Distinct mechanisms of site-specific oxidative DNA damage by doxorubicin in the presence of copper(II) and NADPH-cytochrome P450 reductase

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The anticancer mechanism of doxorubicin (DOX), an anthracycline antibiotic, is believed to involve DNA damage through topoisomerase II inhibition and free radical generation. The free radical generation may also participate in genotoxicity, as well as cardiotoxicity, in normal human cells. The present study showed that DOX generates 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage, in HL-60 cells, but not in H_2O_2-resistant HP100 cells, suggesting the involvement of H_2O_2 in cellular DNA damage. Since DOX has both *p*-quinone and *p*-hydroquinone residues, free radical generation can be initiated by either reduction or oxidation of DOX. To clarify whether the oxidized or reduced form is more important for DOX-induced H_2O_2 generation, we investigated the site-specific DNA damage induced by DOX in the presence of Cu(II), in comparison with that in the presence of cytochrome P450 reductase, using ^{32}P-labeled DNA fragments. DOX caused DNA damage in the presence of Cu(II) or cytochrome P450 reductase. The degree of Cu(II)-mediated DNA damage, including 8-oxodG formation, was much greater than that of cytochrome P450 reductase-mediated DNA damage. DOX plus Cu(II) caused DNA damage specifically at guanine, thymine and cytosine residues, particularly at 5′-GG-3′, 5′-GT-3′ and 5′-TG-3′ sequences. Scavenger experiments suggested the involvement of reactive species generated from H_2O_2 and Cu(I). When cytochrome P450 reductase and NADPH were used instead of Cu(II), every nucleotide was uniformly damaged, suggesting the participation of ·OH. We conclude that DOX may induce carcinostatic and genotoxic effects through oxidation of its *p*-hydroquinone moiety by metal ion rather than through *p*-quinone reduction by cytochrome P450 reductase. (Cancer Sci 2003; 94: 686–691)

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most widely used anticancer drugs. DOX is used for the treatment of human cancers, including a variety of solid cancers.[1)] The main anticancer action of DOX is believed to involve DNA damage through topoisomerase II inhibition and free radical generation.[1–4)] The free radical generation induces oxidative damage and apoptosis,[2,3,5–16)] although the molecular mechanisms are unclear. The free radical generation by DOX may also participate in cardiotoxicity and genotoxicity in normal human cells.[1–3,5)] The genotoxicity can result in induction of secondary cancer.[5)] DOX is, in fact, carcinogenic in animals and is potentially carcinogenic in humans.

DOX has both *p*-quinone and *p*-hydroquinone moieties, so free radical generation can be initiated by both cytochrome P450 reductase-mediated reduction and metal-mediated oxidation of DOX. DOX undergoes a one-electron reduction of the *p*-quinone residue by cytochrome P450 reductase in the presence of NADPH to form the semiquinone radical, resulting in the generation of free hydroxyl radical (·OH), which causes DNA damage.[2,3,6,13)] On the other hand, DNA damage may also be caused by the oxidative activation of DOX, that is, one-electron oxidation of the *p*-OH residue.[17–19)] Thus, DOX can be converted to both oxidized and reduced forms.

To clarify whether the oxidized or reduced form is more important for DOX-induced carcinostatic and genotoxic effects, we compared DOX-induced site-specific DNA damage via metal-mediated oxidation with that via reduction by cytochrome P450 reductase, using ^{32}P-labeled DNA fragments obtained from the human c-Ha-*ras*-1 and *p53* genes. We measured the content of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a marker of oxidative DNA damage, in calf thymus DNA treated with DOX in the presence of Cu(II) by high-performance liquid chromatography with an electrochemical detector (HPLC-ECD). Furthermore, the formation of 8-oxodG was measured in a human leukemia cell line, HL-60, and its hydrogen peroxide (H_2O_2)-resistant clone HP100 cells.

Materials and Methods

Materials. Restriction enzymes (*Apa*I, *Ava*I, *Eco*RI, *Pst*I, *Xba*I and *Hind*III) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). T_4 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*‴-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto). Superoxide dismutase (SOD, 3000 U/mg from bovine erythrocytes) and catalase (45,000 U/mg from bovine liver) were from Sigma Chemical Co. (St. Louis, MO). Doxorubicin hydrochloride was from Wako Pure Chemical Industries (Osaka). 3-(Methylthio)propionaldehyde (methional) was from Tokyo Kasei Co. (Tokyo). Copper chloride ($CuCl_2 \cdot 2H_2O$) and NADPH were from Nacalai Tesque Co. (Kyoto). Cytochrome P450 reductase (10 mg/ml protein from human microsomes) was from Gentest Co. (Woburn, MA).

Measurement of 8-oxodG in cultured cells. Human leukemia cell line HL-60 and its H_2O_2-resistant HP100 cells[20)] were grown in RPMI 1640 supplemented with 6% fetal calf serum at 37°C under 5% CO_2 in a humidified atmosphere. Cells (10^6 cells/ml) were incubated with DOX for 3 h at 37°C and immediately washed three times with PBS, and DNA was extracted using a DNA Extractor WB Kit (Wako Pure Chemical Industries). The DNA was dissolved in water, and digested to nucleosides with nuclease P_1 and bacterial alkaline phosphatase and analyzed by HPLC-ECD.[21)]

Preparation of ^{32}P-labeled DNA fragments. DNA fragments were prepared from the human *p53* tumor suppressor gene.[22)] The ^{32}P-5′-end-labeled 443-bp fragment (*Apa*I 14179–*Eco*RI* 14621) and 211-bp fragment (*Hind*III* 13972–*Apa*I 14182) were obtained as described previously.[23)] DNA fragments were

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also obtained from the human c-Ha-*ras*-1 protooncogene.[24] The 261-bp fragment (*Ava*I* 1645–*Xba*I 1905) and 341-bp fragment (*Xba*I 1906–*Ava*I* 2246) were prepared from the plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA restriction fragment. The asterisk indicates ^{32}P-labeling.

Analysis of DNA damage by DOX in the presence of Cu(II). The standard reaction mixture in a microtube (1.5 ml Eppendorf) contained DOX, 20 μ*M* $CuCl_2$, ^{32}P-labeled DNA fragment and calf thymus DNA (2 μ*M*/base) in 200 μl of 10 m*M* sodium phosphate buffer (pH 7.8) containing 5 μ*M* DTPA, a chelating agent, to remove trace amounts of contaminating metals. After incubation at 37°C for 30 min, the DNA fragments were heated at 90°C in 1 *M* piperidine for 20 min and treated as described previously.[25–27] 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 (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Analysis of DNA damage by DOX in the presence of cytochrome P450 reductase and NADPH. The reaction mixture contained the ^{32}P-labeled DNA fragments, 2 μ*M*/base of calf thymus DNA, DOX, NADPH, and 20 μg/ml of cytochrome P450 reductase in 200 μl of 0.4 *M* Tris-HCl (pH 8.0), and the reaction was run at 37°C for 60 min according to the method of Berlin and Haseltine.[13] After piperidine treatment, the DNA fragments were examined as described above.

Analysis of 8-oxodG formation in calf thymus DNA by DOX in the presence of Cu(II). 8-OxodG formation was determined by a modification of a reported method.[29] Calf thymus DNA fragments (50 μ*M*/base) were incubated with DOX and 20 μ*M* $CuCl_2$ for 2 h at 37°C. After ethanol precipitation, DNA was digested to nucleosides with nuclease P_1 and calf intestine alkaline phosphatase, and analyzed by HPLC-ECD.[21]

Results

Formation of 8-oxodG in human cultured cells by DOX. To investigate the induction of cellular oxidative DNA damage by DOX, we measured the content of 8-oxodG in HL-60 and HP100 cells treated with DOX. DOX treatment resulted in an increase of 8-oxodG content in treated HL-60 cells (Fig. 1). The content of 8-oxodG in HL-60 cells treated with DOX was significantly increased in comparison with that in non-treated control cells. However, DOX did not significantly increase the amount of 8-oxodG in H_2O_2-resistant HP100 cells (Fig. 1).

Damage to ^{32}P-DNA fragments by DOX in the presence of metals. Fig. 2 shows an autoradiogram of DNA fragments treated with DOX in the presence of metals. DOX plus Cu(II) induced DNA damage, whereas DNA damage was not observed in the absence of Cu(II). DOX caused slight DNA damage in the presence of Fe(II), but not in the presence of Fe(III), Zn(II), Ni(II), Mg(II), Co(II) or Mn(II) (Fig. 2).

Comparison of DOX-induced DNA damage in the presence of Cu(II) with that in the presence of cytochrome P450 reductase. Fig. 3 shows an autoradiogram of DNA fragments treated with DOX in the presence of Cu(II). The degree of DNA damage increased depending on DOX concentration. DOX induced weak DNA damage at 5 μ*M* and clear damage above 10 μ*M* in the presence of Cu(II). DNA damage was enhanced by piperidine treatment (data not shown), suggesting that DOX caused not only backbone breakage, but also base damage. In the presence of cytochrome P450 reductase and NADPH, DOX also induced DNA damage even in the absence of metal ions (Fig. 3). This DNA damage was not observed in the absence of cytochrome P450 reductase. The DNA damage was slightly enhanced by the addition of Cu(II) alone. The intensity of Cu(II)-mediated DNA damage in the presence of DOX was much greater than that of cytochrome P450 reductase-mediated DNA damage in the presence of DOX and NADPH (Fig. 3).

Effects of scavengers and bathocuproine on DNA damage by DOX in the presence of Cu(II). Fig. 4 shows the effects of scavengers and bathocuproine on DNA damage induced by DOX in the presence of Cu(II). Typical ·OH scavengers, ethanol, mannitol, sodium formate and DMSO, showed little or no inhibitory effect on DNA damage. SOD and catalase had inhibitory effects on DNA damage, but denatured catalase had no effect. DNA damage was completely inhibited by methional and bathocuproine.

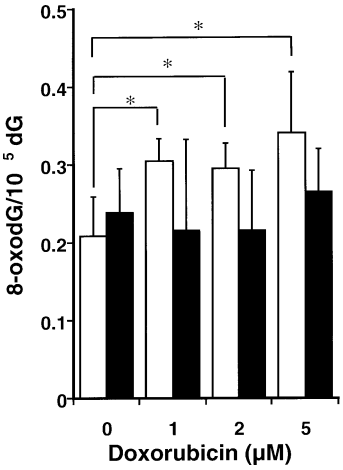

Fig. 1. Formation of 8-oxodG in HL-60 and HP100 cells treated with doxorubicin. HL-60 and HP100 cells (1×10^6 cells/ml) were treated with doxorubicin at 37°C for 3 h. After the treatment, DNA was extracted, subjected to enzyme digestion and analyzed by HPLC-ECD. Values represent means±SD of three independent experiments. Asterisks indicate significant differences compared with the control by *t* test (* $P<0.05$). □ HL-60, ■ HP100.

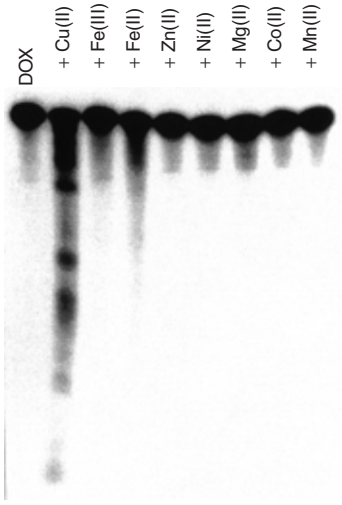

Fig. 2. Effects of metals on DNA damage induced by doxorubicin. The reaction mixture contained the ^{32}P-5′-end-labeled 261-bp fragment (*Ava*I *1645–*Xba*I 1905), 2 μ*M*/base of calf thymus DNA, 20 μ*M* metal and 20 μ*M* doxorubicin in 200 μl of 10 m*M* phosphate buffer (pH 7.8) containing 2.5 μ*M* DTPA. Where indicated, $CuCl_2$, $FeCl_3$, $FeSO_4(NH_4)_2SO_4$, $ZnSO_4$, $NiSO_4$, $MgCl_2$, $CoCl_2$ or $MnCl_2$ was added. The mixture was incubated at 37°C for 30 min. The DNA fragments were treated with 1 *M* piperidine at 90°C for 20 min, and then electrophoresed on an 8% polyacrylamide 8 *M* urea gel. The autoradiogram was obtained by exposing X-ray film to the gel.

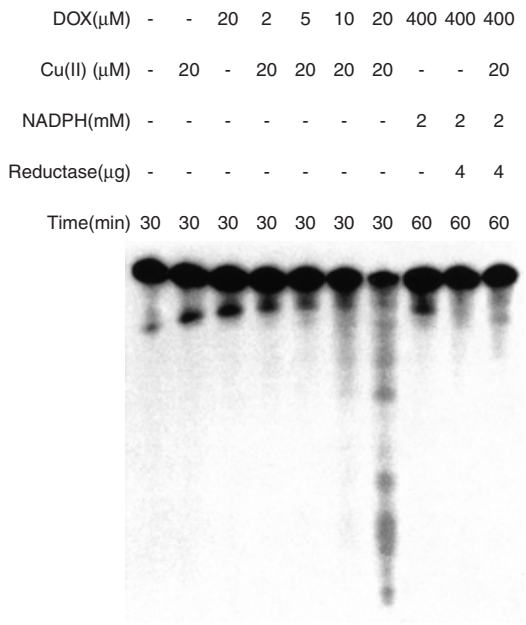

Fig. 3. Autoradiogram of ^{32}P-labeled DNA fragment incubated with doxorubicin in the presence of Cu(II) and cytochrome P450 reductase-NADPH. The reaction mixture contained the ^{32}P-5′-end-labeled 261-bp fragment (*Ava*I ∗1645–*Xba*I 1905), 2 μ*M*/base of calf thymus DNA, 20 μ*M* $CuCl_2$ and doxorubicin in 200 μl of 10 m*M* phosphate buffer (pH 7.8) containing 2.5 μ*M* DTPA. Where indicated, the reaction mixture contained the DNA fragment, 2 μ*M*/base of calf thymus DNA, 400 μ*M* doxorubicin and 2 m*M* NADPH in 200 μl of 0.4 *M* Tris-HCl (pH 8.0). After incubation at 37°C, followed by piperidine treatment, the DNA fragments were analyzed by the method described in the legend to Fig. 2.

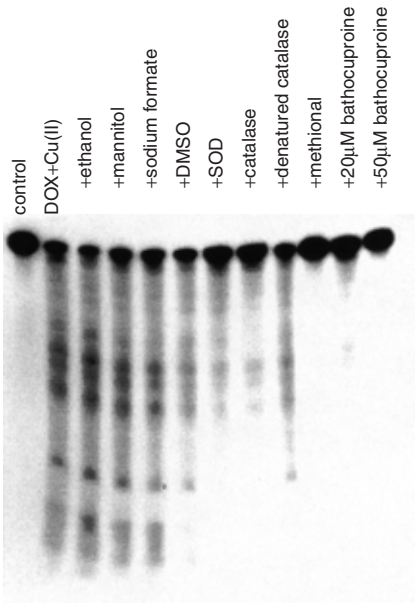

Fig. 4. Effects of scavengers and bathocuproine on DNA damage induced by doxorubicin in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 211-bp fragment (*Hind*III ∗13972–*Apa*I 14182), 2 μ*M*/base of calf thymus DNA, 20 μ*M* $CuCl_2$ and 20 μ*M* doxorubicin in 200 μl of 10 m*M* phosphate buffer (pH 7.8) containing 2.5 μ*M* DTPA. Where indicated, 1.7 *M* ethanol, 0.1 *M* mannitol, 0.1 *M* sodium formate, 0.7 *M* DMSO, 30 units of SOD, 30 units of catalase, 30 units of denatured catalase, 0.1 *M* methional, or 20 or 50 μ*M* bathocuproine was added. After incubation at 37°C for 30 min, followed by piperidine treatment, the DNA fragments were analyzed by the method described in the legend to Fig. 2.

Site specificity of DNA damage by DOX in the presence of Cu(II) and cytochrome P450 reductase. The site specificity of DNA damage induced by DOX was determined by the Maxam-Gilbert procedure.[28] The relative intensity of DNA damage obtained by scanning the autoradiogram with a laser densitometer is shown in Fig. 5. In the presence of Cu(II), DOX caused DNA damage specifically at guanine, thymine and cytosine residues, particularly at the 5′-GG-3′, 5′-GT-3′ and 5′-TG-3′ sequences (Fig. 5A). On the other hand, DNA damage by DOX was observed at every base in the presence of cytochrome P450 reductase and NADPH (Fig. 5B).

Formation of 8-oxodG in calf thymus DNA by DOX in the presence of Cu(II). 8-OxodG is one of the DNA products generated by reactive oxygen species. In the presence of Cu(II), the amount of 8-oxodG increased with increasing DOX concentration (Fig. 6). The formation of 8-oxodG increased about 3.0-fold after DNA denaturation.

Discussion

The present study revealed that DOX induced significant 8-oxodG formation in HL-60 cells, but did not increase 8-oxodG formation in HP100 cells. It was reported that HP100 cells are ~340-fold more resistant to H_2O_2 than the parent cells, HL-60.[20] Therefore, this result suggests that generation of H_2O_2 plays an important role in DOX-induced 8-oxodG formation. Moreover, our finding of DOX-induced 8-oxodG formation in HL-60 cells is consistent with the fact that oxidative DNA damage markers increase in cancer patients treated with DOX or epirubicin, a DOX derivative.[30, 31]

DOX caused DNA damage, including 8-oxodG formation, in the presence of Cu(II), suggesting that this DNA damage is induced by oxidative stress. DOX induced site-specific DNA damage at guanine, thymine and cytosine residues, especially at 5′-GG-3′, 5′-GT-3′ and 5′-TG-3′ sequences, in the presence of Cu(II). SOD and catalase had inhibitory effects on DNA damage, suggesting the involvement of $O_2^{\bullet-}$ and H_2O_2. Bathocuproine, which prevents the activation of H_2O_2 by stabilizing Cu(I),[32, 33] completely inhibited DNA damage, suggesting the involvement of Cu(I). Typical $^{\bullet}$OH scavengers did not inhibit the DNA damage, suggesting that free $^{\bullet}$OH does not play an important role. DNA damage was completely inhibited by methional, which scavenges not only $^{\bullet}$OH, but also other radicals such as metal-oxygen complexes.[34] Therefore, it is considered that reactive oxygen species such as Cu(I)OOH are involved in Cu(II)-mediated DNA damage.

A possible mechanism of DNA damage by DOX in the presence of Cu(II), shown in Fig. 7, is consistent with most of the observations and reported data[17–19] (Fig. 7). We postulate that the major pathway of DNA damage is as follows: DOX undergoes Cu(II)-mediated one-electron oxidation at the *p*-OH residue to generate Cu(I) and the semiquinone radical. Cu(I) reacts with O_2 to generate $O_2^{\bullet-}$ and subsequently H_2O_2. Generated Cu(I) bound to DNA interacts with H_2O_2, resulting in the formation of a DNA-copper-hydroperoxo complex [DNA-Cu(I)OOH]. Although typical $^{\bullet}$OH scavengers did not inhibit the DNA damage, $^{\bullet}$OH may participate in DNA damage through the formation of DNA-Cu(I)OOH, which can release $^{\bullet}$OH in the vicinity of DNA. $^{\bullet}$OH immediately attacks an adjacent constituent of DNA, before it can be scavenged by $^{\bullet}$OH scavengers.[35] In addition, it has been reported that the mutagenicity of DOX is amplified by cupric ion.[36] Several studies have shown that plasma copper concentrations are increased in cancer patients.[37, 38] Copper catalyzes the production of reactive

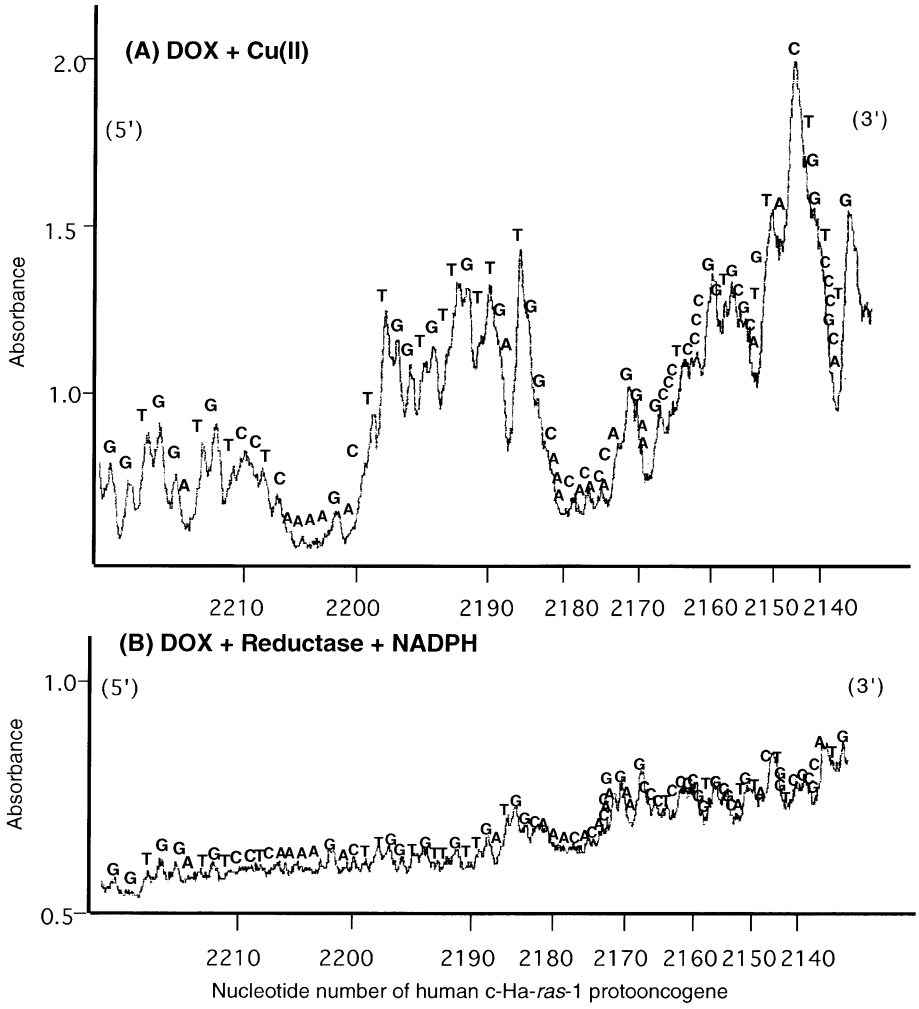

Fig. 5. Comparison of site specificity of DNA cleavage induced by doxorubicin in the presence of Cu(II) or cytochrome P450 reductase. (A) The reaction mixture contained the ^{32}P-5′-end-labeled 341-bp fragment (*Xba*I 1906–*Ava*I ∗2246), 2 μ*M*/base of calf thymus DNA, 20 μ*M* $CuCl_2$ and 20 μ*M* doxorubicin in 200 μl of 10 m*M* phosphate buffer (pH 7.8) containing 2.5 μ*M* DTPA. The mixture was incubated at 37°C for 30 min. (B) The reaction mixture contained the ^{32}P-5′-end-labeled 341-bp fragment (*Xba*I 1906–*Ava*I ∗2246), 2 μ*M*/base of calf thymus DNA, 20 μg/ml of cytochrome P450 reductase, 2 m*M* NADPH and 400 μ*M* doxorubicin in 200 μl of 0.4 *M* Tris-HCl (pH 8.0) containing 2.5 μ*M* DTPA. The mixture was incubated at 37°C for 60 min. After incubation, followed by piperidine treatment, the DNA fragments were electrophoresed on an 8% polyacrylamide/8 *M* urea gel using a DNA-sequencing system, and the autoradiogram was obtained by exposing X-ray film to the gel. The relative amounts of oligonucleotides produced were measured with a laser densitometer (LKB 2222 UltroScan XL). The piperidine-labile sites of the treated DNA were determined by direct comparison with the same DNA fragment after DNA sequencing according to the Maxam-Gilbert procedure. The horizontal axis shows the nucleotide number.

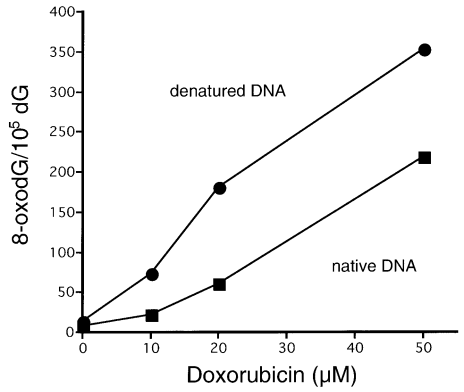

Fig. 6. Formation of 8-oxodG in calf thymus DNA by doxorubicin in the presence of Cu(II). Where indicated, DNA was denatured by heating at 90°C for 5 min, and then chilled on ice before incubation. Calf thymus DNA was incubated with doxorubicin and 20 μ*M* $CuCl_2$ for 2 h at 37°C. After ethanol precipitation, the DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by the HPLC-ECD.

oxygen species to mediate oxidative DNA damage induced by carcinostatics[39, 40] and carcinogens.[23, 41] Copper is an essential component of chromatin and is known accumulate preferentially in the heterochromatic regions.[42, 43] These reports and our results suggest that Cu(II)-mediated DNA damage through the oxidized form of DOX may readily occur in cancer patients.

Many previous studies have shown that the quinone residue of DOX can undergo one-electron reduction at the *p*-quinone residue mediated by NADPH-cytochrome P450 reductase, mitochondrial NADH dehydrogenase and metal compounds.[2, 3, 7–11, 44, 45] The semiquinone radical generated by cytochrome P450 reductase can react with O_2 to generate $O_2^{\bullet-}$, which is dismutated to H_2O_2.[13] The semiquinone radical reacts with H_2O_2 to generate $^{\bullet}OH$, resulting in DNA damage. In this study, we also demonstrated that DOX induced DNA damage at every base in the presence of cytochrome P450 reductase and NADPH, suggesting that $^{\bullet}OH$ is involved in DNA damage.[13, 46, 47] Our comparison of DOX-induced DNA damage via Cu(II)-mediated oxidation with that via reduction by cytochrome P450 reductase, revealed that reduction to the semiquinone radical is a minor pathway of DNA damage. Therefore, the ability of DOX to induce DNA damage under oxidative conditions is much greater than that under reductive conditions. In clinical studies, the maximal plasma DOX concentration detected was approximately 5 μ*M*.[2, 48] Moreover, cytochrome P450 reductase is primarily located in the endoplasmic reticulum but not in chromatin.[49] Thus, it seems likely that DNA damage mediated by cytochrome P450 reductase and NADPH would hardly occur under *in vivo* conditions. In conclusion, DOX-induced site-specific DNA damage in the presence of Cu(II) is considered to be a major mechanism of the carcino-

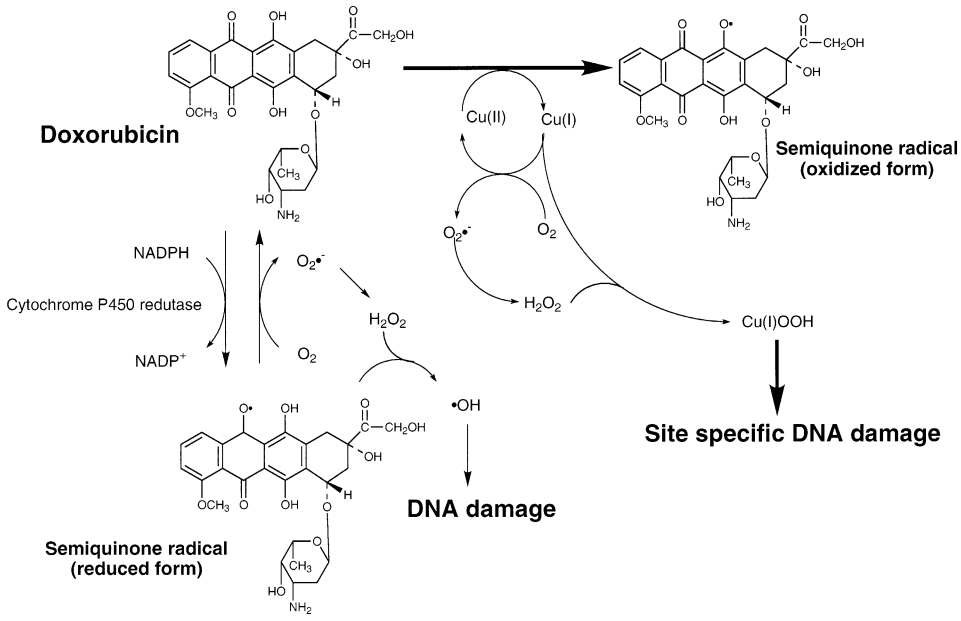

Fig. 7. Proposed mechanisms for DNA damage induced by doxorubicin.

static and genotoxic effects of DOX. This mechanism may play a role in the oxidative DNA damage induced by a variety of anthracycline antibiotics.

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