Effect of Glutathione on λ Deoxyribonucleic Acid Strand Breaks in the Reaction System of Glutathione–Alloxan in the Presence of Fe^{3+}-Ethylenediaminetetraacetic Acid

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Alkaline sucrose density gradient and agarose gel electrophoresis methods were used to observe λ deoxyribonucleic acid (DNA) strand breaks by the reaction system of reduced glutathione (GSH) with alloxan in the presence of Fe^{3+}-ethylenediaminetetraacetic acid (EDTA). When DNA was incubated in the reaction system for 10 min, DNA strand breaks were easily induced. The increasing concentrations of GSH up to 1.0 mM in the reaction system in the presence of 1.0 mM alloxan caused DNA strand breaks in a concentration-dependent fashion and GSH beyond 2.0 mM caused in the strand breaks of DNA by which the fragments with multiple ranges of molecular weight were produced. The strand breaks of DNA in the reaction system containing low concentrations of GSH were protected by catalase and hydroxyl radical (HO·) scavengers but superoxide dismutase (SOD) did not, indicating that such breaks were induced by HO· generated from the Fenton reaction. On the other hand, the strand breaks of DNA at high concentrations of GSH were protected by ethanol and desferrioxamine, but not effectively by SOD and HO· scavengers, suggesting the possible participation of some oxidizing species of iron rather than HO·. These results indicate that HO· or oxidizing species of iron generated in the GSH–alloxan system depending on the concentration of GSH attacks DNA to produce strand breaks.

Keywords alloxan; glutathione; λ DNA strand break; iron; hydroxyl radical; oxidizing iron species; oxygen radical; Fe^{3+}-EDTA

Alloxan selectively damages pancreatic β-cells and thus causes diabetes mellitus in animals.[1–3] Since this effect is inhibited by superoxide dismutase (SOD),[3,4] catalase[3,5] and several scavengers of hydroxyl radical (HO·),[1,3,6] it has been proposed that the cytotoxicity of alloxan is mediated by a sequence of redox cyclic reaction of alloxan involving the production of HO·.[7] Glutathione (GSH) has been shown to play a role in the cyclic reduction of alloxan to dialuric acid,[8,9] suggesting the possibility that GSH, normally regarded as a cellular antioxidant, may promote the formation of oxidizing species, HO·, by facilitating cycling between alloxan and dialuric acid.

We previously demonstrated that GSH had a biphasic action on the generation of alloxan radical (HA·) and HO·, *i.e.* GSH at low concentrations caused a marked generation of HA· and HO·, however, the generation of both radicals were suppressed by increasing the concentration of GSH.[8,10] The cytotoxic effect of alloxan on pancreatic islets which underlies the biphasic action of GSH has not yet been clearly understood.

Several workers have shown with *in vitro* experimental system using isolated pancreatic islets of rats that the first step of alloxan toxicity is the generation of HO· by which the deoxyribonucleic acid (DNA) of pancreatic islets is attacked to produce strand breaks.[11] The present study using λ DNA as one of the targets for HO· is undertaken to clarify the diphasic action of GSH for DNA strand breaks in the GSH–alloxan system in the presence of Fe^{3+}-ethylenediaminetetraacetic acid (EDTA).

Experimental

Materials Alloxan, GSH and Fe^{3+}-EDTA were purchased from Wako Pure Chemical Industries, Ltd., Japan and λ DNA were from Nippon Gene Ltd., Japan. To remove Tris–HCl buffer and EDTA, commerical λ DNA solution was dialyzed against sterilized 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl at 4 °C. The dialysis was continued for 2 d with three changes of the same buffer. SOD (from bovine erythrocytes) and catalase (from bovine liver, thymole free) were from Simga Co., St. Louis, Mo. Thiourea, methylthiourea and dimethylthiourea were from Aldrich Chemical Co. and desferrioxamine was from CIBA Laboratories. All other chemicals used in this experiment were of analytical grade from commercial suppliers.

Reaction System The reaction system consited of 1.0 ml of sterilized 10 mM phosphate buffer, pH 7.4, 50 μg/ml λ DNA, 1.0 mM alloxan, 0.3 mM GSH, 100 μM Fe^{3+}-EDTA and 0.15 M NaCl. The reactions were initiated by the addition of alloxan and then incubated for 10 min at 37 °C.

Detection of DNA Strand Breaks Alkaline sucrose gradients contained 4.5 ml of 5 to 20% (w/v) sucrose in 0.3 N NaOH and 0.01 M EDTA. On the bottom of each gradient was a 0.6 ml 80% (w/v) sucrose layer. Samples (200 μl) containing 10 μg DNA were layered on the top of the gradient. After the centrifugation in a swing type RPS65T rotor (Hitachi 70 P-72 HIMAC) at 37000 rpm at 20 °C for 7 h, the gradient was collected from the bottom at 15 drops for a fraction. DNA in each fraction was precipitated by adding 2.0 ml of 20% trichloroacetic acid with 200 μg bovine serum albumin as carrier. The precipitation was washed three times with 20% trichloroacetic acid and assayed by a fluorometric method using 3,5-diaminobenzoic acid.[12]

Electrophoresis was done in horizonated slab gels containing 0.8% (w/v) agarose dissolved in electrophoresis buffer (40 mM Tris, 2 mM EDTA-2Na and 20 mM glacial acetic acid, pH 8.1). At about 30 min after the gel was poured, samples containing 0.1 μg DNA, 0.025% bromophenol blue and 0.5% glycerol were loaded. After electrophoresis was perfomed at 100 V for about 40 min at room temperature, the gels were soaked for 30 min in ethidium bromide solution (0.5 μg/ml) and photographed in ultraviolet light.

Assay of Fe^{3+}-EDTA Reduction The reduction of Fe^{3+}-EDTA was measured as described previously.[8] Fe^{3+}-EDTA (100 μM) was incubated in 10 mM phosphate buffer, pH 7.4, containing 1.0 mM alloxan, 0.15 M NaCl, various concentrations of GSH and 1.0 mM bathophenanthroline sulfate. The reaction was initiated by the addition of alloxan at 37 °C and continuously monitored at 534 nm. The amount of reduced iron was calculated from the increase in the absorption, based on a coefficient of $22140\,M^{-1}\cdot cm^{-1}$.[13]

Determination of H_2O_2 The generation of H_2O_2 was determined according to the method of Pobiner[14] with minor modifications. The reaction mixtures of 1.0 mM alloxan and various concentrations of GSH in the absence of Fe^{3+}-EDTA were incubated for 10 min at 37 °C. The reaction mixture (0.8 ml) was mixed with 0.25 ml of 20% (w/v) H_2SO_4 and 0.15 ml of 1.0 M $TiSO_4$. The absorbance at 408 nm was measured with a Hitachi model U-2000 spectrophotometer. The amount of produced H_2O_2 was calculated by using an extinction coefficient of $750\,M^{-1}\cdot cm^{-1}$.

Results

DNA Strand Breaks Alkaline sucrose density gradient

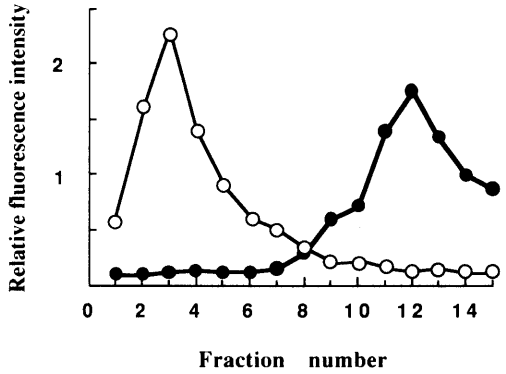

Fig. 1. DNA Strand Breaks in the GSH–Alloxan System in the Presence of Fe^{3+}-EDTA

The reaction system consisted of 1.0 mM alloxan, 0.3 mM GSH, 100 μM Fe^{3+}-EDTA in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. DNA incubated for 10 min at 37 °C in the reaction system was analyzed by an alkaline sucrose gradient as described in Experimental. Fluorescence intensity was measured at 508 nm (excitation was at 408 nm). Fractions are numbered from bottom to top. Each point represents the mean of 3 to 5 experiments.

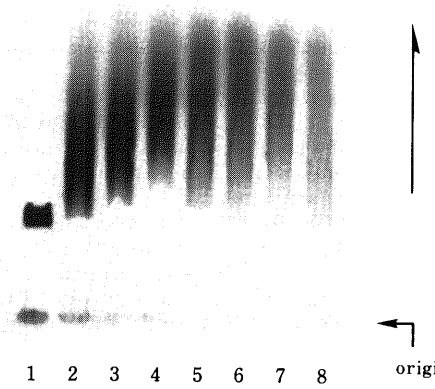

Fig. 2. Effect of Varying Concentrations of GSH on DNA Strand Breaks

The experimental conditions were the same as that in Fig. 1, except for varying the concentration of GSH. Other conditions were described in Experimental. Track 1, untreated DNA; 2, 0.3 mM; 3, 0.5 mM; 4, 1.0 mM; 5, 2.0 mM; 6, 3.0 mM; 7, 5.0 mM; 8, 10.0 mM of GSH.

centrifugation was used to observe DNA strand breaks in the GSH–alloxan system in the presence of Fe^{3+}-EDTA. As shown in Fig. 1, when DNA was incubated in the reaction system for 10 min, DNA was recoverd as a broad peak near the top of the gradient. When DNA was incubated for 0 min, a single peak near the bottom of the gradient was observed at the position of untreated DNA. The DNA incubated without alloxan, GSH or Fe^{3+}-EDTA for 10 min was recovered as a single peak near the bottom of the gradient, the position at which undamaged DNA segmented (data not shown). These results indicate that the DNA strand breaks are easily induced in the GSH–alloxan system in the presence of Fe^{3+}-EDTA.

Effect of GSH-Concentrations Figure 2 shows the effect of varying concentrations of GSH on DNA strand breaks by using gel elecrophoresis on agarose gel. The increasing concentrations of GSH up to 1.0 mM in the reaction system in the presence of 1.0 mM alloxan caused DNA strand breaks in a concentration-dependent fashion. However, high concentrations of GSH beyond 2.0 mM caused DNA strand breaks independently on GSH concentrations. These

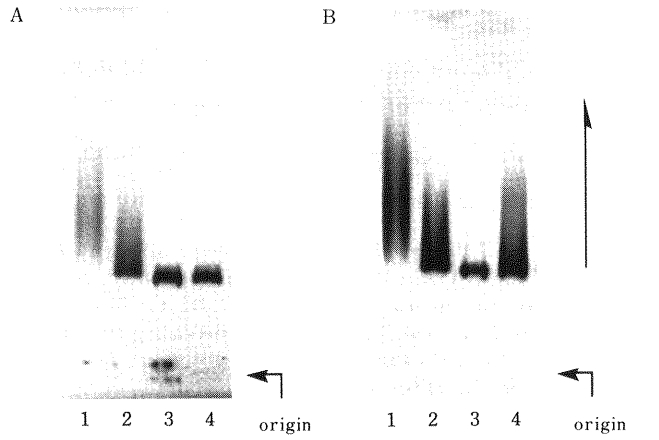

Fig. 3. Effect of SOD, Catalase and HO· Scavenger on DNA Strand Breaks

The reaction system consisted of 1.0 mM alloxan and 100 μM Fe^{3+}-EDTA in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl in the presence of GSH at 0.3 mM (A) and 10.0 mM (B). Other conditions were described in Experimental. Track 1, complete reaction system; 2, +100 U/ml SOD, 3, +50 U/ml catalase; 4, +30 mM thiourea.

results suggest the possibility that GSH at low and high concentrations cause the DNA strand breaks, respectively, in a different manner.

Effect of SOD, Catalase and HO· Scavengers We have previously demonstrated that the generation of HO· in the GSH–alloxan system was inhibited by catalase and HO· scavengers but not by SOD.[8] Therefore, we investigated the possible participation of O_2^-, H_2O_2 and HO· in DNA strand breaks. As shown in Fig. 3A, in the reaction system containing a low concentration of GSH (0.3 mM), catalase (50 U/ml) and thiourea (30 mM), an effective HO· scavenger, almost completely inhibited the DNA strand breaks, however, SOD (100 U/ml) had little effect. Such DNA strand breaks were also strikingly inhibited by other HO· scavengers (30 mM) such as mannitol, benzoate, methylthiourea, dimethylthiourea and dimethyl sulfoxide (data not shown). These results suggest the possible involvement of HO· in the DNA strand breaks in the reaction system in the presence of 0.3 mM GSH.

As can been seen in Fig. 3B, in the reaction system containing a high concentration of GSH (10 mM), catalase (50 U/ml) markedly inhibited the DNA strand breaks but SOD (100 U/ml) and thiourea (30 mM) did not. Moreover, other HO· scavengers employed in this experiment did not inhibit the DNA strand breaks. When DNA was exposed directly to 80 μM H_2O_2, little or no DNA strand breaks were observed (data not shown). These results suggest the possibility that oxygen radicals other than H_2O_2 or HO· may be involved in the DNA strand breaks in the presence of high concentrations of GSH.

Inhibitory Effect of Ethanol and Desferrioxamine Several workers[15] have suggested that an oxidizing iron intermediate is formed in the reaction of Fe^{2+} and H_2O_2. More recently, Yamazaki and Piette[16] have suggested that the oxidizing species of iron which might be assigned as ferryl, FeO_2^+ or Fe(IV)=0 are generated in the Fenton reaction, particularly at high Fe^{2+} concentrations. On the other hand, the oxidizing species of iron is known to efficiently react with ethanol.[15-18] Therefore, we examined the effect of ethanol and desferrioxamine on the

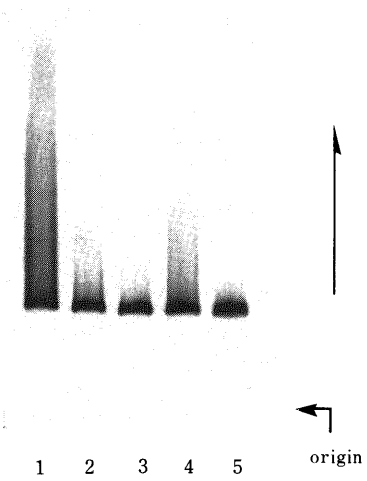

Fig. 4. Effect of Ethanol and Desferrioxamine on DNA Strand Breaks

The experimental conditions were the same as that in Fig. 1, except for the presence of 10.0 mM GSH. Other conditions were described in Experimental. Track 1, complete reaction system; 2, +30 mM ethanol; 3, +70 mM ethanol; 4, +1.0 mM desferrioxamine; 5, +5.0 mM desferrioxamine.

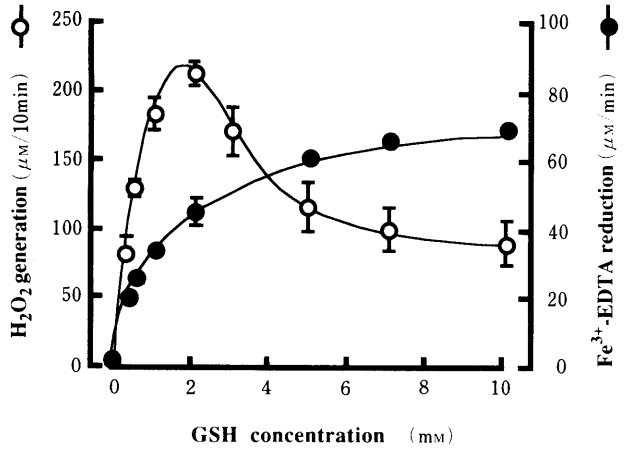

Fig. 5. Generation of H_2O_2 and Reduction of Fe^{3+}-EDTA in the Reaction of GSH with Alloxan

The reaction system consisted of 1.0 mM alloxan, 0.3 mM GSH in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. The generation of H_2O_2 and the reduction of Fe^{3+}-EDTA were measured as described in Experimental. Each point represents the mean ± S.E. of triplicate experiments.

DNA strand breaks to elucidate the possible involvement of the oxidizing species of iron. As shown in Fig. 4, the DNA strand breaks in the reaction of alloxan with 10 mM GSH were strongly protected by ethanol and desferrioxamine in a concentration-dependent fashion, suggesting the possibility that DNA strand breaks in the presence of high concentrations of GSH are mediated by the oxidizing species of iron.

Generation of H_2O_2 and Reduction of Fe^{3+}-EDTA Figure 5 shows the generation of H_2O_2 and the reduction of Fe^{3+}-EDTA in the GSH–alloxan system in the presence of various concentrations of GSH. The amount of H_2O_2 generated in response to GSH revealed a biphasic profile, *i.e.* the addition of GSH up to 2.0 mM caused a marked increase and increasing GSH beyond 2.0 mM caused a decrease in the generation of H_2O_2. On the other hand, the rates of Fe^{3+}-EDTA reduction increased with increasing concentrations of GSH up to 10.0 mM. The reduction of Fe^{3+}-EDTA was scarcely observed under the experimental conditions in the presence of desferrioxamine (5 mM) (data not shown). These result indicate that the ratio of $[H_2O_2]/[Fe^{2+}$-EDTA] varies depending on the concentrations of GSH in the reaction system.

Discussion

Diabetogenic action of alloxan is thought to be initiated by the generation of HO·[2,3] and induced DNA strand breaks in isolated pancreas islet.[11] The present study demonstrated that in the GSH–alloxan system containing a low concentration of GSH (0.3 mM) in the presence of Fe^{3+}-EDTA, DNA strand breaks were strongly inhibited by various HO· scavengers (Fig. 3A). Little DNA strand breaks were observed in the GSH (0.3 mM)–alloxan system substituting the phosphate buffer by 30 mM Tris–HCl buffer, pH 7.4 (data not shown). It has been reported that Tris is a powerful scavenger of HO·.[19,20] These results indicate that HO· acts as an active species for DNA strand breaks at low conentrations of GSH. In general, HO· is known to be generated *via* the iron-catalyzed Haber–Weiss reaction[7] as follows:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^- \quad (2)$$

The reaction is essentially O_2^--dependent, so SOD should inhibit the generation of HO·. However, the strand breaks of DNA in the GSH–alloxan system in the presence of low concentrations of GSH were strongly inhibited by catalase, but not effectively by SOD (Fig. 3A). Moreover, the marked generation of H_2O_2 and reduction of Fe^{3+}-EDTA were observed in the GSH–alloxan system in the presence of a low concentration of GSH (Fig. 5). These results suggest that the DNA strand breaks are induced by HO· generated *via* the reaction of H_2O_2 with Fe^{2+}-EDTA, namely, the Fenton reaction (reaction 2)[21] but not *via* the Haber–Weiss reaction.[7]

We reported previously that HO· was generated in the reaction of low concentrations of GSH with alloxan in the presence of Fe^{3+}-EDTA through the Fenton reaction, in which Fe^{3+}-EDTA was reduced by the alloxan radical (AH·),[8] but not in the reaction system containing high concnetrations of GSH.[10] In this paper, however, we showed that DNA strand breaks were induced in the reaction of high concentrations of GSH with alloxan in the presence of Fe^{3+}-EDTA. Such DNA strand breaks were inhibited by catalase but not effectively by SOD and HO· scavenger (Fig. 3B). DNA strand breaks were clearly observed in the GSH–alloxan system in 30 mM Tris–HCl buffer, pH 7.4, only in high concentratios of GSH (data not shown). These results suggest that DNA strand breaks at high concentrations of GSH are induced by certain active oxygens other than HO·. Moreover, desferrioxamine, an iron-chelating agent, also inhibited DNA strand breaks (Fig. 4) and Fe^{3+}-EDTA reduction to produce Fe^{2+}-EDTA (data not shown), suggesting the possible participation of Fe^{2+}-EDTA in such DNA strand breaks. Yamazaki and Piette[16] have demonstrated on the nature of the Fenton reaction that as the Fe^{2+} concentrations are increased, the efficiency of HO· formation decreases drastically while the oxidizing species of iron which might

be assigned as ferryl, FeO_2^+ or Fe(IV)=0 predominate. The present study showed that the rate of Fe^{3+}EDTA reduction increased with increasing concentrations of GSH up to 10 mM in the GSH–alloxan system while H_2O_2 generation decreased with increased GSH beyond 2.0 mM (Fig. 5), indicating that the radio of $[H_2O_2]/[Fe^{2+}$-EDTA] varied depending on the concentration of GSH. From these results, we presumed that the oxidizing species of iron were generated in the GSH–alloxan system in the presence of high concentrations of GSH. DNA strand breaks at high concentrations of GSH were protected by ethanol (Fig. 4) which is known to react with oxidizing species of iron,[16-18] suggesting the possible participation of oxidizing species of iron in DNA strand breaks. However, the chemical nature of oxidizing species of iron and the exact mechanism of its generation has not been revealed because of its high reactivity.

GSH is known to protect against tissues damaged by oxygen radicals. For example, GSH can scavenge HO·[8,22] and plays a key role in scavenging oxygen radicals in lipid peroxidation.[23] The results presented here showed *in vitro* that GSH enhanced the DNA strand breaks. Uchiagata *et al.*[11] have also demonstrated that the co-existance of GSH with alloxan results in more extensive islet DNA strand breaks than that induced by alloxan alone. Further, GSH directly induces the one-electron reduction of quinone to semiquinone radicals which lead to the formation of various active oxygens.[24] As previously reported, GSH reduces alloxan, which is a quinoidal compound, to AH· or dialuric acid, and these two reduced forms of alloxan react rapidly with dioxygen and Fe^{3+}-EDTA to form active oxygen and Fe^{2+}-EDTA.[8,25] The results present here show that HO· or oxidizing species of iron induce DNA strand breaks depending on the concentrations of GSH. The diabetogenic action of alloxan has been reported to be protected by the injection of GSH to mice,[26] although the exact protective mechanism of GSH *in vivo* is not fully understood. The present paper serves to delineate the reaction of DNA with active oxygens generated from the reaction of alloxan with GSH and should assist to clarify the mode of action of this diabetogenic agent.

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