A Role of Iron in λ DNA Strand Breaks in the Reaction System of Alloxan with Reduced Glutathione: Iron(III) Binding to the DNA

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λ DNA strand breaks were easily induced in a reaction system involving alloxan with reduced glutathione (GSH) in the presence of FeCl_3 in a HEPES–NaOH buffer, pH 7.4. Increasing concentrations of FeCl_3 in the reaction system caused DNA strand breaks in a concentration-dependent fashion, suggesting that iron is required to induce the DNA strand breaks. Catalase, scavengers of hydroxyl radicals ($\text{HO}\cdot$) and iron-chelators almost completely inhibited the DNA strand breaks, but superoxide dismutase (SOD) did not do so, suggesting that the $\text{HO}\cdot$, formed by a Fenton-type reaction, was the species responsible for the DNA strand breaks. The addition of FeCl_3 to the solution containing DNA caused the formation of a DNA–Fe(III) complex, in which Fe(III) was reduced by an alloxan radical ($\text{HA}\cdot$) but not by a superoxide radical. Only when apotransferrin was added to the reaction mixtures before the addition of FeCl_3 , were both the DNA strand breaks and the reduction of Fe(III) strongly inhibited. These results suggest that the Fe(III) bound to DNA catalyzes the DNA strand breaks which may be caused by the generation of site-specific $\text{HO}\cdot$ via an $\text{HA}\cdot$ -dependent Fenton-type reaction.

Keywords alloxan; reduced glutathione (GSH); DNA strand break; hydroxyl radical; DNA–iron(III) complex; alloxan radical

Alloxan is widely used in studies of experimental diabetes because this agent destroys pancreatic β -cells with a specific selectivity.^{1,2)} Since this cytotoxic action is inhibited by superoxide dismutase (SOD), catalase and several scavengers of hydroxyl radicals ($\text{HO}\cdot$), it has been proposed that the cytotoxicity of alloxan is mediated by some active oxygens.^{3–5)} Several workers have shown, with *in vitro* experimental systems using isolated pancreatic islets of rats, that the primary step of alloxan toxicity is the generation of $\text{HO}\cdot$ by which the DNA of pancreatic islets is attacked to produce strand breaks.^{6,7)} More recently, we showed that λ DNA strand breaks were easily induced by $\text{HO}\cdot$ generated via an alloxan radical ($\text{HA}\cdot$) through a Fenton-type reaction in the reaction system of alloxan with reduced glutathione (GSH) in the presence of Fe^{3+} -EDTA.⁸⁾

The involvement of iron in the alloxan-induced cytotoxicity has been indicated by several lines of studies, *in vivo*^{9,10)} and *in vitro*.^{5,11)} However, under physiological conditions, the concentration of free iron capable of inducing these toxicity is probably low, because iron is tightly controlled and regulated.¹²⁾ On the other hand, $\text{HO}\cdot$ can attack and destroy biomolecules such as DNA at diffusion-control rates.¹³⁾ These facts suggest that iron bound to the biomolecules will lead to the site-specific damage of the biomolecules. The present study shows that iron bound to DNA catalyzes the radical reaction that leads to DNA strand breaks in a reaction system of alloxan with GSH.

MATERIALS AND METHODS

Materials Alloxan, GSH, α,α' -dipyridyl, thiourea, FeCl_3 , sodium benzoate and disodium-1,2-dihydroxybenzene-3,5-disulfonate (Tiron) were purchased from Wako Pure Chemical Industries, Ltd., Japan. A solution of FeCl_3 was made up freshly just before use. Apotransferrin, transferrin (the iron saturation was ap-

proximately 98%), SOD (from bovine erythrocytes) and catalase (from bovine liver, thymol-free), were obtained from Sigma Co., St. Louis, MO, U.S.A. Apotransferrin was dissolved in 10 mM HEPES–NaOH buffer, pH 7.4, containing 5 mM NaHCO_3 . SOD and catalase were used without further purification. λ DNA (M.W. 31.5×10^6 Da, 48502 base pairs) was from Nippon Gene, Ltd., Japan; bathophenanthroline disulfonate from Dojindo Laboratories Co., Ltd.; dimethylthiourea (DMTU) from Aldrich Chemical Co., Inc.; diethylenetriaminepentaacetic acid (DETAPAC) from Kanto Chemical Co., Inc.; Sephadex G-50 DNA grade column from Pharmacia Co.; and desferrioxamine from CIBA Laboratories. Other reagents used in the experiments were of the highest grade.

Detection of DNA Strand Breaks To remove the Tris–HCl buffer and EDTA, the commercial λ DNA solution was dialyzed against sterilized 10 mM HEPES–NaOH buffer, pH 7.4, containing 0.15 M NaCl at 4 °C for 2 d with three changes of the same buffer. The reaction mixtures consisted of 1 mM alloxan, 2 mM GSH, 20 $\mu\text{g}/\text{ml}$ λ DNA and 10–100 μM FeCl_3 (nucleotide : iron = approximate 6 : 1–10) in 10 mM HEPES–NaOH buffer, pH 7.4, containing 0.15 M NaCl. The reactions were initiated by the addition of alloxan and then incubated for 10 min at 37 °C. The DNA strand breaks were observed as described previously by using the electrophoresis method,⁸⁾ and were photographed in ultraviolet (UV) light.

Binding of Iron to DNA The mixture (100 μl) containing 20 $\mu\text{g}/\text{ml}$ λ DNA and 40 μM FeCl_3 in 10 mM HEPES–NaOH buffer, pH 7.4, was applied to a Sephadex G-50 DNA grade column equilibrated with Tris–HCl buffer, pH 7.4. The column was eluted with Tris–HCl buffer, pH 7.4, and then eluted with the same buffer containing 1 mM Tiron. Each fraction of 400 μl was collected, and the concentrations of iron were measured with an atomic absorption flameless spectrophotometer (Hitachi, 180-80). The concentrations of DNA in the eluted fractions were determined by UV spectrophotometry using

an A_{260} ($E_{1\text{cm}}^{1\%} = 200$).

Assay of Iron Reduction The reduction of Fe(III) bound to DNA was measured as described previously.¹⁴⁾

Detection of Alloxan Radical The generation of $\text{HA}\cdot$ was observed as described previously by using an ESR (JEOL model JES-RE1X) at room temperature.¹⁴⁾ The relative intensity of $\text{HA}\cdot$ was determined by measuring the peak height at the center hyperfine line of the spectrum.

RESULTS

Effect of Iron on DNA Strand Breaks As shown in Fig. 1, under aerobic conditions, when λ DNA was incubated in the reaction system of alloxan with GSH in the presence of various concentrations of FeCl_3 , DNA strand breaks were caused in an iron concentration-dependent fashion. However, the DNA incubated with iron alone or without iron did not show any strand breaks. When alkaline sucrose density gradient centrifugation was used to observe the DNA strand breaks in the reaction system of alloxan with GSH in the presence of $40\text{ }\mu\text{M}$ of FeCl_3 , DNA strand breaks were observed as shown in the previous results.⁸⁾ These results indicate that the DNA strand breaks are easily induced in the reaction system in the presence of

Fig. 1. Effect of Iron Concentrations on DNA Strand Breaks in the Reaction System of Alloxan with GSH

The reaction mixture consisted of 1 mM alloxan, 2 mM GSH, 0.15 M NaCl, and various concentrations of FeCl_3 in 10 mM HEPES-NaOH buffer, pH 7.4. λ DNA ($20\text{ }\mu\text{g/ml}$) were incubated for 10 min at 37°C in the reaction mixture. The addition of FeCl_3 were as follows: track 1, $0\text{ }\mu\text{M}$; 2, $10\text{ }\mu\text{M}$; 3, $20\text{ }\mu\text{M}$; 4, $40\text{ }\mu\text{M}$; 5, $60\text{ }\mu\text{M}$; 6, $80\text{ }\mu\text{M}$; 7, $100\text{ }\mu\text{M}$.

FeCl_3 .

Effect of SOD, Catalase, $\text{HO}\cdot$ Scavengers and Iron-Chelators To clarify the participation of oxygen radicals in DNA strand breaks, we examined the effect of SOD, catalase, several $\text{HO}\cdot$ scavengers and iron-chelators. As shown in Fig. 2, catalase and $\text{HO}\cdot$ scavengers (thiourea, DMTU and benzoate) almost completely inhibited the DNA strand breaks, but SOD had little effect, suggesting the participation of H_2O_2 and $\text{HO}\cdot$ in DNA strand breaks.

On the other hand, desferrioxamine and DETAPAC as a ferric iron-chelator, and bathophenanthroline and α,α' -dipyridyl as a ferrous iron-chelator, strikingly inhibited the DNA strand breaks (tracks 8–11). These results suggest a possibility that both ferrous and ferric iron are involved in DNA strand breaks under the experimental conditions. The addition of a Fe(III)-binding protein, apotransferrin (4 mg/ml), which has $100\text{ }\mu\text{M}$ Fe binding capacity, to the reaction system did not inhibit the DNA strand breaks (track 7).

Iron Binding to DNA In order to confirm the interaction of Fe(III) with λ DNA, the sample containing Fe(III) and λ DNA was applied to a Sephadex G-50 DNA grade column. Studies of Fe(III) binding to calf thymus DNA by Sephadex chromatography have been reported previously.¹⁵⁾ As shown in Fig. 3, Fe(III) was eluted together with λ DNA at the void volume of the column. On the other hand, in the sample containing only Fe(III), Fe(III) was detected as a broad peak at around fraction number 9 only when the column was eluted with Tris-HCl buffer containing 1 mM Tiron. More than 99% of iron and λ DNA applied to the column were recovered in each fraction. These results indicate a strong interaction between Fe(III) and λ DNA to form a DNA-Fe(III) complex at pH 7.4. The insert of Fig. 3 shows that the added iron caused a linear increase in the formation of DNA-Fe(III) complex in a concentration-dependent fashion up to about $150\text{ }\mu\text{M}$.

Reduction of Fe(III) Bound to DNA Figure 4 shows the time course of the reduction of Fe(III) bound to λ DNA in the reaction system of alloxan with GSH. Fe(III) was rapidly reduced and the amount of ferrous iron increased with the time of incubation. Neither alloxan nor GSH alone reduced Fe(III) under the experimental conditions employed. The addition of SOD ($50\text{ }\mu\text{g/ml}$) to the reaction system had no significant effect, while catalase ($50\text{ }\mu\text{g/ml}$) brought about a slight increase in the reduction of Fe(III).

Fig. 2. Effect of SOD, Catalase, $\text{HO}\cdot$ Scavengers and Iron-Chelators on DNA Strand Breaks

The experimental conditions were the same as given in Fig. 1, except that the concentration of FeCl_3 was $40\text{ }\mu\text{M}$. Track 1, control; 2, $+50\text{ }\mu\text{g/ml}$ SOD; 3, $+50\text{ }\mu\text{g/ml}$ catalase; 4, $+10\text{ mM}$ thiourea; 5, $+10\text{ mM}$ DMTU; 6, $+10\text{ mM}$ benzoate; 7, $+4\text{ mg/ml}$ apotransferrin; 8, $+0.1\text{ mM}$ desferrioxamine; 9, $+0.1\text{ mM}$ DETAPAC; 10, $+0.1\text{ mM}$ bathophenanthroline; 11, $+0.7\text{ mM}$ α,α' -dipyridyl.

Fig. 3. Binding of Iron to DNA

After FeCl_3 ($40 \mu\text{M}$) was mixed with (○) or without (●) λDNA ($20 \mu\text{g/ml}$) in 10 mM HEPES–NaOH buffer, pH 7.4, each mixture was applied to a Sephadex G-50 DNA grade column. Each point represents the mean of triplicate experiments. The insert shows the binding of Fe(III) to λDNA in the reaction of $20 \mu\text{g/ml}$ of λDNA with various concentrations of FeCl_3 .

Fig. 4. Reduction of Fe(III) in the Reaction System of Alloxan with GSH in the Presence of DNA

(A) The reaction mixture consisted of 1 mM alloxan, 2 mM GSH, $40 \mu\text{M}$ FeCl_3 and 1 mM bathophenanthroline sulfonate in 10 mM HEPES–NaOH buffer, pH 7.4, in the presence of $20 \mu\text{g/ml}$ λDNA . ●, control; △, + $50 \mu\text{g/ml}$ SOD; ▲, + $50 \mu\text{g/ml}$ catalase.

These results suggest that O_2^- and H_2O_2 generated in the reaction system are not directly involved in the reduction of Fe(III) bound to DNA but H_2O_2 could reoxidize Fe(II) to Fe(III).

Generation of the Alloxan Radical As previously reported, the alloxan radical ($\text{HA}\cdot$) generated in the reaction system of alloxan with GSH could reductively release iron from ferritin.¹⁶⁾ This finding suggests the possible participation of $\text{HA}\cdot$ in the reduction of Fe(III) bound to DNA. As shown in Fig. 5a, the ESR spectrum of $\text{HA}\cdot$ obtained in the reaction system of alloxan with GSH in the presence of λDNA ($a_N = a_H = 0.45\text{G}$, $g = 2.005$) was similar to that reported in the previous paper.¹⁴⁾ When GSH was omitted from the reaction mixtures, no generation of $\text{HA}\cdot$ was observed (data not shown). The addition of the DNA–Fe(III) complex to the reaction system resulted in a marked decrease in the signal intensity of $\text{HA}\cdot$ depending on the concentrations of Fe(III). These

Fig. 5. Decrease in Alloxan Radical Generation by the Addition of DNA–Fe(III)

The reaction mixtures consisted of 1 mM alloxan and 0.2 mM GSH in 10 mM HEPES–NaOH buffer, pH 7.4, containing 0.15 M NaCl. λDNA $20 \mu\text{g/ml}$ (a), λDNA –Fe(III) $40 \mu\text{M}$ (as Fe) (b) and λDNA –Fe(III) $120 \mu\text{M}$ (as Fe) (c) were added to the reaction system, respectively, and then ESR was measured. Other conditions were as described in Material and Methods.

results indicate that $\text{HA}\cdot$ directly reacts with Fe(III) bound to DNA, which is easily reduced to Fe(II).

Effect of Apotransferrin The addition of apotransferrin to the reaction mixtures was unable to inhibit the DNA strand breaks (Fig. 2, track 7). This result was unexpected; therefore, further studies were performed on the effect of apotransferrin on the DNA strand breaks and the reduction of Fe(III). As shown in Fig. 6, when apotransferrin (4 mg/ml) was premixed with DNA immediately before the addition of FeCl_3 to the reaction mixtures, the DNA strand breaks were almost completely inhibited at FeCl_3 concentrations up to $60 \mu\text{M}$ (A). In contrast, when apotransferrin was added after the addition of FeCl_3 to the reaction mixtures, DNA strand breaks were scarcely observed at any concentration of FeCl_3 up to $120 \mu\text{M}$ (B).

As shown in Fig. 7, the initial rates of Fe(III)-reduction

Fig. 6. Effect of Apotransferrin on DNA Strand Breaks

Apotransferrin (4 mg/ml) was added to the reaction mixture before (A) or after (B) the addition of FeCl_3 at various concentrations. Other conditions were the same as described in Fig. 1. FeCl_3 concentrations were as follows: track 1, 0 μM ; 2, 40 μM ; 3, 60 μM ; 4, 100 μM ; 5, 120 μM .

Fig. 7. Effect of Apotransferrin on the Reduction of Fe(III) Bound to DNA

The experimental conditions were the same as given in Fig. 4, except for varying the concentration of FeCl_3 (●). Apotransferrin (4 mg/ml) was added to the reaction mixture before (□) or after (○) the addition of FeCl_3 .

in the reaction system increased with increasing concentrations of Fe(III) up to 120 μM . When apotransferrin (4 mg/ml) was premixed with DNA before the addition of Fe(III) , the rates of Fe(III) -reduction were markedly inhibited at all concentrations of FeCl_3 . But when apotransferrin was added after the addition of FeCl_3 , there was no effect. Although data were not shown, the reduction of Fe(III) bound to the transferrin (the iron-saturation was approximately 98%) was scarcely observed in the reaction system without DNA. These results suggest a possible requirement that Fe(III) be previously bound to DNA to produce DNA strand breaks, and that Fe(III) bound to apotransferrin, having a high affinity for Fe(III) , is inactive in promoting the DNA strand breaks.

DISCUSSION

It is generally believed that a highly reactive species such

as $\text{HO}\cdot$ is involved in cytotoxicity, and that a transition metal such as iron is strictly required in the generation of the active oxygens.^{17,18)} We reported previously that $\text{HO}\cdot$ and oxidizing species of iron generated in the GSH-alloxan system in the presence of Fe^{3+} -EDTA attacks DNA to produce strand breaks. However, under the experimental conditions used here, various $\text{HO}\cdot$ scavengers almost completely inhibited the DNA strand breaks (Fig. 2), suggesting that the strand breaks of λ DNA were induced by $\text{HO}\cdot$ generated in the reaction system of alloxan with GSH in the presence of FeCl_3 . In our previous works with the reaction system of alloxan with GSH in the presence of Fe^{3+} -EDTA, we suggested that $\text{HO}\cdot$ was generated by $\text{HA}\cdot$ through a Fenton-type reaction as follows¹⁴⁾:

Several studies have been carried out to determine what forms of iron are able to generate $\text{HO}\cdot$ under physiological conditions, demonstrating that iron ions bound to adenine nucleotide, citrate, adriamycin, bleomycin or deoxyribose could participate in Fenton-type reactions.^{19,20)} Iron bound to DNA has also been reported to be attributed to DNA damage mediated by the Fenton reaction.²¹⁾ In the present studies, the interaction between λ DNA and FeCl_3 to form a DNA- Fe(III) complex at pH 7.4 was demonstrated by column chromatography (Fig. 3), suggesting that the iron bound to DNA could play a role in DNA strand breaks in the reaction system of alloxan with GSH. Since the DNA strand breaks were inhibited by either ferric or ferrous iron-chelators (Fig. 2), the redox-cycling of iron bound to DNA appears to occur

during the generation of $\text{HO}\cdot$.

The reduction of Fe(III) bound to DNA could not be inhibited by the addition of SOD or catalase (Fig. 4), indicating that O_2^- or H_2O_2 are not involved in the reduction of Fe(III) bound to DNA. Moreover, the addition of a DNA- Fe(III) complex to the reaction system resulted in a decrease in the signal intensity of $\text{HA}\cdot$ depending on concentrations of Fe(III) bound to DNA (Figs. 4 and 5). In our previous works, $\text{HA}\cdot$ generated in the reaction system of alloxan with GSH directly reduces Fe^{3+} -EDTA to Fe^{2+} -EDTA⁸⁾ and reductively releases iron from ferritin.²²⁾ These results suggest that $\text{HA}\cdot$ directly reduces Fe(III) to Fe(II) . We have observed, although data are not shown, that the addition of ferrous iron (FeSO_4) to the solution of λ DNA in the presence of H_2O_2 causes the DNA strand breaks. Floyd has also shown that DNA binds to ferrous iron in a manner such that $\text{HO}\cdot$ formation from H_2O_2 is enhanced, as compared to the amount formed in the absence of DNA.²³⁾ Thus, the DNA strand breaks induced by alloxan may depend on the formation of a DNA- Fe(III) complex that catalyzes the site-specific production of $\text{OH}\cdot$ through the Fenton reaction; the target site to which Fe(III) are bound will tend to be preferentially attacked by $\text{HO}\cdot$.

The Fe(III) -binding proteins, apotransferrin or apolactoferrin, have two separate binding sites to which Fe(III) attaches tightly at physiological pH, preventing the reaction of iron-dependent $\text{HO}\cdot$ generation.²⁴⁾ The present study, however, demonstrated that apotransferrin did not inhibit DNA strand breaks when apotransferrin was added after the addition of FeCl_3 to the reaction system (Fig. 6). These results suggest that the apotransferrin was unable to remove iron from the DNA- Fe(III) complex previously formed under the experimental conditions, probably because apotransferrin ($\text{pI}=5.6$) negatively charged at physiological pH²⁵⁾ made it difficult to attain close proximity to the Fe(III) binding site of the DNA molecule. On the other hand, apotransferrin pre-mixed with DNA before the addition of FeCl_3 inhibited the DNA strand breaks (Fig. 6). The binding constant of an iron-DNA complex and transferrin is reported to be 2.1×10^{14} ¹⁵⁾ and 4.7×10^{20} ²⁵⁾ at pH 7.4, respectively. Therefore, most Fe(III) probably forms a complex with apotransferrin and not DNA. The results presented here suggest that Fe(III) bound to DNA could participate in the generation of $\text{HO}\cdot$ and lead to DNA strand breaks in the reaction system of alloxan with GSH.

Samuni *et al.* proposed a "site-specific" Fenton-type reaction in which the binding of a transition metal such as iron or copper to the target is a prerequisite for the production of $\text{HO}\cdot$.²⁶⁾ On the other hand, alloxan has

been known to induce DNA strand breaks to cause experimental diabetes mellitus²⁷⁾ which is protected by the administration of several iron-chelators.^{9,10)} These findings suggest that the diabetogenic action of alloxan may be initiated through the involvement of Fe(III) bound to DNA. The source of the iron is unknown; however, the rat pancreas has been reported to contain a significant amount of ferritin.²⁸⁾ We previously reported that the reactive iron was reductively released from ferritin by alloxan.²²⁾ Further studies are required to elucidate the exact mechanism of the diabetogenic action of alloxan.

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