrates phototoxicity. The mechanism of damage to biomacromolecules induced by photoirradiated MTX was examined using ^{32}P-labeled DNA fragments obtained from a human gene. Photoirradiated MTX caused DNA cleavage specifically at the underlined G in 5′-GG and 5′-GGG sequences in double-stranded DNA only when the DNA fragments were treated with piperidine, which suggests that DNA cleavage was caused by base modification with little or no strand breakage. With denatured single-stranded DNA the damage occurred at most guanine residues. The amount of formation of 8-hydroxy-2′-deoxyguanosine (8-oxodGuo), an oxidative product of 2′-deoxyguanosine, in double-stranded DNA exceeded that in single-stranded DNA. These results suggest that photoirradiated MTX participates in 8-oxodGuo formation at the underlined G in 5′-GG and 5′-GGG sequences in double-stranded DNA through electron transfer, and then 8-oxodGuo undergoes further oxidation into piperidine-labile products. Fluorescence measurement, high-pressure liquid chromatography and mass spectrometry have demonstrated that photoexcited MTX is hydrolyzed into 2,4-diamino-6-(hydroxymethyl)pteridine (DHP). DNA damage induced by DHP was observed in a similar manner as was the damage induced by MTX. The extent of DNA damage and the formation of 8-oxodGuo by DHP were much larger than those induced by MTX. The kinetic analysis, based on the time course of DNA oxidation by photoirradiated MTX, suggests that DNA damage is caused by photoexcited DHP rather than by photoexcited MTX. In conclusion, photoexcited MTX undergoes hydrolysis through intramolecular electron transfer, resulting in the formation of DHP, which exhibits a phototoxic effect caused by oxidation of biomacromolecules through photoinduced electron transfer.

¶Posted on the web site on 28 August 2002.
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Abbreviations: dGuo, 2′-deoxyguanosine; DHP, 2,4-diamino-6-(hydroxymethyl) pteridine; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; HPLC-ECD, high-pressure liquid chromatography equipped with an electrochemical detector; MTX, methotrexate; $^1O_2$, singlet oxygen; 8-oxodGuo, 8-hydroxy-2′-deoxyguanosine (also known as 8-oxo-7,8-dihydro-2′-deoxyguanosine).
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INTRODUCTION

The photochemical stability and the excited state property of pharmaceutical substances should be related to the occurrence of phototoxicity as an adverse side effect after drug administration to patients (1). Methotrexate (MTX), a strong inhibitor of dihydrofolate reductase, has been widely used for chemotherapy in many types of cancer (2). MTX also has been used in the treatment of psoriasis. However, the phototoxic effects observed after administration of MTX to patients have been reported as adverse side effects (3–6). Furthermore, after administration, MTX also causes phototoxic effects as adverse side effects of PUVA therapy (4–6). Because MTX has an absorption band in the UVA region, the UVA-induced damage to biomacromolecules may participate in the phototoxicity of MTX.

Photoinduced damage to biomacromolecules has been investigated using DNA as one of the target molecules (7–25). We have previously reported the mechanism of DNA damage induced by photoexcited drugs (20–25). In this study, to approach the clarification of the mechanism of biomacromolecules damage by photoirradiated MTX, we examined MTX-mediated photolesions of ^{32}P-labeled DNA fragments obtained from a human gene as a target molecule. We also measured the content of 8-hydroxy-2′-deoxyguanosine (8-oxodGuo) (26,27), an oxidative product of 2′-deoxyguanosine (dGuo), formed by photoirradiating MTX with an electrochemical detector coupled with high-performance liquid chromatography (HPLC-ECD). Photochemical degradation of MTX was investigated by fluorescence measurements. Furthermore, the photolysis product of MTX was analyzed by HPLC and mass spectrometry. The DNA-damaging ability of 2,4-diamino-6-(hydroxymethyl) pteridine (DHP), which is a photoproduct of MTX, also has been examined.

MATERIALS AND METHODS

Materials. Restriction enzymes (*Apa*I and *Hin*dIII) and T_4 polynucleotide kinase were purchased from New England Biolabs (Berverly, MA). A restriction enzyme (*Eco*RI) and calf intestine phosphatase were from Boehringer Mannheim GmbH (Mannheim, Germany). MTX was from Aldrich Chemical Co. (Milwaukee, WI). [γ-^{32}P]-ATP (222 TBq mmol^{-1}) was from New England Nuclear (Boston, MA). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) was from Dojin Chemicals Co. (Kumamoto, Japan). Calf thymus DNA was from Sigma Chemical Co. (St. Louis, MO). Nuclease P_1 was from Yamasa Shoyu Co. (Chiba, Japan). DHP was from Acros Organics (New Jersey).

Preparation of ^{32}P-5′–end-labeled DNA fragments. The DNA fragment of the human *p53* tumor suppressor gene was prepared from pUC18

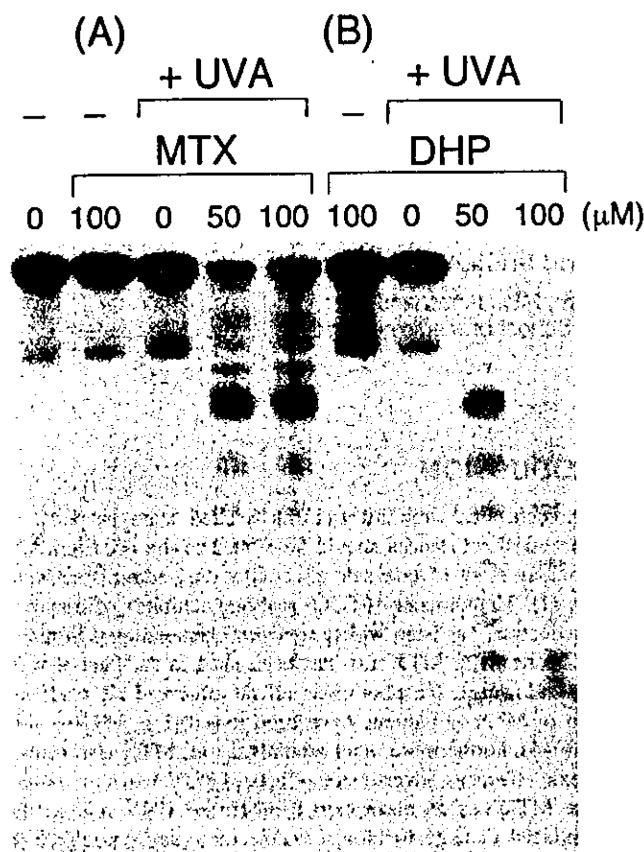


Figure 1. Autoradiogram of DNA fragments exposed to UVA light in the presence of MTX or DHP. The reaction mixture contained the ^{32}P-labeled 443-base pair (*Apa*I 14179–*Eco*RI* 14621) fragment, 5 μM/base of calf thymus DNA, and the indicated concentration of MTX (A) or DHP (B) in 100 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The reaction mixtures were exposed to 7 J cm^{-2} UVA light (λ_{max} = 365 nm). Then, the DNA fragments were treated with piperidine and analyzed by the method described in Materials and Methods.

plasmid, ligated fragments containing exons of the *p53* gene (28). The 5′-end-labeled 650-base pair (*Hin*dIII* 13972–*Eco*RI* 14621) fragment was obtained as described previously (29). This fragment was further digested with *Apa*I to obtain the singly labeled 443-base pair (*Apa*I 14179–*Eco*RI* 14621) and 211-base pair (*Hin*dIII* 13972–*Apa*I 14182) fragments.

Detection of DNA damage induced by photoirradiated MTX or DHP. The standard reaction mixture in a microtube (1.5 mL Eppendorf) contained MTX or DHP, ^{32}P-labeled DNA fragment and calf thymus DNA in 100 μL of 10 mM sodium phosphate buffer (pH 7.8). Denatured single-stranded DNA fragments were prepared by heating DNA fragments at 90°C for 10 min, followed by quick chilling before exposure to UVA light. The mixtures were exposed to 6 or 7 J cm^{-2} UVA light by using 10 W UV lamps (λ_{max} = 365 nm, UVP, Inc., California) placed at a distance of 20 cm. After irradiation, the DNA fragments were treated with 1 M piperidine at 90°C for 20 min and treated as described previously (30). The DNA fragments were subjected to electrophoresis on an 8 M urea and 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 (31) by using a DNA sequencing system (LKB2010 Macrophor, Pharmacia Biotech, Uppsala, Sweden). A laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech) was used for measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Measurement of 8-oxodGuo formation induced by photoirradiated MTX or DHP. The reaction mixtures containing 100 μM/base calf thymus DNA and MTX or DHP in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were exposed to UVA light (λ_{max} = 365 nm). 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 (23).

Fluorescence measurements of MTX and DHP. The fluorescence spectra of MTX and DHP were measured with an RF-5300PC spectrophotometer (Shimadzu, Kyoto, Japan). All samples for the fluorescence spectra were measured in 10 mM sodium phosphate buffer (pH 7.8).

Assay of photolysis of MTX. MTX (200 μM) in 10 mM phosphate buffer (pH 7.8) was irradiated with UVA light (λ_{max} = 365 nm, 1.16 mW cm^{-2}) and analyzed by HPLC consisting of an LC-6A pump (Shimadzu) and equipped with a Wakopak ODS column (i.d. 4.6 × 150 mm, Wako, Osaka, Japan). The mobile phase consisted of 89.8% (vol/vol) of water, 10.0% (vol/vol) of acetonitrile and 0.2% (vol/vol) of acetic acid. The analysis was carried out at a column temperature of 25°C and a flow rate of 0.5 mL min^{-1}. The HPLC eluate was routed directly into a photodiode array UV-visible detector (SPD-M10A, Shimadzu), and the spectrum of the eluate was measured. The concentration of detected MTX and photoproducts were estimated by using absorbance at 340 nm.

Mass spectra measurements. Laser desorption mass spectrometry was performed on a Voyager MALDI-TOFMS (Applied Biosystems, Foster City, CA) equipped with a nitrogen laser (337 nm, 3 ns pulse) to determine the molecular weight of photoproducts of MTX. The fraction isolated from the UVA-irradiated MTX solution by HPLC (retention time: 2.8 min) was air-dried on a stainless steel probe tip. No matrix solution was added to the sample. Mass spectrum was obtained to sum 50 laser shots.

RESULTS

Damage to DNA fragments induced by photoirradiated MTX or DHP

Figure 1 shows the autoradiogram of ^{32}P-5′-end-labeled DNA fragments exposed to UVA light (λ_{max} = 365 nm) in the presence of MTX or DHP. UVA irradiation caused DNA damage in the presence of MTX (Fig. 1A), whereas UVA irradiation alone caused little or no DNA damage under the conditions used. DHP also induced DNA photodamage (Fig. 1B), and the extent of DNA damage induced by DHP was greater than the extent of damage induced by MTX. DNA photodamage was observed when the DNA fragment was treated with piperidine, suggesting that the base modification was induced by photoirradiated MTX or DHP (data not shown).

Sequence specificity of DNA damage by photoirradiated MTX or DHP

Figure 2 shows the sequence specificity of DNA damage induced by photoirradiated MTX or DHP. Photoirradiated MTX caused damage to double-stranded DNA fragments at guanine residues, especially at the underlined G of 5′-GG-3′ and 5′-GGG-3′ (5′-TGGG-3′) sequences (Fig. 2A). The difference in reactivity of GG doublets may be due to the difference in the oxidation potential of GG, depending on the neighboring sequences. When replacing MTX with DHP, the result was almost the same (Fig. 2A,C). When denatured single-stranded DNA fragments were used, MTX or DHP plus photoirradiation caused DNA damage at single guanines and consecutive guanine residues (Fig. 2B,D). The sequence specificity of DNA damage by photoirradiated MTX in double-stranded DNA was almost consistent with that of DNA damage by photoexcited riboflavin, a Type-I photosensitizer (data not shown).

Formation of 8-oxodGuo induced by photoirradiated MTX or DHP

Figure 3 shows 8-oxodGuo formation induced by photoirradiation in the presence of MTX or DHP. It has been reported that

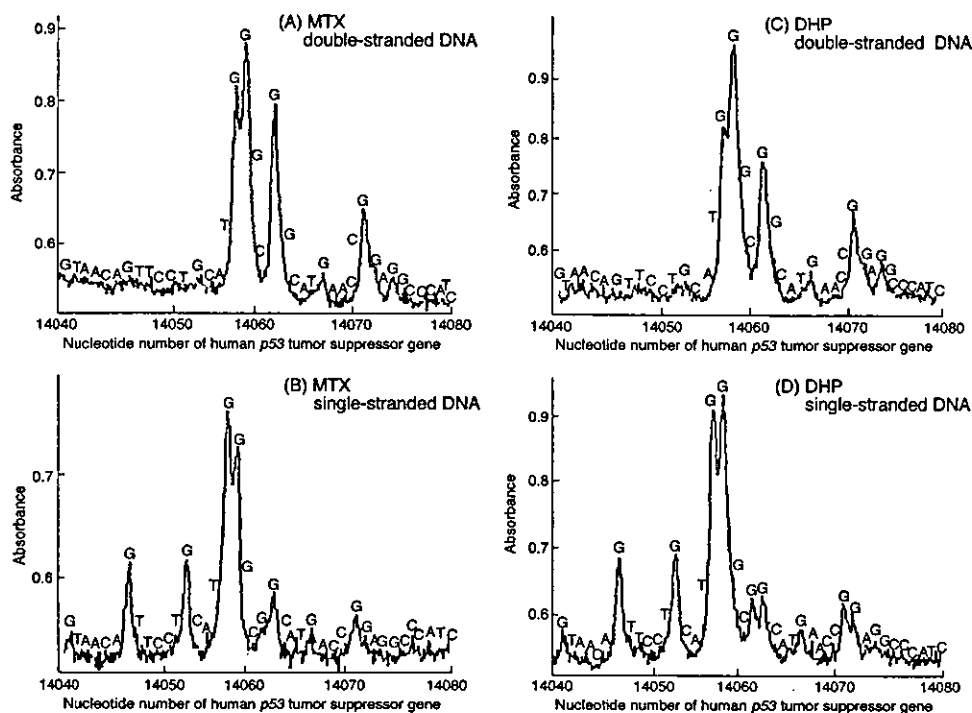


Figure 2. Sequence-specificity of DNA damage induced by photoirradiation in the presence of MTX or DHP. The reaction mixture contained the ^{32}P-labeled 211-base pair (*Hin*dIII* 13972–*Apa*I 14182) fragment, 5 μM/base of calf thymus DNA, and 100 μM MTX (A,B) or 50 μM DHP (C,D) in 100 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Where indicated, the ^{32}P-labeled and calf thymus DNA fragments were denatured by heating at 90°C for 5 min, followed by quick chilling on ice (B,D). The reaction mixtures were exposed to 6 J cm^{-2} UVA light (λ_{max} = 365 nm). 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 Materials and Methods. Horizontal axis shows the nucleotide numbers of the *p53* tumor suppressor gene.

formation of 8-oxodGuo can cause DNA misreplication that may lead to mutation or cancer (32,33). The amount of 8-oxodGuo formation increased in a dose-dependent manner up to 50 μM of MTX or DHP and decreased at concentrations of over 50 μM, suggesting that the 8-oxodGuo formed is converted into further oxidative products. The level of 8-oxodGuo in double-stranded DNA exceeded that in single-stranded DNA. The formation of 8-oxodGuo induced by DHP was about twice as much as that induced by MTX.

Photolysis of MTX into DHP

Fluorescence intensity of the pteridine moiety of MTX was very weak compared with that of DHP (Fig. 4), though pteridine is a strong fluorescent molecule. Relative fluorescence quantum yield (Φ_r) of MTX, estimated from the comparison with that of DHP, is 4.4 × 10^{-3}. When MTX was previously treated with photoirradiation, the fluorescence intensity increased depending on the extent of photoirradiation (Fig. 4), suggesting that UVA light can convert MTX into a stronger fluorescent molecule. The fluorescence spectrum of UVA-irradiated MTX was similar to that of DHP, suggesting that the photoexcited MTX is hydrolyzed into DHP.

Figure 5A shows the chromatogram of MTX. The peak height of MTX was decreased by photoirradiation, and the peak of a photolysis product was observed. The retention time and the absorption spectrum of the photolysis product were similar to those of DHP (data not shown). Furthermore, the photoproduct of MTX was analyzed by using a mass spectrometer. Mass spectrum with a molecular ion at *m/e* 192 (M^+) was obtained, confirming the formation of DHP. The concentration of MTX decreased, whereas that of DHP increased, depending on the photoirradiation dose (Fig. 5B). Another photohydrolysis product (absorption maximum at 279 nm, retention time of 6.4 min) was detected by HPLC analysis, and the intensity increased in a dose-dependent manner (data not shown). The absorption spectrum of this product was similar to that of a *p*-(*N*-methylamino)benzoyl compound. These findings indicate that photoirradiated MTX generates DHP and a *p*-(*N*-methylamino)benzoyl compound, possibly *p*-(*N*-methylamino)benzoyl-L-glutamic acid.

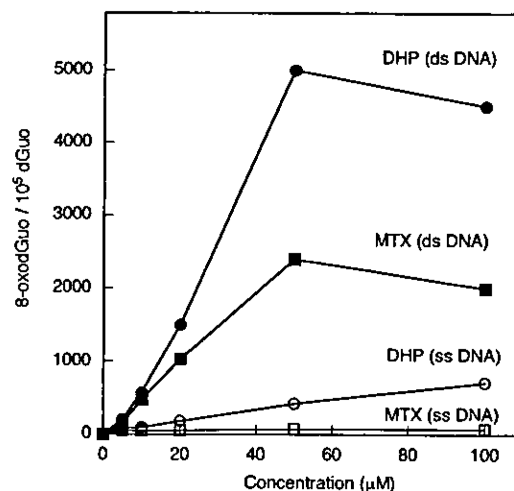


Figure 3. Formation of 8-oxodGuo induced by photoirradiated MTX or DHP. The reaction mixture containing 100 μM/base double-stranded (ds) or single-stranded (ss) calf thymus DNA, and the indicated concentration of MTX or DHP in 100 μL of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA was exposed to 6 J cm^{-2} UVA light (λ_{max} = 365 nm). Where indicated, the DNA fragment was denatured by heating for 5 min at 90°C followed by chilling on ice before photoirradiation. After irradiation, DNA was treated, and the amount of 8-oxodGuo was measured by the methods described in Materials and Methods.

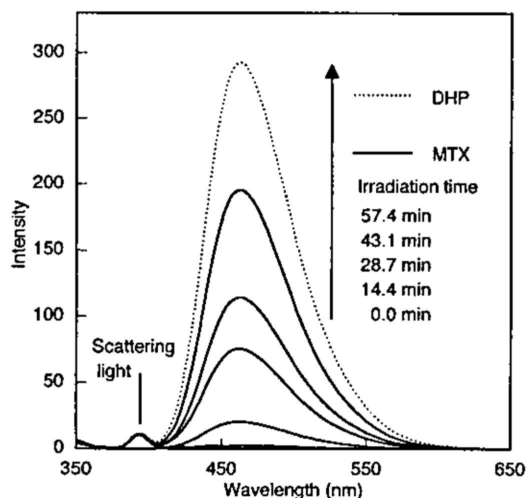


Figure 4. Fluorescence spectra of MTX previously treated with photoirradiation (solid line) and DHP (dotted line). MTX (200 μM) in 10 mM phosphate buffer (pH 7.8) was previously irradiated with UVA light (λ_{max} = 365 nm, 1.16 mW cm^{-2}) for the time indicated and diluted into 2 μM solution for measurement. The concentration of DHP was 1 μM. These spectra were measured in 10 mM sodium phosphate buffer (pH 7.8) at 340 nm excitation.

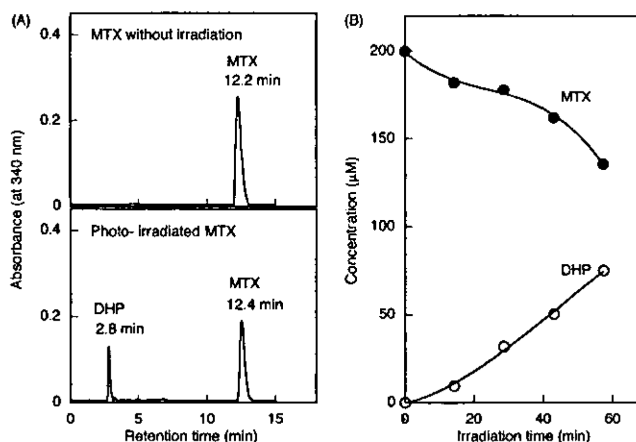


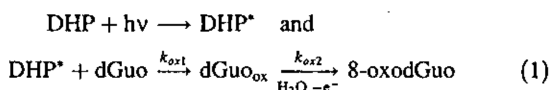
Figure 5. A: Chromatogram of MTX with or without photoirradiation. B: The time course of photolysis of MTX. MTX (200 μM) in 10 mM phosphate buffer (pH 7.8) was irradiated with UVA light (λ_{max} = 365 nm, 1.16 mW cm^{-2}) and analyzed by HPLC using a mobile phase consisting of 89.8% (vol/vol) of water, 10.0% (vol/vol) of acetonitrile and 0.2% (vol/vol) of acetic acid. A: The irradiation time was 57.4 min. B: The symbols are the concentrations of MTX (•) and DHP (o) detected by HPLC.

Measurement and simulation of the time course of the 8-oxodGuo formation by photoirradiated MTX or DHP

Formation of 8-oxodGuo by MTX or DHP increased depending on the photoirradiation time (Fig. 6). 8-oxodGuo formation by DHP was significantly larger than that by MTX. An induction period (approximately 15 min) was observed in the 8-oxodGuo formation by MTX, suggesting that MTX itself could cause little or no DNA oxidation and that reactive species generated from photoirradiated MTX induced DNA oxidation.

The curves in this figure were obtained by simulation according to the following procedure. The 8-oxodGuo formation is roughly expressed as

$$\text{DHP} + h\nu \longrightarrow \text{DHP}^{*} \quad \text{and}$$



$$\text{DHP}^{*} + \text{dGuo} \xrightarrow{k_{ox1}} \text{dGuo}_{ox} \xrightarrow[\text{H}_2\text{O},-e^-]{k_{ox2}} \text{8-oxodGuo} \qquad (1)$$

where DHP*, $dGuo_{ox}$, and k_{ox1} and k_{ox2} are photoexcited DHP, oxidized dGuo (intermediate into 8-oxodGuo) and reaction rate constants, respectively. The rate equation can be practically expressed as follows:

$$[\text{dGuo}_{ox}]/dt = k_{ox1}[\text{DHP}^{*}][\text{dGuo}] = k_{ox1}a[\text{DHP}][\text{dGuo}] \quad \text{and}$$
$$d[\text{8-oxodGuo}]/dt = k_{ox2}[\text{dGuo}_{ox}] \qquad (2)$$

where [dGuo], [$dGuo_{ox}$], [8-oxodGuo], [DHP] and [DHP*] are concentrations of dGuo, $dGuo_{ox}$, 8-oxodGuo, ground state of DHP and excited state of DHP, respectively. The nondimensional coefficient a depends on the number of photons absorbed by DHP. The fitting curve of time course of 8-oxodGuo formation has been obtained by numerical calculation. The values of $a \times k_{ox1}$ and k_{ox2} were determined by the time course of 8-oxodGuo formation by DHP as shown in Fig. 6 and were 0.12 M^{-1} s^{-1} and 7.4 × 10^6 s^{-1}, respectively. Based on the assumption that photoexcited DHP generated from MTX oxidizes dGuo, although photoexcited MTX does not, the time course of 8-oxodGuo formation was calculated from Eq. 2 using the $a \times k_{ox1}$ and k_{ox2} values mentioned above. The value of [DHP] formed from MTX was obtained from the curve fitting of the plots in Fig. 5. The curve obtained in Fig. 6 clearly fits with the experimental results, supporting the idea that DNA oxidation is caused by DHP generated from MTX.

DISCUSSION

Guanine-specific oxidation can be caused by excited photosensitizers through Type-I (electron transfer) mechanism, Type-II (singlet oxygen [$^1O_2$]) mechanism (7,8,20), or both. Cadet and co-workers (9,10) have reported that imidazolone and oxazolone are the major oxidation products of guanine formed by the Type-I mechanism, whereas 8-oxodGuo was found to be the main $^1O_2$-mediated guanine oxidation product (8). The present study has demonstrated that photoirradiated MTX and DHP induce base oxidation, especially at the underlined G in 5′-G͟G-3′ and 5′-GG͟G-3′ (5′-TGG͟G-3′) sequences in double-stranded DNA. We previously observed a similar DNA cleavage pattern when a Type-I photosensitizer, riboflavin, was used (23). The amount of 8-oxodGuo formed by photoirradiated MTX in double-stranded DNA was much larger than that in single-stranded DNA. We have previously reported that the sequence specificity of DNA damage by $^1O_2$ is quite different from that by the Type-I mechanism (20–22). Therefore, the present results can be reasonably explained by assuming that nucleobase oxidation is induced by the activated photosensitizer mainly through electron transfer (Type-I mechanism), although $^1O_2$ participates in DNA damage to some extent. Guanine is the most easily oxidized among the four DNA bases because the oxidation potential of guanine is lower than those of the other DNA bases (7,34). Molecular orbital calculations have revealed that stacking of two guanine bases in double-stranded DNA lowers the ionization potential significantly, and electron-loss centers are localized on the 5′-G of the 5′-GG-3′ sequence through charge transfer (16). Recently, it has been reported that the ionization potential of GGG is lower than that of GG and that the central G is the most reactive in the 5′-TGG͟G-3′ sequence (19). These reports support the proposed mechanism by which the photoirradiated photosensi-

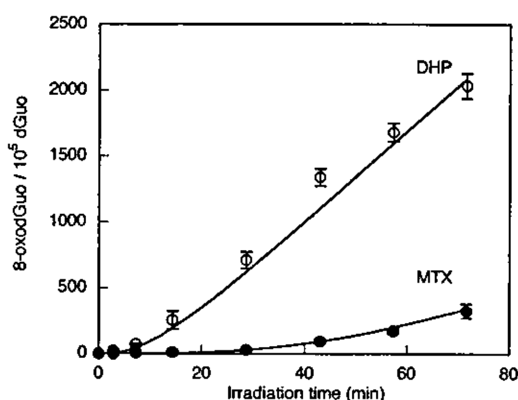


Figure 6. Time course of the formation of 8-oxodGuo induced by photoirradiated MTX or DHP. The reaction mixture containing 100 μM/base calf thymus DNA, 50 μM MTX (•) or DHP (o) in 100 μL of 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA was exposed to UVA light (λ_{max} = 365 nm, 1.16 mW cm^{-2}). After irradiation, DNA was treated, and the amount of 8-oxodGuo was measured by the methods described in Materials and Methods. Results represent means ± SD of four independent experiments. The curves in this figure were simulated as described in Results.

tizer causes the poly-G–specific oxidation in double-stranded DNA through electron transfer. Guanine cation radicals formed in DNA are finally localized on the underlined sites of the 5′-GG-3′ and 5′-GGG-3′ (5′-TGGG-3′) sequences and react with the water molecule to form the C-8 OH adduct radical, followed by oxidation, leading to the formation of 8-oxodGuo (7,8,13–15). Although the 8-oxodGuo site is not efficiently cleaved under piperidine treatment (15), 8-oxodGuo is more easily oxidized than dGuo is and can be converted into piperidine-labile products (*e.g.* imidazolone, oxazolone, or both [7–11]) through further oxidation (7,17,18,35,36).

Pteridine is a strong fluorescent molecule, but the pteridine moiety of MTX shows weak fluorescence, possibly due to an intramolecular electron transfer from the *p*-(*N*-methylamino)-benzoyl residue to the photoexcited pteridine moiety. This study showed that photoirradiation converted MTX into the fluorescent molecule, with the fluorescence spectrum similar to that of DHP. HPLC and mass spectrum measurements have also shown that photoexcited MTX generates DHP depending on the irradiation dose. We proposed the photolysis of MTX into DHP and a *p*-(*N*-methylamino)benzoyl compound, possibly *p*-(*N*-methylamino)benzoyl-L-glutamic acid, through an intramolecular electron transfer after hydrolysis (Fig. 7).

Sequence specificity of DNA damage induced by photoexcited DHP was similar to that of photoirradiated MTX, and the extent of DNA damage and 8-oxodGuo formation by DHP was much larger than in the case of MTX. The time course of 8-oxodGuo formation by photoirradiated MTX has been reasonably explained by assuming that DNA damage is caused by photoactivation of DHP generated from MTX and that MTX itself has little or no DNA-damaging ability. In the photoexcited MTX the intramolecular electron transfer leading to DHP formation competes with the intermolecular reaction with DNA. The intramolecular electron transfer rate coefficient (k_{et1}) has been roughly estimated from the following equation:

$$k_{et1} = (1/\Phi_r - 1) \times 1/\tau_0 \quad (3)$$

where τ_0 is the lifetime of the photoexcited pteridine moiety (ranges almost from 10^{-9} to 10^{-8} s^{-1}) (37). Because this study has

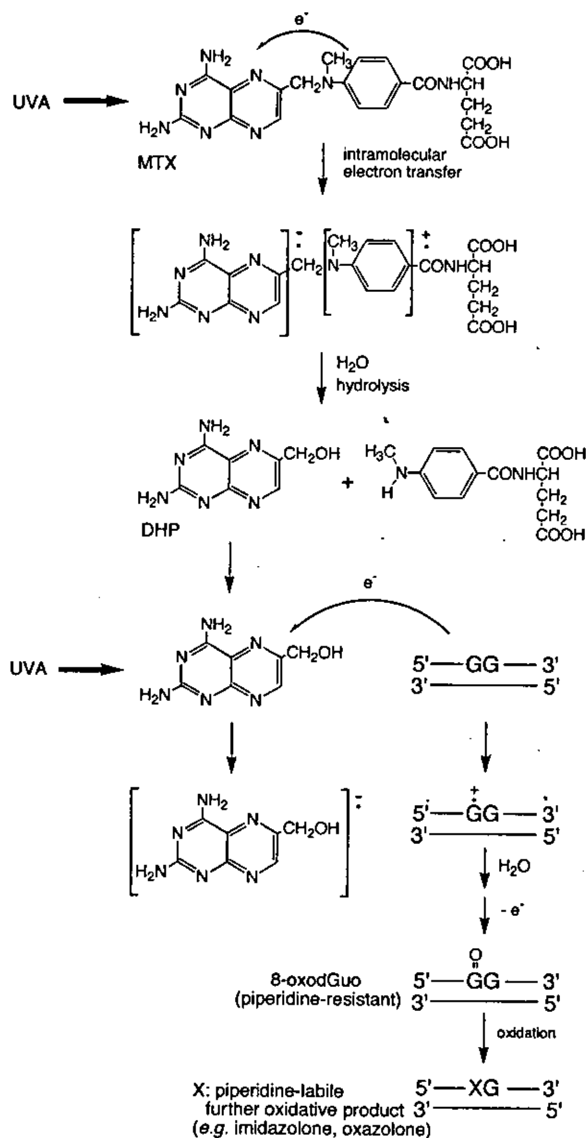


Figure 7. Proposed mechanism of DNA damage induced by photoirradiated MTX.

estimated that the value of Φ_r is 4.4 × 10^{-3}, the value of k_{et1} can be estimated to be almost 10^{10}–10^{11} s^{-1}. On the other hand, k_{et2}, the collision rate coefficient of one photoexcited MTX molecule with DNA, has been estimated from the equation of diffusion controlled reaction:

$$k_{et2} = 8RT[dGuo]/3\eta \quad (4)$$

where R is the gas constant, T is the absolute temperature, [dGuo] is 25 μM and η is the viscosity of water (0.891 × 10^{-3} kg m^{-1} s^{-1}). The k_{et2} is calculated to be 1.8 × 10^5 s^{-1} and is markedly smaller than k_{et1}. Therefore, in the photoexcited MTX, intramolecular electron transfer preferentially occurs rather than DNA oxidation by photoexcited MTX itself.

In summary, the present study has demonstrated that photoirradiated MTX induces poly-G–specific DNA oxidation. The reactive species that induces DNA oxidation is photoexcited DHP, which is generated from photoexcited MTX through hydrolysis after intramolecular electron transfer. This study suggests that the

phototoxicity of MTX is due to the oxidation of biomacromolecules, mainly through electron transfer by photoexcited DHP.

Acknowledgement—This work was supported by Grants-in-Aid for Scientific Research granted by the Ministry of Education, Science, Sports and Culture of Japan.

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