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Involvement of Oxidative DNA Damage and Apoptosis in Antitumor Actions of Aminosugars

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We investigated the mechanisms of apoptosis and DNA damage induced by aminosugars in relation to their antitumor actions. The order of cytotoxic effects of aminosugars was D-mannosamine (ManN) ≫ D-galactosamine (GalN) > D-glucosamine (GlcN). A comparison of the frequency of apoptotic cells showed the same order. DNA ladders were formed by only ManN and the formation of DNA ladders was inhibited by a caspase inhibitor. Pulsed-field gel electrophoresis showed that ManN caused cellular DNA cleavage at a lower concentration than those causing apoptosis. Cellular DNA cleavage was inhibited by catalase and enhanced by a catalase inhibitor. Flow cytometry showed that ManN enhanced the production of intracellular peroxides. These results suggest that ManN-induced apoptosis is preceded by H_2O_2-mediated DNA damage. The order of the extent of damage to ^{32}P-labeled DNA fragments by aminosugars plus Cu(II) was ManN ≫ GalN > GlcN. The DNA damage was inhibited by catalase and bathocuproine, suggesting that H_2O_2 reacts with Cu(I) to form the metal-peroxide complex capable of causing DNA damage. Two mechanisms of H_2O_2 generation from aminosugars were proposed: one is the major pathway to form a dioxo compound and NH_4^+; the other is the minor pathway to form a pyrazine derivative through the condensation of two molecules of an aminosugar. The order of reactivity to generate these products was ManN ≫ GalN > GlcN. On the basis of these results, it is concluded that aminosugars, especially ManN, produce H_2O_2 to cause DNA damage, which mediates apoptosis resulting in tumor growth inhibition.

Keywords: DNA damage, apoptosis, D-mannosamine, hydrogen peroxide, copper, reactive oxygen species

Abbreviations: ManN, D-mannosamine; ROS, reactive oxygen species; GalN, D-galactosamine; GlcN, D-glucosamine; Z-Asp-CH_2-DCB, benzyloxycarbonyl-Asp-$CH_2OC(O)$-2,6-dichlorobenzene; ESR, electron spin resonance; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; SOD, superoxide dismutase; POBN, α-(1-oxy-4-pyridyl)-N-*tert*-butylnitrone; FCS, fetal calf serum; PBS, phosphate-buffered saline

INTRODUCTION

Many chemotherapeutic drugs show toxic effects on tumor cells to inhibit tumor growth. Aminosugars have inhibitory effects on tumor growth, like a number of antitumor drugs. Quastel and Cantero reported that a certain aminosugar suppressed the tumor growth and decreased the mortality rate of mice.[1] Aminosugars showed inhibitory effects on viability and

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transplantability of ascites tumor cells.[2] Aminosugars inhibited the biosynthesis of protein, RNA and DNA in neoplastic tissues,[3–5] and provoked cytoplasmic and nuclear changes in tumor cells.[6] The combination of an aminosugar D-mannosamine (ManN) and fatty acids showed cytotoxic effects on human leukemia cell lines due to changes in membrane structure.[5,7] ManN inhibited the biosynthesis of glycosylphosphatidylinositol, which anchors membrane proteins.[8–10] However, not only alteration in cell membrane but also DNA damage would be caused by aminosugars. Aminosugars induced strand breakage of plasmid DNA in the presence of copper ions.[11–13] It has been suggested that reactive oxygen species (ROS) are involved in DNA damage induced by aminosugars.[13,14] However, it has not been clarified whether DNA damage is involved in antitumor effects of aminosugars.

Suppression of tumor growth by antitumor drugs is contributed by apoptosis.[15–17] Apoptosis is characterized by morphological and biochemical changes, including chromatin condensation, nuclear fragmentation and the formation of DNA ladder due to endonuclease-catalyzed DNA degradation at internucleosomal sites. The participation of DNA damage in apoptosis, which is triggered by diverse cytotoxic signals, has been discussed.[15–17] Recently, the involvement of ROS in apoptosis has been suggested.[18–26] Some chemicals and UV radiation induce apoptosis by generating ROS.[24–26] ROS can cause DNA damage, which may mediate apoptosis. The relationship among apoptosis, DNA damage and ROS attracts our interest in relation to the mechanism of inhibitory effects of aminosugars on tumor growth.

In the present study, we studied the mechanism of antitumor effects by aminosugars using ManN, D-galactosamine (GlcN) and D-glucosamine (GalN). Inhibitory effect of each aminosugar on the growth of human cultured cells was compared. We examined apoptotic changes, such as internucleosomal DNA fragmentation and morphological changes in cells. We also examined the involvement of caspases in apoptosis using an effective caspase inhibitor benzyloxycarbonyl-Asp-$CH_2OC(O)$-2,6-dichlorobenzene (Z-Asp-CH_2-DCB), which inhibits apoptosis induced by antitumor drugs.[27] Caspases play important roles to mediate apoptosis induced by chemotherapeutic agents.[27,28] DNA strand breaks in human cultured cells were investigated with pulsed-field gel electrophoresis, and the involvement of H_2O_2 in DNA strand breaks was examined by using catalase and its inhibitor. The production of intracellular H_2O_2 and other peroxides was analyzed with a flow cytometer. To clarify the mechanism of cellular DNA damage, we examined DNA damage induced by aminosugars in the presence of copper ions using ^{32}P-5′-end-labeled DNA fragments obtained from the human c-Ha-*ras*-1 proto-oncogene and the *p53* tumor suppressor gene. Free radicals generated from aminosugars were detected by the electron spin resonance (ESR) spin-trapping technique. We also investigated the reactivity of each aminosugar by measuring the products generated during its autoxidation, a dioxo compound, NH_4^+ and a pyrazine derivative.

MATERIALS AND METHODS

Materials Aminosugars (ManN, GalN and GlcN), proteinase K and 3-aminotriazol were from Nacalai Tesque Inc. (Kyoto, Japan). RNase and a restriction enzyme StyI were from Boehringer Mannheim. Restriction enzymes (AvaI, XbaI and PstI) and T_4 polynucleotide kinase were purchased from New England Biolabs. [γ-^{32}P]-ATP (222 TBq/mmol) was from New England Nuclear. Z-Asp-CH_2-DCB was from Takara Shuzo Co. (Otsu, Japan). Acridine orange was from Chroma-Gesellschaft Schmid GmbH Co. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes, Inc. Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were

from Dojin Chemicals Co. (Kumamoto, Japan). Superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver) were from Sigma Chemical Co. α-(1-Oxy-4-pyridyl)-N-*tert*-butylnitrone (POBN) was from Aldrich Chemical Co. (Milwaukee, WI). 2,3-Diaminonaphthalene was from Tokyo Kasei Co. (Tokyo, Japan).

Effects of Aminosugars on Growth of Cultured Cells HL60 cells (2×10^5 cells/mL) were incubated with 5 mM aminosugars in 5 mL of RPMI 1640 (Gibco Laboratories, NY) supplemented with 6% fetal calf serum (FCS, Whittaker Bioproducts) for 3 days at 37°C. Cell viability was determined by trypan blue exclusion and counting in a hemocytometer. HP100 cells were also used to examine the involvement of H_2O_2 in cytotoxic action of ManN. HP100 cells were created by treatment of HL60 cells with 100 μM H_2O_2 and selection of H_2O_2-resistant cells as reported previously.[29,30] Catalase synthesis and its activity in HP100 cells were 4- and 18-fold higher than those in HL60 cells, respectively.[29] For statistical analysis of the data, Student's *t*-test was used at a significance level of $p < 0.05$. The data represent means ± SD of three independent experiments.

Detection of DNA Ladder Formation Induced by Aminosugars HL60 cells (2×10^5 cells/mL) were incubated with aminosugars in 5 mL of RPMI 1640 supplemented with 6% FCS for 6 h at 37°C. In a certain experiment, 55 μM Z-Asp-CH_2-DCB, a caspase inhibitor, was added before the incubation. After the incubation, the media were removed and the cells were washed twice with phosphate-buffered saline (PBS). The cells were lysed and treated with RNase and proteinase K as described previously.[31] Subsequently, the DNA was extracted with phenol–chloroform and water–saturated ether, and then precipitated with ethanol. The DNA was electrophoresed on a 1.4% agarose gel containing ethidium bromide.

Identification of Apoptotic Cells HL60 cells (5×10^5 cells/mL) were incubated with aminosugars in RPMI 1640 supplemented with 6% FCS for 24 h at 37°C. The cells were stained with acridine orange. Apoptotic cells were identified by chromatin condensation and nuclear fragmentation by using a fluorescence microscope.

Detection of Cellular DNA Damage by Pulsed-Field Gel Electrophoresis For the determination of DNA strand breaks, HL60 cells (2×10^5 cells/mL) were incubated with ManN in 5 mL of RPMI 1640 containing 6% FCS for 6 h at 37°C. In a certain experiment, catalase, 3-aminotriazol (a catalase inhibitor) or Z-Asp-CH_2-DCB (a caspase inhibitor) was added before the incubation. After the incubation, the media were removed and the cells were washed twice with PBS and resuspended in 35 μL of PBS. The cell suspension was solidified with agarose gel, and subsequently the cells in agarose plugs were lysed and deproteinized with proteinase K according to the method as described previously.[32] The DNA was electrophoresed on a 1% agarose gel in Tris-borate EDTA buffer with a CHEF-DRII pulsed-field electrophoresis system (Bio-Rad) at 200 V at 14°C. Switch time was 60 s for 15 h followed by a 90 s switch time for 9 h. The DNA in the gel was visualized in ethidium bromide.

Flow Cytometric Detection of Peroxides Produced in Cultured Cells Treated with Aminosugars HL60 cells (2×10^5 cells/mL) were incubated with aminosugars in RPMI 1640 containing 6% FCS for 3.5 h at 37°C. DCFH-DA, a sensitive fluorimetric probe of peroxides,[33,34] was dissolved in ethanol, and 5 μM DCFH-DA was added to the media and the cells were incubated for 30 min at 37°C. After the incubation, the cells were washed with PBS once and suspended in 1 mL of PBS. The cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA).

Detection of DNA Damage Using c-Ha-ras-1 and p53 DNA Fragments DNA fragments were prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the human c-Ha-*ras*-1 protooncogene.[35,36] The singly labeled 261-base pair (*Ava*I* 1645–*Xba*I 1905), 341-base pair (*Xba*I 1906–*Ava*I* 2246) and 337-base pair (*Pst*I 2345–*Ava*I*

2681) fragments were obtained according to the method described previously.[35,36] The asterisk indicates ^{32}P-labeling. Nucleotide numbering starts with the *Bam*HI site.[37] DNA fragments were also obtained from the human *p53* tumor suppressor gene.[38] DNA fragments were prepared from the pUC18 plasmid containing exons of the *p53* gene. The singly labeled 348-base pair (*Sty*I 13160–*Eco*RI* 13507) fragment was obtained according to the method described previously.[39]

The standard reaction mixture in a microtube (1.5-mL Eppendorf) contained an aminosugar, 20 μM $CuCl_2$, [^{32}P]DNA fragment and sonicated calf thymus DNA (2 μM/base) in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. In a certain experiment, [^{32}P]DNA fragment and calf thymus DNA were added after incubation. After incubation at 37°C for indicated durations, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min and treated as described previously.[35,36]

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[40] using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Determination of Dioxo Compounds and NH_4^+ Generated from Aminosugars in the Presence of Cu(II) The reaction mixture, which contained an aminosugar and 20 μM $CuCl_2$ in 50 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA, was incubated for 4 h at 37°C. After the incubation, 200 μM DTPA was added to stop the reaction. Dioxo compounds formed by aminosugars were measured using the procedure reported previously.[41,42] 2,3-Diaminonaphthalene (1 mM) in ethanol solution was added to the reaction mixture, which was heated for 1 h at 60°C to generate benzoquinoxaline derivatives. UV-visible spectra of the benzoquinoxaline derivatives were measured, and the concentration of dioxo compounds was calculated using the molecular extinction coefficient of a certain benzoquinoxaline derivative, $\varepsilon = 6370$ ($M^{-1} \times cm^{-1}$) at 365 nm.[41] NH_4^+ generation was measured using a commercial kit, Monotest ammonia (Boehringer Mannheim) as described previously.[42]

Detection of Free Radicals Derived from Aminosugars with ESR Spectrometry ESR spectra were recorded to detect free radicals derived from aminosugars. The 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 16 mW and a modulation amplitude of 0.1 mT.

Analysis of Pyrazine Derivatives Generated from Aminosugars in the Presence of Cu(II) The reaction mixture, containing 10 mM aminosugar and 20 μM $CuCl_2$ in 50 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA, was incubated for 10 h at 37°C. To detect the pyrazine derivatives generated by aminosugars, HPLC was carried out on an LC-10A HPLC system (Shimadzu, Kyoto, Japan) using a Cosmosil column (4.6 mm i.d. × 150 mm), flow rate of 0.5 mL/min, linear gradient in 10 min of 0–10% acetonitrile. The HPLC eluate was routed directly into a photodiode array UV-visible detector (SPD-M10A, Shimadzu), and the spectrum of the eluate was obtained. To determine molecular weights of the pyrazine derivatives, laser desorption mass spectrometry was performed. The HPLC eluate was analyzed with a Kompact MALDI III equipped with a nitrogen laser (337 nm, 3-ns pulse).

We calculated the concentration of the pyrazine derivatives in the reaction mixture, which contained an aminosugar, 20 μM $CuCl_2$ and 5 μM DTPA in 50 mM sodium phosphate buffer (pH 7.8). The reaction mixture was incubated for 4 h at 37°C, and then UV-visible spectra were measured with a UV-Vis-NIR recording spectrophotometer (Shimadzu UV-365). The concentration of

pyrazine derivatives was calculated using $\varepsilon = 7360\ (M^{-1} \times cm^{-1})$ at 276 nm.[43]

RESULTS

Inhibitory Effects of Aminosugars on Cell Growth and Viability Figure 1 shows the effects of aminosugars on growth and viability of human HL60 cells. ManN showed much more potent cytotoxic effect than GalN and GlcN. After the 1-day treatment, ManN showed a significant inhibitory effect on cell growth ($p < 0.05$, Figure 1A) and viability ($p < 0.001$, Figure 1B) compared with the control. After the 2-day treatment, ManN dramatically decreased the viability (20.6 ± 2.71%) and cell number. GalN also significantly decreased viability after the 1-day treatment ($p < 0.001$), whereas the inhibitory effect of GalN on cell growth was not significant. Although GalN significantly inhibited cell growth by the 2- and 3-day treatments, GalN showed weaker effect on cell growth and slower reduction in viability than ManN. GlcN had no significant effect on cell growth and viability, although GlcN slightly inhibited cell growth after the 3-day treatment. These results suggest that the order of cytotoxic effects of aminosugars is ManN ≫ GalN > GlcN.

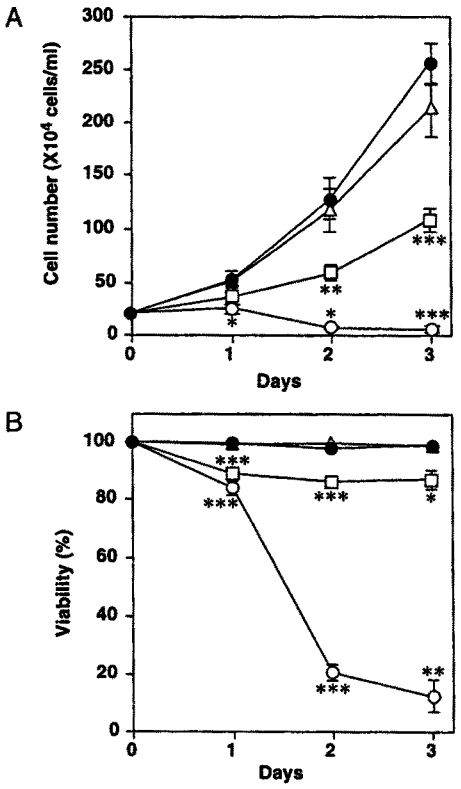

FIGURE 1 Effects of aminosugars on the growth of cultured cells. HL60 cells (2×10^5 cells/mL) were incubated without aminosugar (●) or with 5 mM aminosugar [ManN (○), GalN (□) or GlcN (△)] for 3 days at 37°C. Cell number (A) and viability (B) were determined by trypan blue exclusion and counting in a hemocytometer. Values represent means ± SD of three independent experiments. Statistically significant compared with the control at $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

HP100 cells, HL60-derived cells in which catalase activity is elevated, were used to examine the involvement of H_2O_2 in cytotoxicity of ManN. The viability of HP100 cells was not decreased by ManN for 3 days, although the growth was slightly inhibited by the 3-day treatment with ManN ($p < 0.05$, data not shown). The extent of ManN-induced cytotoxicity in HP100 was much smaller than that in HL60 cells. These results suggest that cytotoxicity caused by ManN involves the generation of H_2O_2.

Apoptotic Changes in Cultured Cells Treated with Aminosugars Figure 2 shows DNA ladder formation, due to internucleosomal DNA fragmentation, in cultured cells treated with aminosugars. ManN formed slight DNA ladder at 3 mM, and prominent ladders at 4–5 mM (Figure 2A). GalN and GlcN formed little or no DNA ladder under the condition used (Figure 2A). Z-Asp-CH_2-DCB, a caspase inhibitor, inhibited the formation of DNA ladder induced by ManN (Figure 2B). We also observed morphological changes in apoptosis in the cells treated with aminosugars. Chromatin condensation and nuclear fragmentation were observed in 36%, 6% and 0.5% of the cells treated with ManN, GalN and GlcN, respectively.

Detection of DNA Damage in Cultured Cells Treated with Aminosugars Using Pulsed-Field Electrophoresis Figure 3A shows DNA strand

breaks in cultured cells treated with ManN, detected by pulsed-field gel electrophoresis. ManN induced slight DNA cleavage to produce 1–2-Mb DNA fragments at 2 mM, and apparent cleavage at 3 mM. The 1–2-Mb DNA fragments disappeared at 5 mM ManN, probably due to further fragmentation of DNA into smaller fragments with multiples of approximately 180 base pairs, as shown in Figure 2A. Figure 3B shows effects of a caspase inhibitor (Z-Asp-CH_2-DCB),

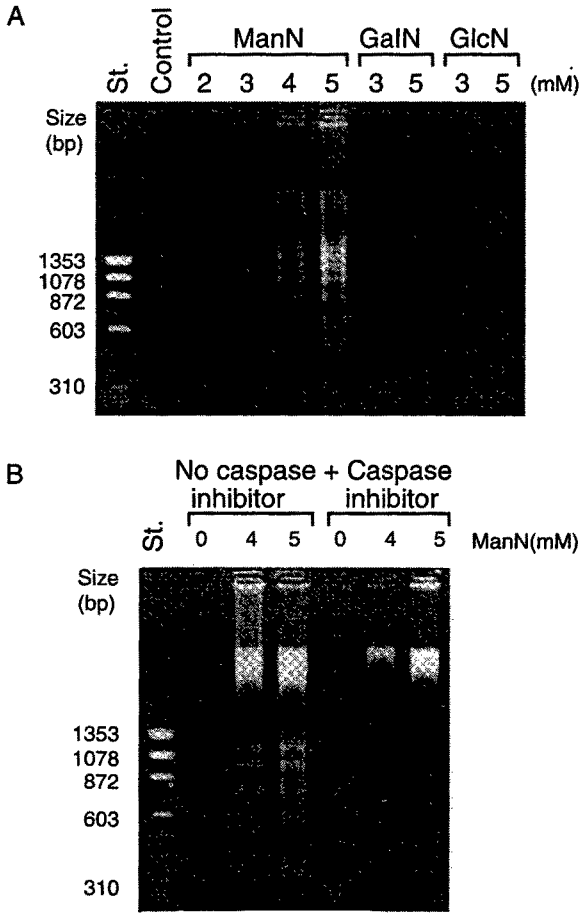

FIGURE 2 DNA ladder formation in cultured cells treated with aminosugars. A, DNA ladder formation induced by aminosugars. HL60 cells were treated with aminosugars for 6 h at 37°C. The cells were lysed and DNA was extracted as described in "Experimental procedures". The DNA was electrophoresed in a 1.4% agarose gel containing ethidium bromide. B, Effect of a caspase inhibitor on the formation of DNA ladder by ManN. HL60 cells were treated with ManN and 55 μM Z-Asp-CH_2-DCB (a caspase inhibitor) for 6 h at 37°C. The cells were treated and the electrophoresis was performed as described above. Lane St., size marker DNA (ϕX 174/*Hae* III digest). Control contained no aminosugar.

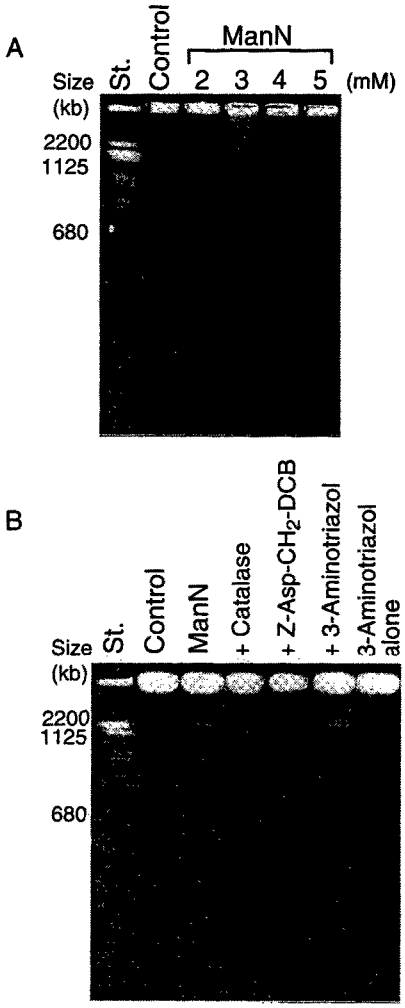

FIGURE 3 DNA cleavage in cultured cells treated with aminosugars. A, Detection of ManN-induced cellular DNA cleavage by pulsed-field gel electrophoresis. HL60 cells were treated with ManN for 6 h at 37°C. The cells were prepared into agarose plugs, lysed and subjected to pulsed-field gel electrophoresis through a 1% agarose gel. The gel was stained with ethidium bromide after the electrophoresis. B, Effects of a caspase inhibitor, catalase and its inhibitor on DNA cleavage by ManN. HL60 cells were treated with 3 mM ManN for 6 h at 37°C. In addition to ManN, 200 units/mL catalase, 55 μM Z-Asp-CH_2-DCB or 5 mM 3-aminotriazol was added as indicated. The cells were treated and the electrophoresis was performed as described above. Lane St., size marker DNA (*Saccharomyces cerevisiae*). Control contained no aminosugar.

catalase and its inhibitor (3-aminotriazol) on DNA strand breaks in HL60 cells treated with ManN. Cellular DNA strand breaks induced by ManN were inhibited by catalase and enhanced by 3-aminotriazol, indicating that H_2O_2 is required for the DNA strand breaks. Z-Asp-CH_2-DCB did not inhibit the DNA strand breaks, and resulted in appearance of approximately 50-kb DNA fragments. This suggests that cleavage of the 50-kb DNA fragments into smaller fragments is catalyzed by caspases, but caspases are not involved in production of the 1–2-Mb fragments.

Detection of Intracellular H_2O_2 and Peroxides Generated from Aminosugars Figure 4 shows flow cytometric distribution of cells treated with aminosugars, and subsequently treated with DCFH-DA, a probe to detect the production of peroxides in cells.[33,34] In the cells treated with ManN, the fluorescence intensity, which means the production of intracellular peroxides, increased in a dose-dependent manner (Figure 4A). ManN showed the largest fluorescence intensity among the aminosugars tested (Figure 4B), suggesting that ManN produces peroxides more efficiently than GalN and GlcN.

Damage to ^{32}P-labeled DNA Fragments Induced by Aminosugars plus Cu(II) Figure 5 shows the autoradiogram of DNA damage induced by aminosugars. When DNA fragments were added to the reaction mixture after the reaction of aminosugars with Cu(II), ManN caused the strongest DNA damage among the aminosugars tested (Figure 5). Aminosugars induced little or no DNA damage in the absence of metal ion and in the presence of Fe(II), Fe(III) or Mn(II) (data not shown). These results suggest that ManN is more reactive to generate reactive species causing Cu(II)-dependent DNA damage than other aminosugars. DNA damage induced by aminosugars plus Cu(II) was enhanced by piperidine treatment (data not shown), indicating that aminosugars cause not only DNA strand breakage but also base modification and/or liberation. Glucose, mannose and galactose caused only slight DNA damage even in the presence of Cu(II) (data not shown).

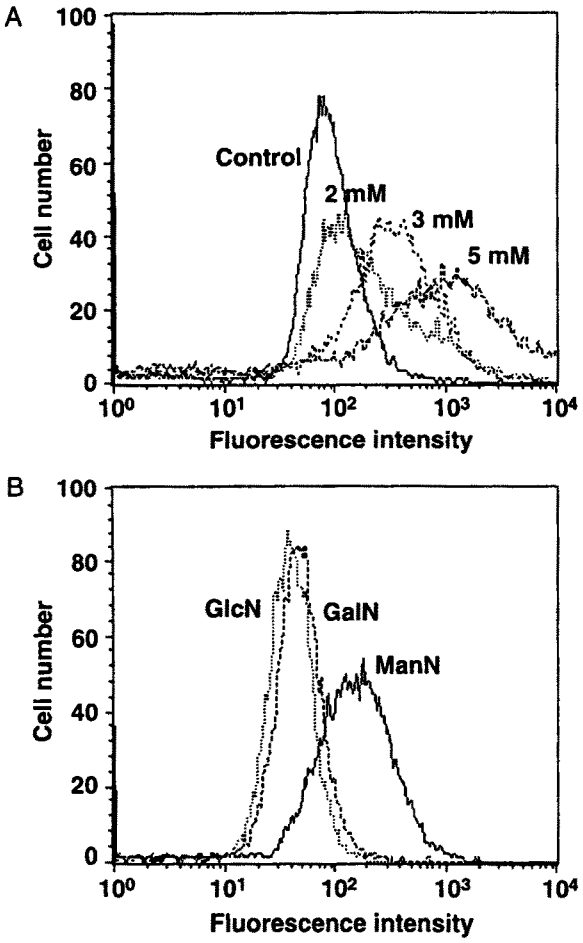

FIGURE 4 Flow cytometric fluorescence distributions of cultured cells treated with aminosugars. HL60 cells were treated with aminosugars for 3.5 h at 37°C. Subsequently, 5 μM DCFH-DA was added to the media and the cells were incubated for 30 min at 37°C. The cells were analyzed with a flow cytometer. A, cells treated with various concentrations of ManN. Control contained no aminosugar. B, cells treated with 5 mM ManN, GalN and GlcN. *Abscissa*, relative fluorescence intensity; *ordinate*, cell number.

Effects of Scavengers and Bathocuproine on DNA Damage by Aminosugars The effects of scavengers and bathocuproine, a Cu(I)-specific chelator, on DNA damage induced by aminosugars were investigated to clarify the reactive species involved in DNA damage. Figure 6 shows the effects of scavengers and bathocuproine on

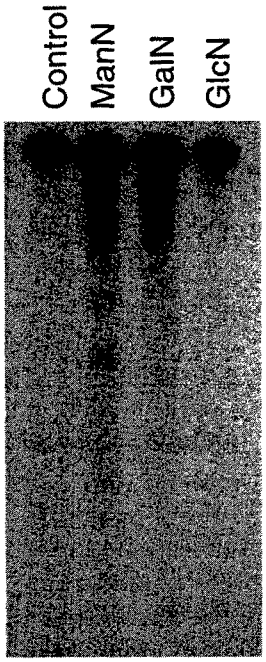

FIGURE 5 Autoradiogram of ^{32}P-labeled DNA fragments incubated with aminosugars in the presence of Cu(II). The reaction mixture, which contained 5 mM aminosugar and 20 μM $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA, was incubated for 1 h at 37°C. Then the ^{32}P-5′-end-labeled 348-base pair (*Sty*I 13160-*Eco*RI* 13507) DNA fragment and 2 μM/base of sonicated calf thymus DNA were added to the reaction mixture, and the mixture was incubated for 10 min at 37°C. The DNA fragments were treated with 1 M piperidine for 20 min at 90°C, and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel. Control contained no aminosugar.

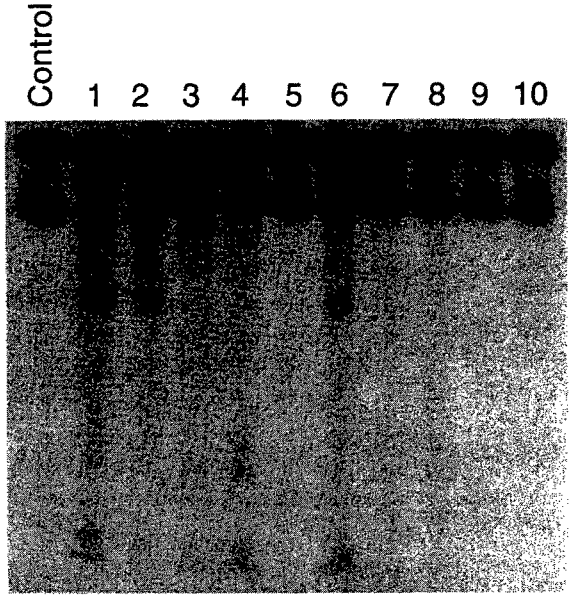

FIGURE 6 Effects of scavengers and bathocuproine on DNA damage induced by ManN plus Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 341-base pair DNA fragment (*Xba*I 1906–*Ava*I* 2246), 2 μM/base of sonicated calf thymus DNA, 2 mM ManN and 20 μM $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 1 h at 37°C. The DNA fragments were treated with piperidine, and analyzed by the method described in the legend to Figure 5. Scavenger or bathocuproine was added as follows; Lane 1, no scavenger; Lane 2, 5% (v/v) ethanol; Lane 3, 0.1 M mannitol; Lane 4, 0.1 M sodium formate; Lane 5, 150 units/mL of catalase; Lane 6, 150 units/mL of SOD; Lane 7, 10 μM bathocuproine; Lane 8, 20 μM bathocuproine; Lane 9, 50 μM bathocuproine; Lane 10, 100 μM bathocuproine. Control contained neither ManN nor $CuCl_2$.

DNA damage induced by ManN plus Cu(II). Typical $^{\bullet}$OH scavengers, ethanol, mannitol and sodium formate, showed weak inhibitory effects on DNA damage (Lanes 2–4). Catalase (Lane 5) and bathocuproine (Lanes 7–9) inhibited DNA damage, suggesting the involvement of H_2O_2 and Cu(I) in DNA damage induced by ManN plus Cu(II). SOD showed little or no effect on DNA damage (Lane 6). These scavengers and bathocuproine showed similar effects on DNA damage induced by other aminosugars in the presence of Cu(II). Whereas catalase and bathocuproine showed inhibitory effects on DNA damage, $^{\bullet}$OH scavengers and SOD showed little effect (data not shown).

Site Specificity of DNA Damage Induced by Aminosugars plus Cu(II) The relative intensity of ManN-induced DNA cleavage was obtained by scanning the autoradiogram with a laser densitometer. ManN induced piperidine-labile sites at thymine, cytosine and guanine residues in the presence of Cu(II) (Figure 7), although there remains a possibility that certain base damage might be over- or underrepresented, depending on their sensitivity to piperidine. Other aminosugars showed similar patterns of

DNA damage (data not shown). Without piperidine treatment, the site specificity of DNA damage by ManN was not clearly observed (data not shown). This result suggests that direct breakage of the deoxyribose phosphate backbone by aminosugars occurs at every nucleotide.

Generation of Free Radicals by Autoxidation of Aminosugars Figure 8 shows ESR spectra of a spin adduct observed when POBN was incubated with aminosugar and Cu(II). The signals ($a_N = 1.55$ mT; $a_H = 0.254$ mT) can be assigned to the carbon-centered radical adduct of POBN by the reference to the hyperfine splitting constants previously reported.[44] The order of the signal intensity was ManN $\gg$ GalN > GlcN. The signal intensity was dramatically reduced in the absence of Cu(II) (data not shown). No signal was observed with Cu(II) alone (data not shown).

Formation of the Products during the Autoxidation of Aminosugars Figure 9 shows the generation of ManN-derived products, dioxo compounds, NH_4^+, and pyrazine derivatives.

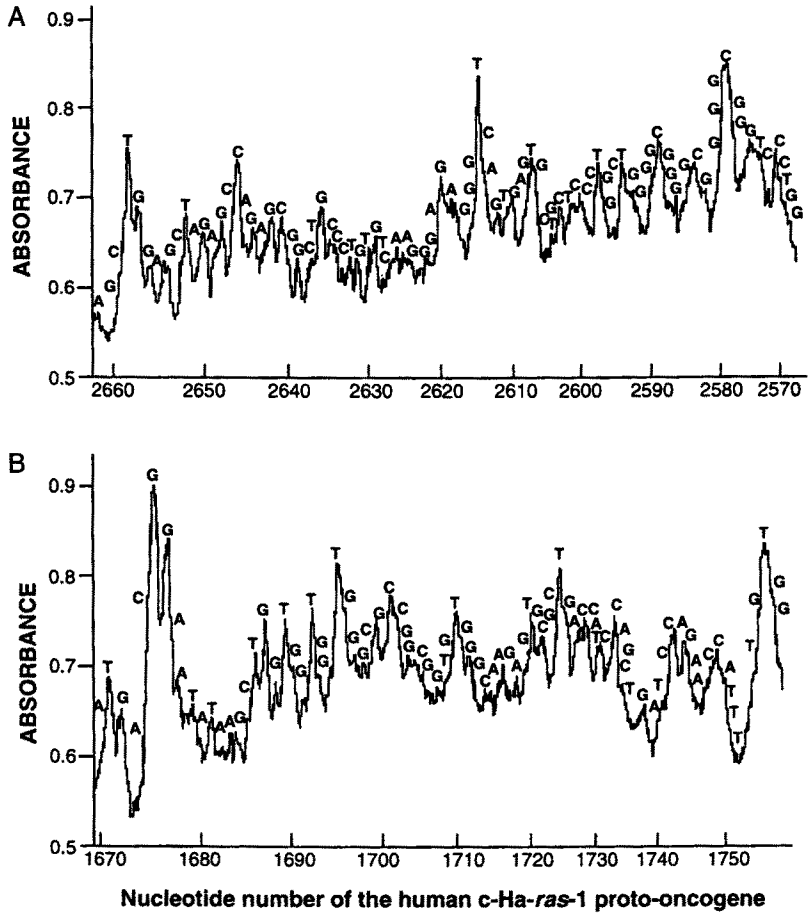

FIGURE 7 Site specificity of DNA cleavage induced by ManN in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end labeled 337-base pair (*Pst*I 2345–*Ava*I* 2681; A) or 261-base pair (*Ava*I* 1645–*Xba*I 1905; B) DNA fragment, 2 μM/base of sonicated calf thymus DNA, 2 mM ManN and 20 μM $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 1 h at 37°C. The DNA fragments were treated with piperidine and analyzed by the method described in the legend to Figure 5. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). *Abscissa*, nucleotide number of the human c-Ha-*ras*-1 proto-oncogene starting with the *Bam*HI site.[36]

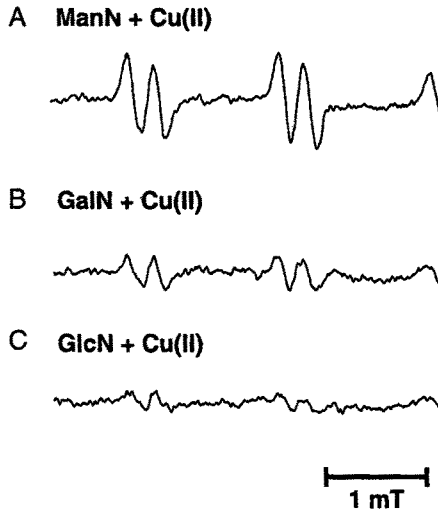

FIGURE 8 ESR spectra of the carbon-centered radical adduct of POBN produced by aminosugars plus Cu(II). The reaction mixtures, containing 10 mM aminosugar [ManN (A), GalN (B) or GlcN (C)], 100 mM POBN and 20 μM $CuCl_2$ in 50 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA, were incubated for 4 h at 37°C. The ESR spectra were measured immediately after the incubation.

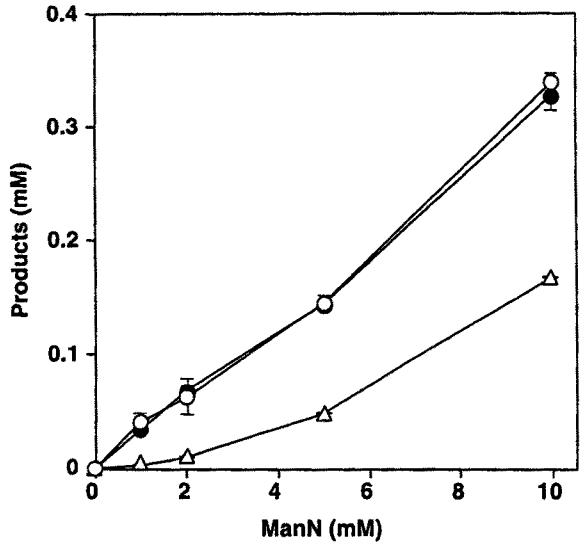

FIGURE 9 Production of dioxo compounds, NH_4^+ and pyrazine derivatives during the autoxidation of ManN. The reaction solution, containing ManN and 20 μM $CuCl_2$ in 50 mM sodium phosphate buffer (pH 7.8), was incubated for 4 h at 37°C. Production of dioxo compounds (○), NH_4^+ (●) and pyrazine derivatives (△) was assayed as described in "Experimental procedures". Values represent means ± SD of three independent experiments.

The generation of these products increased in a dose-dependent manner. The yield of the products from ManN was larger than the products from GalN and GlcN (data not shown). The amount of dioxo compounds was almost as large as that of NH_4^+. The ratio of pyrazine derivatives to aminosugars increased depending on the concentration of aminosugars, suggesting that the production of pyrazine derivatives is more important at higher concentrations of aminosugars. The pyrazine derivatives were identified with an HPLC equipped with a photodiode array. The spectrum of a product derived from ManN, eluting at 3.8 min, has a maximum absorption at 276 nm, suggesting the formation of a pyrazine derivative (data not shown). The order of the reactivity to form the pyrazine derivative was ManN ≫ GalN > GlcN. The eluent at 3.8 min, containing the pyrazine derivative, was analyzed with a mass spectrometer. Mass spectrum with molecular ion at m/e 321 (M^+) was obtained (data not shown), suggesting that 2,5-bis (tetrahydroxybutyl)pyrazine (fructosazine) was formed.

DISCUSSION

We investigated apoptosis and DNA damage induced by aminosugars to clarify the mechanism of their antitumor effects. ManN dramatically decreased the viability after the 2-day treatment. GalN also significantly inhibited cell growth and reduced viability, but to a lesser extent than ManN. GlcN had little or no cytotoxic effect under the condition used. The order of cytotoxic effects of aminosugars was ManN ≫ GalN > GlcN. The order of the frequency of apoptotic cells in which chromatin condensation and nuclear fragmentation were observed was the same. These results suggest that the cytotoxic effects of these aminosugars depend on their abilities to cause apoptosis. DNA ladder formation was observed when the cells were treated with ManN, whereas GalN and GlcN formed no DNA ladders under the

condition used. ManN formed DNA ladders at 4–5 mM, whereas cellular DNA strand breaks, detected by pulsed-field gel electrophoresis, were induced at 3 mM. The observation that ManN induced DNA strand breaks at a lower concentration than inducing apoptosis supports the idea that DNA damage precedes apoptosis. The formation of DNA ladders by ManN was inhibited by a caspase inhibitor Z-Asp-CH_2-DCB, indicating that caspases were involved in ManN-induced apoptosis. On the other hand, DNA strand breaks were not inhibited by this caspase inhibitor, suggesting that caspases do not play an important role in DNA cleavage to produce 1–2-Mb fragments. DNA strand breaks were inhibited by catalase and enhanced by 3-aminotriazol, a catalase inhibitor, indicating that H_2O_2 played a main role in causing cellular DNA damage. Flow cytometry showed that the order of the generation of intracellular peroxides was ManN $\gg$ GalN > GlcN. Little or no cytotoxic effect of ManN was observed in HP100 cells, HL60-derived cells in which catalase activity is much higher than that in HL60 cells.[29] On the basis of these results, it is speculated that ManN generates peroxides, mainly H_2O_2, causing cellular DNA damage, which is followed by the activation of caspases to mediate apoptotic cell death. However, DNA damage may not be the only trigger for ManN-induced apoptosis. Previous studies have suggested that ManN causes a cytotoxic effect by changing the membrane structure.[5,7] ManN has been reported to inhibit the biosynthesis of glycosylphosphatidylinositol that anchors membrane proteins.[8–10] Recently, it has been reported that changes in cell membrane trigger apoptosis.[45] Therefore, the possibility that alteration in membrane structure is implicated in ManN-induced apoptosis cannot be excluded.

Recently, it has been suggested that ROS contribute to apoptosis. ROS participate in both DNA damage-dependent and -independent pathways of apoptosis.[18–22] Some chemicals and UV radiation induced apoptosis through DNA damage by generating ROS.[24–26] Cell death in response to DNA damage in many instances has been shown to result from apoptosis.[15–17] These reports attract our interests to study whether ROS contribute to DNA damage-dependent mechanism of apoptosis induced by aminosugars.

To study the mechanism of cellular DNA damage, we investigated damage to DNA fragments obtained from the human c-Ha-*ras*-1 proto-oncogene. The order of the extent of DNA damage induced by aminosugars was ManN $\gg$ GalN > GlcN, when DNA was added after the reaction of an aminosugar with Cu(II). This suggests that ManN is more reactive in generating reactive species causing DNA damage than GalN and GlcN, as reported previously.[11,12] Aminosugars plus Cu(II) induced DNA damage at thymine, cytosine and guanine residues. Catalase and bathocuproine inhibited the DNA damage, indicating the involvement of H_2O_2 and Cu(I). Involvement of ROS in aminosugar-induced DNA damage has been suggested.[13] Typical $^{\bullet}$OH scavengers showed only weak inhibitory effects on the DNA damage, suggesting that $^{\bullet}$OH may not play an important role. The pattern of DNA cleavage induced by aminosugars is quite similar to that induced by H_2O_2 plus Cu(I).[46] This supports the involvement of reactive species generated from H_2O_2 plus Cu(I), other than $^{\bullet}$OH, which causes DNA cleavage at every nucleotide with little site specificity.[47,48] Recently, we have reported that in Fe(III)-dependent oxidative DNA damage, $^{\bullet}$OH generated through the Fenton reaction acts as the main reactive species, whereas Cu(II)-dependent DNA damage involves the formation of copper-peroxide complexes with a reactivity similar to $^{\bullet}$OH.[49] On the basis of these findings, it can be speculated that Cu(II) binds to DNA in a site-specific manner,[50,51] and is reduced to Cu(I), which reacts with H_2O_2 generated from aminosugars to form a DNA–metal-peroxide complex to release $^{\bullet}$OH, which immediately attacks an adjacent DNA constituent before being scavenged by $^{\bullet}$OH scavengers.

To clarify the mechanisms for H_2O_2 generation from aminosugars, we analyzed the aminosugar-derived products, dioxo compounds, NH_4^+, and pyrazine derivatives. ManN showed the highest reactivity to generate these products among the aminosugars tested. The amount of dioxo compounds was almost as large as that of NH_4^+, suggesting that NH_4^+ is released from ManN to produce dioxo compounds. A spectrum with a maximum absorption at 276 nm was obtained by the analysis using an HPLC equipped with a photodiode array, suggesting the formation of pyrazine derivatives through autocondensation. The analysis with mass spectrometry showed that the pyrazine derivative is fructosazine. ManN was more reactive to form fructosazine than other aminosugars as reported previously.[52,53] ESR spin-trapping method revealed that the order of the signal intensity was ManN ≫ GalN > GlcN, suggesting that the radical-generating capability of each aminosugar is consistent with the ability of generating H_2O_2 and the intensity of DNA damage.

On the basis of our results, we propose possible mechanisms of DNA damage induced by ManN plus Cu(II) as shown in Figure 10. The

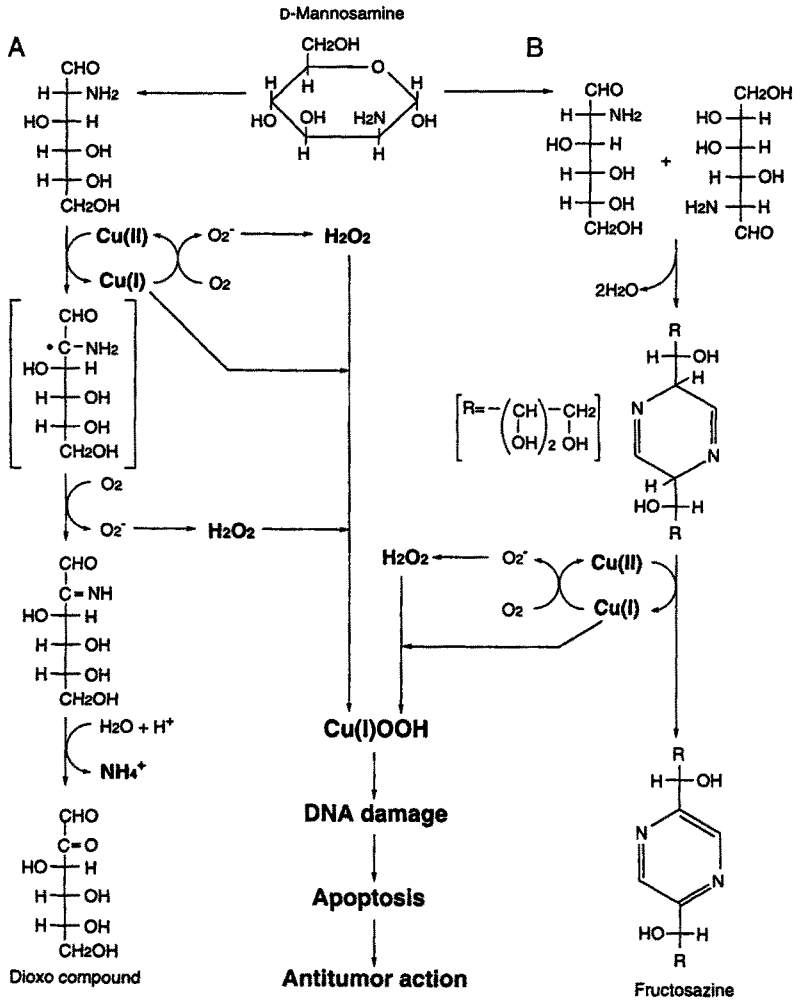

FIGURE 10 Proposed mechanisms of DNA damage and apoptosis induced by ManN in the presence of Cu(II).

mechanisms of DNA damage consist of two pathways. One of them is the major pathway in which dioxo compounds and NH_4^+ are generated (Figure 10A). ManN undergoes Cu(II)-mediated autoxidation into carbon-centered radicals, and Cu(II) is reduced to Cu(I) concomitantly. The radical formation was confirmed by the result of the ESR spin-trapping experiment. These radicals react with O_2 to generate O_2^-, which is dismutated to H_2O_2, followed by the formation of dioxo compounds and NH_4^+. The other is the minor pathway in which a pyrazine derivative fructosazine is formed through the condensation of ManN (Figure 10B). Two molecules of ManN condense through the amino-carbonyl reaction to form a dihydropyrazine derivative, which undergoes the autoxidation into fructosazine. H_2O_2 is generated during the autoxidation. ManN-induced DNA damage is caused by the interaction of Cu(I) and H_2O_2 to form a metal-oxygen complex, such as the Cu(I)-peroxide complex. The carbon-centered radical generated during Cu(II)-mediated autoxidation cannot be the primary species to react with DNA, because our results showed that DNA damage was not caused without H_2O_2 or Cu(I).

A number of antitumor drugs show cytotoxic effects and inhibit tumor growth. Inhibitory effects of these drugs on tumor growth are contributed by apoptosis.[15–17] Many antitumor drugs are capable of causing DNA cleavage. Stimuli capable of causing DNA damage induce apoptosis through the activation of cysteine proteases, caspases.[54–56] Selective activation of caspases is dependent on external stimuli, such as UV light and a number of chemical compounds.[54–58] These studies and our results suggest that the activation of caspases would be mediated by ManN-caused DNA damage. In apoptosis caused by DNA-damaging chemotherapy, activation of caspase-3 or caspase-3-like proteases play important roles.[59,60] Recently, we have reported that apoptosis mediated by oxidative DNA damage involves caspase-3 activation.[58] In this study, it is likely that ManN-treated cells undergo apoptosis through oxidative DNA damage and subsequent caspase-3 activation.

The present study showed that the orders of cytotoxic effects and the abilities to cause apoptosis and to generate intracellular peroxides were ManN $\gg$ GalN > GlcN. In addition, the orders of the reactivity to generate aminosugar-derived products and the extent of damage to isolated DNA were also ManN $\gg$ GalN > GlcN. These results suggest that the ability of each aminosugar to inhibit tumor growth depends on its reactivity to generate the products, such as dioxo compounds and fructosazine, and ROS during the autoxidation. Finally, it is concluded that aminosugars, especially ManN, produce H_2O_2 to cause DNA damage, which mediates apoptosis resulting in tumor growth inhibition.

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