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Hydroxyurea Induces Site-specific DNA Damage via Formation of Hydrogen Peroxide and Nitric Oxide

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Hydroxyurea is a chemotherapeutic agent used for the treatment of myeloproliferative disorders (MPD) and solid tumors. The mutagenic and carcinogenic potential of hydroxyurea has not been established, although hydroxyurea has been associated with an increased risk of leukemia in MPD patients. To clarify whether hydroxyurea has potential carcinogenicity, we examined site-specific DNA damage induced by hydroxyurea using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* and *p16* tumor suppressor genes and the c-Ha-*ras*-1 protooncogene. Hydroxyurea caused Cu(II)-mediated DNA damage especially at thymine and cytosine residues. NADH efficiently enhanced hydroxyurea-induced DNA damage. The DNA damage was almost entirely inhibited by catalase and bathocuproine, a Cu(I)-specific chelator, suggesting the involvement of hydrogen peroxide (H_2O_2) and Cu(I). Typical free hydroxyl radical scavengers did not inhibit DNA damage by hydroxyurea, but methional did. These results suggest that crypto-hydroxyl radicals such as Cu(I)-hydroperoxo complex (Cu(I)-OOH) cause DNA damage. Formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) was induced by hydroxyurea in the presence of Cu(II). An electron spin resonance spectroscopic study using *N*-(dithiocarboxy)sarcosine as a nitric oxide (NO)-trapping reagent demonstrated that NO was generated from hydroxyurea in the presence and absence of catalase. In addition, the generation of formamide was detected by both gas chromatography-mass spectrometry (GC-MS) and time-of-flight-mass spectrometry (TOF-MS). A high concentration of hydroxyurea induced depurination at DNA bases in an H_2O_2-independent manner, and endonuclease IV treatment led to chain cleavages. These results suggest that hydroxyurea could induce base oxidation as the major pathway of DNA modification and depurination as a minor pathway. Therefore, it is considered that DNA damage by hydroxyurea participates in not only anti-cancer activity, but also carcinogenesis.

Key words: Hydroxyurea — DNA damage — Copper — Hydrogen peroxide — Nitric oxide

Hydroxyurea is an effective chemotherapeutic agent for neoplastic diseases. It is commonly used for patients with clonal myeloproliferative disorders (MPD), including chronic myelogenous leukemia, as well as for malignant solid tumors.[1–3] Recently, hydroxyurea has been investigated for use in the treatment of human immunodeficiency virus (HIV) disease.[4, 5] Hydroxyurea inhibits ribonucleoside diphosphate reductase,[6, 7] thereby blocking DNA synthesis and repair.

Larger studies have documented an increased risk of leukemia for patients with MPD on hydroxyurea therapy.[8–10] De Simone *et al.* described the occurrence of multiple squamous cell skin carcinomas in a patient treated with hydroxyurea for chronic myelogenous leukemia.[11] Hydroxyurea has been experimentally shown to have clastogenic,[12] teratogenic,[13] and, in some settings, mutagenic effects.[14] Recently, it has been reported that hydroxyurea increases DNA mutations in young patients with sickle cell disease.[15] Hydroxyurea produces chromosome damage and mutation in cultured cells,[16, 17] whereas it is an inactive mutagen in bacteria.[18] Although hydroxyurea does not bind to DNA,[18] it has been reported that nitric oxide (NO) is generated from it.[19–21] Therefore, there remains a possibility that hydroxyurea induces DNA damage via the formation of reactive nitrogen and oxygen species.

In this study, we have examined whether hydroxyurea causes DNA damage in the presence of metal ion using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p16* and *p53* tumor suppressor genes and the c-Ha-

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Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine (also known as 8-oxo-7,8-dihydro-2′-deoxyguanosine); HPLC-ECD, an electrochemical detector coupled to a high-pressure liquid chromatograph; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; DTCS, *N*-(dithiocarboxy)sarcosine; H_2O_2, hydrogen peroxide; NO, nitric oxide; DMSO, dimethylsulfoxide; ESR, electron spin resonance; CIP, calf intestine phosphatase; SOD, superoxide dismutase; MPD, myeloproliferative disorders; HIV, human immunodeficiency virus; UDG, uracil-DNA glycosylase; GC-MS, gas chromatography-mass spectrometry; TOF-MS, time-of-flight-mass spectrometry.

ras-1 protooncogene. We investigated the effects of scavengers and metal chelators on the DNA damage induced by hydroxyurea in order to clarify the reactive species causing the DNA damage. We also analyzed the formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a characteristic oxidative DNA lesion, by using an electrochemical detector coupled to a high-pressure liquid chromatograph (HPLC-ECD). Furthermore, to clarify the mechanism of DNA damage, we studied the Cu(II)-mediated autoxidation process of hydroxyurea by using an electron spin resonance (ESR) spectrometer. We analyzed the products generated by Cu(II)-mediated hydroxyurea oxidation by using gas chromatography-mass spectrometry (GC-MS) and time-of-flight-mass spectrometry (TOF-MS).

MATERIALS AND METHODS

Materials Restriction enzymes (*Sma*I, *Bss*HII, *Eco*RI, *Apa*I, *Hin*dIII, *Xba*I, *Mro*I, *Pst*I and *Sty*I) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). T_4 polynucleotide kinase was from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), *N*-(dithiocarboxy)sarcosine (DTCS) and bathocuproine disulfonic acid were from Dojin Chemical Co. (Kumamoto). Acrylamide, dimethylsulfoxide (DMSO), bisacrylamide and piperidine were from Wako (Osaka). $CuCl_2$, ethanol, D-mannitol and sodium formate were from Nacalai Tesque (Kyoto). NADH, calf thymus DNA, calf intestine phosphatase (CIP), superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45 000 units/mg from bovine liver) were from Sigma Chemical Co. (St. Louis, MO). Nuclease P_1 (400 units/mg) was from Yamasa Shoyu Co. (Chiba). Hydroxyurea was from Aldrich Chemical Co. (Milwaukee, WI). Endonuclease IV and uracil-DNA glycosylase (UDG) were from Trevigen, Inc. (Gaithersburg, MD).

Preparation of ^{32}P-5′-end-labeled DNA fragments Exon-containing DNA fragments were prepared from the human *p53* tumor suppressor gene.[22] The 5′-end-labeled 443-bp fragment (*Apa*I 14 179–*Eco*RI* 14 621) and the 211-bp fragment (*Hin*dIII* 13 972–*Apa*I 14 182) were obtained according to the method described previously.[23] DNA fragments were also obtained from the human *p16* tumor suppressor gene.[24] The singly labeled 328-bp fragment (*Eco*RI* 5841–*Mro*I 6168) was obtained as described previously.[25] Furthermore, we prepared DNA fragments from the human c-Ha-*ras*-1 protooncogene.[26] The DNA fragments were obtained from the plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the c-Ha-*ras*-1 gene.[27] The singly labeled 341-bp fragment (*Xba*I 1906–*Ava*I* 2246) and the 337-bp fragment (*Pst*I 2345–*Ava*I* 2681) were prepared as described previously.[27] Nucleotide numbering for the human c-Ha-*ras*-1 protooncogene starts with the *Bam*HI site.[26] (The asterisk indicates ^{32}P labeling.)

Detection of DNA damage by hydroxyurea in the presence of metal ion The standard reaction mixture (in a 1.5 ml Eppendorf microtube) contained hydroxyurea, ^{32}P-5′-end-labeled DNA fragments, calf thymus DNA (20 μM per base) and 20 μM $CuCl_2$ in 200 μl of 10 m*M* sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 60 min, the double-stranded DNA fragments were heated at 90°C in 1 *M* piperidine for 20 min[28] or treated with 2 units of endonuclease IV in 10 μl of TE buffer (pH 7.5) containing 50 m*M* NaCl at 37°C for 60 min. Then the DNA fragments were electrophoresed on an 8% polyacrylamide/8 *M* urea gel. The autoradiogram was obtained by exposing X-ray film on 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[29] using a DNA-sequencing system (LKB 2010 Macrophor, Pharmacia Biotech, Uppsala, Sweden). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech).

Analysis of 8-OHdG formation in calf thymus DNA by hydroxyurea The amount of 8-OHdG was measured by a modification of the method of Kasai *et al.*.[30] Calf thymus DNA fragments (100 μM) were incubated with hydroxyurea and 20 μM $CuCl_2$ in 200 μl of 4 m*M* sodium phosphate buffer (pH 7.8) containing 5 μM DTPA for 60 min at 37°C. After ethanol precipitation, the DNA fragments were digested to the nucleosides with nuclease P_1 and CIP, and analyzed by HPLC-ECD, as described previously.[31]

ESR spin-trapping studies ESR spectra were measured at room temperature (25°C) by using a JES-TE-100 spectrometer (JEOL, Tokyo) with 100-kHz field modulation. $Fe(DTCS)_3$ was used as a spin-trapping agent.[32] The reaction mixture contained 100 m*M* hydroxyurea, 20 μM $CuCl_2$, $Fe(DTCS)_3$ ([Fe]=10 m*M*, [DTCS]=30 m*M*) in 10 m*M* sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 1 h, reaction mixtures were taken up into capillary tubes and the spectra were measured at room temperature. Spectra were recorded with a microwave power of 16 mW, a modulation amplitude of 0.05 mT, a receiver gain of 500, a time constant of 0.1 s and a sweep time of 4 min. The magnetic fields were calculated based on the splitting of Mn(II) in MgO (ΔH_{3-4}=8.69 mT).

GC-MS and TOF-MS analyses The reaction mixture, containing 10 m*M* hydroxyurea and 20 μM Cu(II) in 10 m*M* sodium phosphate buffer (pH 7.8) containing 5 μM DTPA, was incubated for 2 h at 37°C. GC-MS analysis was performed on a GC-17A gas chromatograph (Shimadzu, Kyoto) equipped with a CAM capillary column

(0.325 mm×15 m; J&W Scientific, Köln, Germany). The constant flow rate was 19.0 ml/min. The injection (injection volume: 1 μl) was performed in the splitless mode with the temperature of the injection port set at 230°C. The temperature of the GC oven was maintained at 50°C for 1 min and then raised to 215°C at a rate of 15°C/min, and finally left at the latter temperature for 4 min. Detection of positive ions was provided by a QP-5000 mass detector using electron impact ionization (Shimadzu). TOF-MS analysis was also performed on a Voyager B-RP (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm, 3 ns pulse) to determine formamide formation. A matrix solution (α-cyano-4-hydroxycinnamic acid) was added to the sample.

RESULTS

Damage to ^{32}P-labeled DNA fragments by hydroxyurea in the presence of metal ions The extent of damage to double-stranded DNA induced by hydroxyurea in the presence of Cu(II) was estimated by gel electrophoretic analysis (Fig. 1). Oligonucleotides were detected on the autoradiogram as a result of DNA damage. Hydroxyurea induced DNA damage in a dose-dependent manner. Even without piperidine treatment, oligonucleotides were formed by hydroxyurea (Fig. 1A), indicating breakage of the deoxyribose phosphate backbone. The amount of oligonucleotides was increased by piperidine treatment (Fig. 1B). Since an altered base is readily removed from its sugar by piperidine treatment, it is considered that the base modification was induced by hydroxyurea. The DNA damage was significantly enhanced by the addition of 100 μM NADH (Fig. 1C). NADH is a reductant existing at high concentrations (100–200 μM) in cells.[33] The magnitude of the DNA damage induced by 10 μM hydroxyurea with NADH was greater than that caused by 100 μM hydroxyurea without NADH. Hydroxyurea did not induce DNA damage in the presence of Co(II), Ni(II), Mn(II), Mn(III), Fe(III) or Fe(III)EDTA (data not shown).

Effects of scavengers and bathocuproine on DNA damage induced by hydroxyurea Fig. 2 shows the effects of scavengers and bathocuproine on DNA damage induced by hydroxyurea in the presence of Cu(II). Catalase and

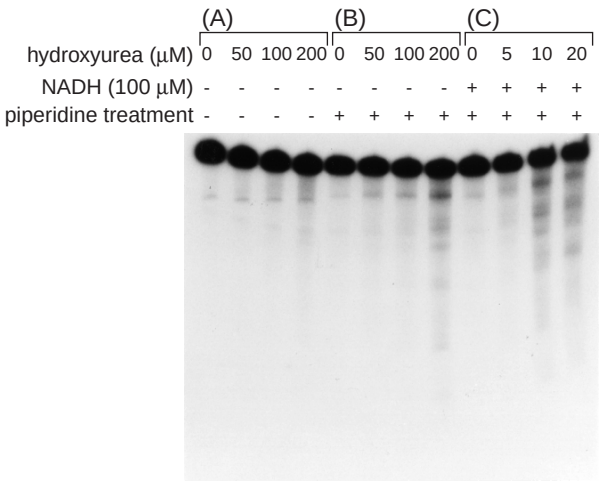

Fig. 1. Autoradiogram of ^{32}P-labeled DNA fragments incubated with hydroxyurea in the presence of NADH and Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 211-bp DNA fragments, 20 μM per base of calf thymus DNA, indicated concentrations of hydroxyurea, 20 μM $CuCl_2$ and 100 μM NADH in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 60 min, followed by piperidine treatment (B, C) or without piperidine treatment (A), the treated DNA fragments were electrophoresed on an 8% polyacrylamide per 8 M urea gel (12×16 cm), and the autoradiogram was obtained by exposing X-ray film to the gel.

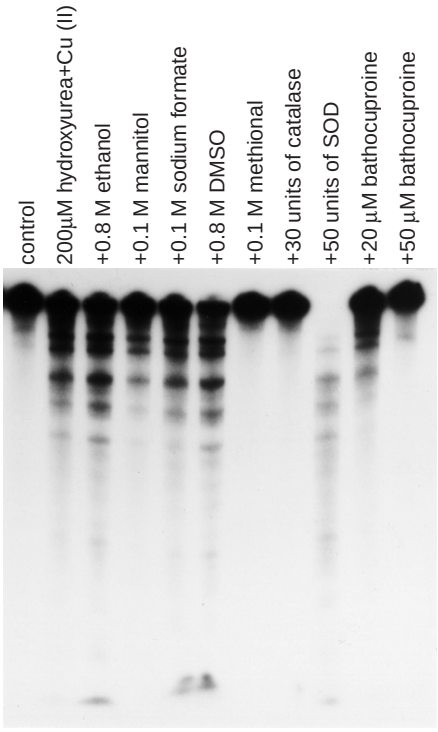

Fig. 2. Effects of scavengers and bathocuproine on DNA damage induced by hydroxyurea in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 443-bp DNA fragment, 20 μM per base of sonicated calf thymus DNA, 200 μM hydroxyurea, scavenger and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After the incubation at 37°C for 60 min, followed by the piperidine treatment, the DNA fragments were analyzed by the method described in the legend to Fig. 1.

bathocuproine, a Cu(I)-specific chelator, inhibited the DNA damage, suggesting the involvement of hydrogen peroxide (H_2O_2) and Cu(I). Typical free hydroxyl radical (•OH) scavengers, ethanol, mannitol, sodium formate and DMSO, showed no or little inhibitory effect on the DNA damage, whereas methional was inhibitory. Methional can scavenge not only •OH, but also species with weaker reactivity than •OH.[34] SOD enhanced the DNA damage.

Abasic site formation by hydroxyurea in the presence of Cu(II) and catalase Fig. 3 shows an autoradiogram of DNA fragments treated with a high concentration of hydroxyurea in the presence of Cu(II) and catalase, followed by endonuclease IV treatment. Endonuclease IV is a DNA repair enzyme responsible for excision, which cleaves DNA strand at an abasic site.[35] DNA cleavage increased with increasing concentrations of hydroxyurea (Fig. 3A). Little or no oligonucleotide was produced without endonuclease IV (data not shown). On the other hand, intensity of damage to the double-stranded DNA fragments treated with endonuclease IV plus UDG, which is a DNA repair enzyme responsible for excision of uracil,[36] was similar to that with endonuclease IV alone (Fig. 3B). These results suggest that hydroxyurea induces formation of abasic sites rather than deamination.

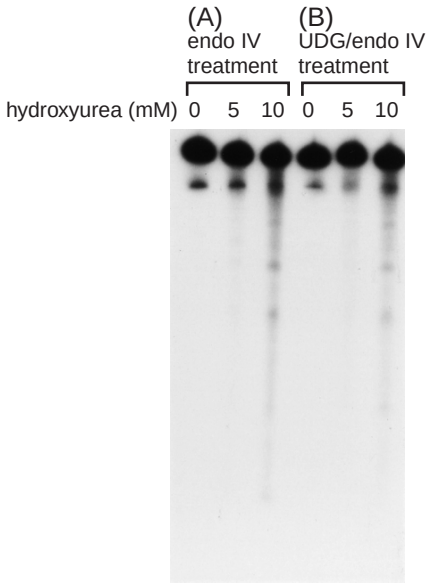

Fig. 3. Detection of abasic sites in DNA induced by hydroxyurea. The reaction mixture contained the ^{32}P-5′-end-labeled 211-bp DNA fragment, hydroxyurea, 20 μM $CuCl_2$, 30 units of catalase and 0.1 μM per base of calf thymus DNA in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C for 120 min, the DNA fragments were treated with endonuclease IV (A) or UDG and endonuclease IV (B), and analyzed by the method described in the legend to Fig. 1.

Site specificity of DNA cleavage by hydroxyurea The patterns of DNA cleavage induced by hydroxyurea in the presence of Cu(II) were determined by the Maxam-Gilbert procedure.[29] The relative intensity of DNA cleavage obtained by scanning autoradiogram with a laser densitometer is shown in Fig. 4. Hydroxyurea frequently generated piperidine-labile sites at thymine and cytosine residues in double-stranded DNA fragments obtained from the human *p53* (Fig. 4A) and *p16* (Fig. 4B) tumor suppressor genes and the c-Ha-*ras*-1 protooncogene (data not

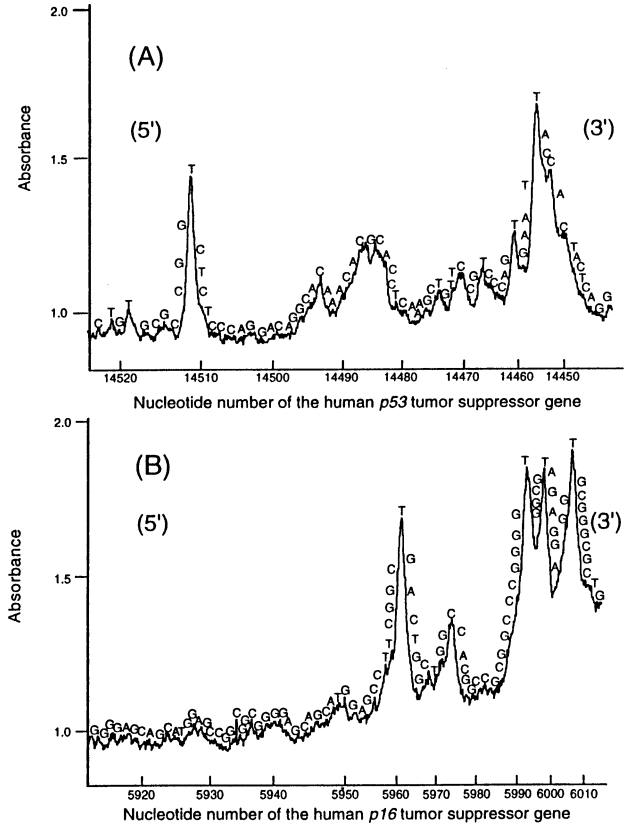

Fig. 4. Site specificity of DNA cleavage induced by hydroxyurea in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 443-bp fragment (*Apa*I 14 179–*Eco*RI* 14 621) of the *p53* tumor suppressor gene (A) or the 328-bp fragment (*Eco*RI* 5841–*Mro*I 6168) of the *p16* tumor suppressor gene (B), 20 μM per base of calf thymus DNA, 200 μM hydroxyurea and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Reaction mixtures were incubated at 37°C for 60 min. After piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide per 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 oligonucleotide were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech).

shown). Similar DNA cleavage patterns were observed in the presence of NADH in the same system (data not shown). When endonuclease IV treatment was performed instead of piperidine treatment, DNA damage was observed mainly at guanine residues (data not shown).

Formation of 8-OHdG in calf thymus DNA by hydroxyurea in the presence of Cu(II) and NADH Using HPLC-ECD, we measured the content of 8-OHdG, an indicator of oxidative base damage,[37–39] in calf thymus DNA treated with hydroxyurea in the presence of Cu(II). The amount of 8-OHdG increased with increasing hydroxyurea concentration (Fig. 5). The addition of NADH enhanced hydroxyurea plus Cu(II)-induced 8-OHdG formation. Hydroxyurea did not cause 8-OHdG formation in the absence of Cu(II) (data not shown).

Production of nitric oxide and formamide from hydroxyurea We analyzed the ESR signal generated from hydroxyurea in the presence of Cu(II) using DTCS as a NO spin-trapping agent. As shown in Fig. 6A, the ESR spectra showed distinct triplet signals with a_N=1.27 mT and g_{iso}=2.040, reasonably assigned to the $Fe(DTCS)_2(NO)$.[32] Catalase did not inhibit NO production from hydroxyurea (Fig. 6B).

The formation of formamide was also measured by both GC-MS and TOF-MS. GC-MS analysis indicated a peak eluting at 6.09 min corresponding to formamide with the expected molecular ion M^+ at m/z 45 (data not shown). Mass spectral analysis using TOF-MS also confirmed formamide formation by comparison of the mass spectrum with that of an authentic standard; m/z=46 $(M+H)^+$. These results suggest that NO and formamide were generated from hydroxyurea.

DISCUSSION

The present study has demonstrated that hydroxyurea caused DNA damage in the presence of Cu(II). Furthermore, Cu(II)-mediated 8-OHdG formation increased with increasing concentration of hydroxyurea in the presence of Cu(II). When NADH, an endogenous reductant, was added, oxidative DNA damage was enhanced. 8-OHdG is

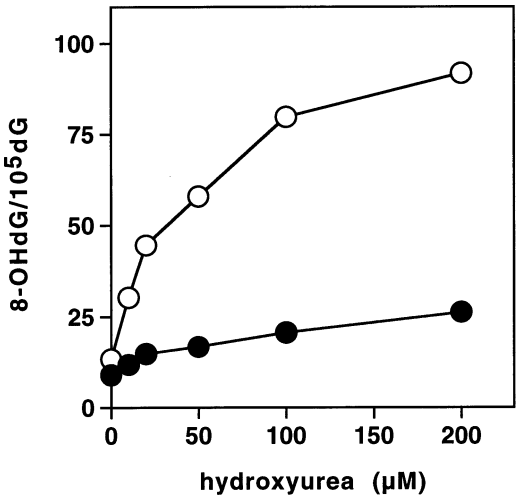

Fig. 5. Formation of 8-OHdG by hydroxyurea in the presence of Cu(II). Calf thymus DNA (100 μM per base) was incubated with hydroxyurea, 100 μM NADH and 20 μM $CuCl_2$ in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA at 37°C for 60 min. After ethanol precipitation, the DNA was subjected to enzyme digestion and analyzed by HPLC-ECD as described under "Materials and Methods." Open circles indicate the reaction with NADH, and closed circles indicate that without NADH.

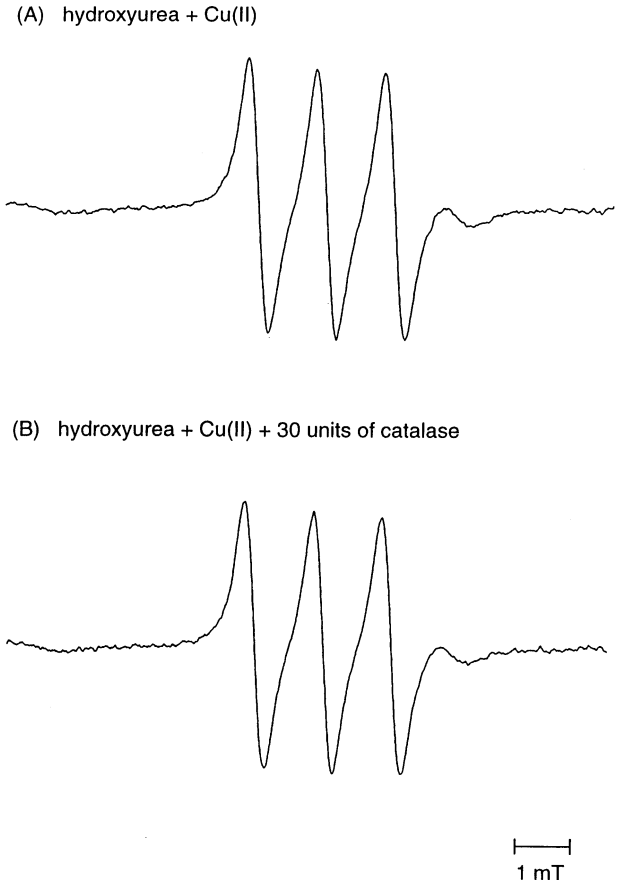

Fig. 6. Detection of $Fe(DTCS)_2(NO)$ complex derived from hydroxyurea in the presence of Cu(II). The reaction mixture contained 100 mM hydroxyurea, 20 μM $CuCl_2$, $Fe(DTCS)_3$ solution ([Fe]=10 mM, [DTCS]=30 mM) in 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The reaction mixture was taken up a capillary tube and the spectra were measured at room temperature.

an indicator of oxidative stress and has been well-characterized as a premutagenic lesion in mammalian cells.[37–39] Therefore, it is considered that Cu(II)-mediated oxidative DNA damage may participate in mutagenic and carcinogenic processes caused by hydroxyurea.

In order to clarify what kinds of reactive oxygen species cause oxidative DNA damage, the effects of various scavengers on DNA damage and its site specificity were examined. Inhibitory effects of catalase and bathocuproine on DNA damage suggest the involvement of H_2O_2 and Cu(I). Furthermore, typical •OH scavengers showed little or no inhibitory effect on DNA damage, whereas methional inhibited it. Methional scavenges not only •OH, but also a variety of reactive species other than •OH.[34] Thus, •OH does not appear to play an important role in DNA damage. The predominant DNA cleavage sites were thymine and cytosine residues. This result supports the involvement of reactive species other than •OH, because •OH causes DNA damage at all nucleotides with little site specificity.[40, 41] However, the possibility that •OH participates in DNA damage cannot be ruled out. Cu(II) binds to DNA in a site-specific manner and is reduced to Cu(I) to react with H_2O_2 generated from hydroxyurea, resulting in formation of DNA-Cu(I)-hydroperoxo complexes such as DNA-Cu(I)OOH. This complex may release •OH, which would immediately attack adjacent DNA constituents before being scavenged by •OH scavengers.[42]

A high concentration of hydroxyurea induced depurination at DNA bases in the presence of catalase, and endonuclease IV treatment led to chain cleavages. Furthermore, ESR spectrometry revealed that NO was generated from hydroxyurea and that catalase did not inhibit the formation of NO. The GC-MS and TOF-MS analyses demonstrated that hydroxyurea non-enzymatically produced formamide in addition to O_2^-. Overall, it is considered that hydroxyurea can generate NO and formamide. Several papers have reported that NO can induce deamination of DNA.[43–47] Under these experimental conditions, however, UDG treatment did not enhance DNA damage, suggesting little or no deamination. It has been reported that peroxynitrite generated from NO plus O_2^- participates in not only 8-OHdG formation,[48] but also depurination of guanine via 8-nitroguanine formation.[49, 50] An apurinic site, which is probably produced by depurination of guanine in DNA, is potentially mutagenic, and induces G:C→T:A transversions.[50–52] Therefore, it is considered that NO may react with O_2^- to cause DNA damage and participate in mutation by a high concentration of hydroxyurea.

Possible mechanisms of oxidation and depurination of DNA bases induced by hydroxyurea are shown in Fig. 7, and could account for most of the observations. The oxidation pathway in which hydroxyurea itself causes Cu(II)-mediated oxidative DNA damage is the major pathway. During the autoxidation of hydroxyurea, Cu(II) is reduced

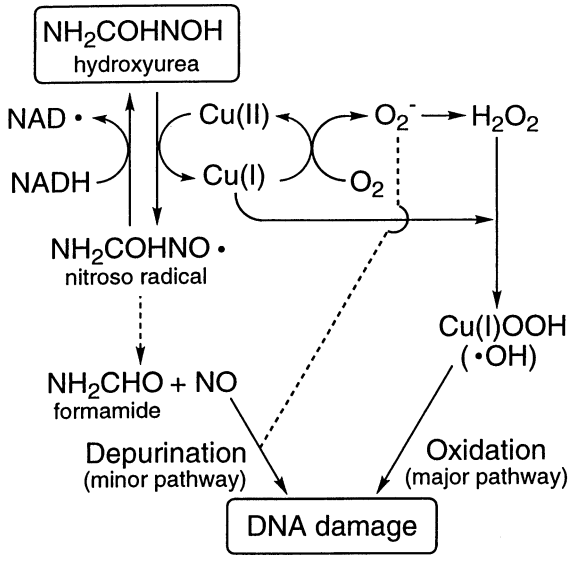

Fig. 7. Possible mechanisms of DNA damage induced by hydroxyurea in the presence of Cu(II).

to Cu(I), which reacts with O_2 to generate O_2^- and subsequently H_2O_2. H_2O_2 reacts with Cu(I) to form crypto-hydroxyl radicals, such as the Cu(I)-hydroperoxo complex, capable of causing site-specific DNA damage. Endogenous reductants, such as NADH, reduce the nitroso radical to form a redox cycle resulting in enhancement of oxidative DNA damage. The concentration of NAD(P)H in certain tissues was estimated to be as high as 50–200 μM.[33] The possibility that chemicals are non-enzymatically reduced by NAD(P)H *in vivo* has been proposed.[53, 54] There is a minor pathway called the depurination pathway. Hydroxyurea is autoxidized by Cu(II) into the nitroso radical, followed by the production of NO and formamide. Several reports have revealed that hydroxyurea is metabolized to release NO.[19–21] Although Sato *et al.* reported the formation of NO from hydroxyurea in the presence of H_2O_2 and metal,[20] we demonstrated that a small amount of NO was generated from hydroxyurea in an H_2O_2-independent manner.

The biological significance of copper has recently drawn much interest in connection with carcinogenicity and mutagenicity. Copper is an essential component of chromatin and is known to accumulate preferentially in the heterochromatic regions.[55, 56] A case-cohort study showed a U-shaped relation between plasma copper levels and the risk of developing breast cancer.[57] Copper sulfate showed clastogenic effects on the bone marrow chromosomes of mice *in vivo*.[58] Copper has the ability to catalyze the production of reactive oxygen species to mediate oxidative DNA damage.[59–62] The present work suggests that copper is an important factor in DNA damage by hydroxyurea.

Hydroxyurea is an often-used chemotherapeutic drug for various malignancies and MPD.[63, 64] It is thought to have lower mutagenic potential than alkylating agents. The mutagenic and carcinogenic potential of hydroxyurea, however, may be a serious risk associated with long-term therapy. Hydroxyurea blocks DNA synthesis and DNA repair *in vitro*.[65] Furthermore, in this study, we demonstrated that hydroxyurea could induce metal-mediated DNA damage. Therefore, it is considered that hydroxyurea participates in not only anti-cancer activity, but also carcinogenesis via DNA damage. Careful consideration of safety is required when hydroxyurea is used as a chemotherapeutic drug.

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