DNA cleavage induced by glycation of Cu,Zn-superoxide dismutase

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Human Cu,Zn-superoxide dismutase (Cu,Zn-SOD) undergoes site-specific and random fragmentation by non-enzymic glycosylation (glycation). Released Cu^{2+} from the glycated Cu,Zn-SOD probably facilitates a Fenton reaction to convert H_2O_2 into hydroxy radical, which then participates in the non-specific fragmentation [Ookawara et al. (1992) J. Biol. Chem. **267**, 18505–18510]. In the present study, we investigated the effects of glycated Cu,Zn-SOD on cloned DNA fragments and nuclear DNA and analysed the formation of 8-hydroxydeoxyguanosine (8-OH-dG). Incubation of cloned DNA fragments with Cu,Zn-SOD and reducing sugars resulted in cleavage of the DNA. The extent of the cleavage corresponded to the reducing capacity of the sugar. Metal-chelating reagents, EDTA and bathocuproine, and an H_2O_2 scavenger, catalase, inhibited the DNA cleavage. Hydroxy radical scavengers and aminoguanidine, an inhibitor of glycation, also inhibited the reaction. Moreover, the glycation of Cu,Zn-SOD caused the substantial formation of 8-OH-dG in DNA. When isolated nuclei were incubated with $CuCl_2$ plus H_2O_2, nuclear DNA cleavage was observed. Incubation of isolated nuclei with Cu,Zn-SOD that had been pre-incubated with glucose also resulted in nuclear DNA cleavage. These results suggest that hydroxy radical is produced through a Fenton reaction by Cu^{2+} and H_2O_2 released from the glycated Cu,Zn-SOD, and participates in nuclear DNA cleavage. This mechanism may partly explain the deterioration of organs under diabetic conditions.

INTRODUCTION

Involvement of the glycation reaction in the pathogenesis of diabetic complications has been suggested [1,2]. Several groups have reported that Schiff base or Amadori products generate reactive oxygen species [3–5]. Brownlee's group [3] described a possible connection between the production of superoxide anion by the glycation of protein and atherogenesis or diabetic complications. Transition metals, especially Cu^{2+} and Fe^{3+}, have also been implicated in the production of reactive oxygen species [4–6].

Cu,Zn-superoxide dismutase (Cu,Zn-SOD) is an anti-oxidant enzyme and is fairly abundant in most tissues. Levels of glycated Cu,Zn-SOD are increased in patients with diabetes mellitus [7,8]. Our previous studies have indicated that human Cu,Zn-SOD is inactivated by glycation at specific lysine residues, Lys^{122} and Lys^{128} [9], and that glycation of Cu,Zn-SOD initially brings about site-specific cleavage of the molecule between Pro^{62} and His^{63} followed by random fragmentation [10]. We speculated that reactive oxygen species produced from the glycated protein are involved in both fragmentation steps.

Recently, the significance of reactive oxygen species in damage to many biological molecules including DNA has drawn much attention. Cleavage of purified DNA has been efficiently induced by direct treatment with several kinds of metals plus H_2O_2 [11–15]. DNA-strand breaks and DNA fragmentation induced by alloxan in pancreatic islet cells seem to play an important role in the development of diabetes [16]. This DNA fragmentation is also thought to result from the accumulation of reactive oxygen species produced directly from alloxan [17,18]. Culture of human endothelial cells in a high-glucose medium has resulted in nuclear DNA damage [19]; several reducing sugars and advanced glycation end-products have been shown to have mutagenic effects [20–24]. 8-Hydroxydeoxyguanosine (8-OH-dG) is considered to be one of the oxidative DNA products induced by reactive oxygen species [25]. It has drawn much attention that the formation of 8-OH-dG causes misreplication of DNA that might lead to mutation [26,27].

In the present study, we evaluated the potential roles in DNA damage of products resulting from glycation of Cu,Zn-SOD. We found that both cloned DNA and nuclear DNA were cleaved by incubation with glycated Cu,Zn-SOD, and that 8-OH-dG was formed during the process. This is the first report that DNA fragmentation and 8-OH-dG formation are induced by the glycation of a metal-containing protein.

MATERIALS AND METHODS

Materials

Recombinant human Cu,Zn-SOD was a kind gift from Ube Industries, Japan. The amino acid sequence of the recombinant enzyme is the same as that of the human erythrocyte enzyme except that the N-terminus Ala is not acetylated. Restriction enzymes (*Ava*I, *Bam*HI, *Sal*I), T_4 polynucleotide kinase, and calf intestine alkaline phosphatase were supplied by Toyobo Biochemicals. [γ-^{32}P]ATP (3000 Ci/mmol) was supplied by Amersham. Human myelogenous leukaemia cell line K562 and hamster pancreatic β-cell line HIT were obtained from the Japanese Cancer Research Bank and the American Type Culture Collection respectively. Bovine liver catalase and proteinase K were purchased from Boehringer. D-Glucose, D-fructose, D-sorbitol, EDTA, dimethyl sulphoxide (DMSO), and aminoguanidine were purchased from Wako Chemicals. Bathocuproine, mannitol, ethanol, methionine, sodium formate, Nonidet P-40 (NP-40),

Abbreviations used: Cu,Zn-SOD, Cu,Zn-superoxide dismutase; DMSO, dimethyl sulphoxide; F-6-P, fructose 6-phosphate; F-1,6-P_2, fructose 1,6-bisphosphate; G-6-P, glucose 6-phosphate; PBS, phosphate-buffered saline; 8-OH-dG, 8-hydroxydeoxyguanosine.

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and SDS were purchased from Nacalai Chemicals. BSA, glucose 6-phosphate (G-6-P), fructose 6-phosphate (F-6-P), fructose 1,6-bisphosphate (F-1,6-P_2), ribose, ADP-ribose, and RNAase A were purchased from Sigma. A disposable cellulose-nitrate filter, DISMIC, was purchased from Advantec. Other reagents were of the highest grade available. In all glycation experiments, potassium phosphate buffer and PBS were autoclaved and all solutions were sterilized by filtration through a DISMIC filter.

Collection of DNA samples

Cloned Mn-SOD genomic DNA was ligated into a *Sal*I/*Bam*HI-site in Bluescript II plasmid and then transferred to *Escherichia coli* JM109. Amplification of the plasmid was carried out in Luria–Bertani medium. Closed circular DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

Preparation of ^{32}P 5′ end-labelled DNA fragments

The plasmid Bluescript II was digested with *Ava*I, and the resulting DNA fragments were fractionated by electrophoresis on 5% (w/v) acrylamide gels. A 743 bp DNA fragment and a 570 bp DNA fragment were obtained. These DNA fragments were dephosphorylated with calf intestine alkaline phosphatase and labelled at the 5′ termini with [γ-^{32}P]ATP and T_4 polynucleotide kinase.

Analysis of DNA fragmentation induced by glycation of Cu,Zn-SOD

The standard reaction mixture contained 100 mM reducing sugar, 1 mg/ml Cu,Zn-SOD, and ^{32}P-labelled DNA fragments (0.6 pmol) in 20 μl of 50 mM potassium phosphate buffer and 0.025% (w/v) NaN_3, pH 7.4. After incubation at 37 °C for 1–5 days, the reaction was terminated by adding a stop solution [95% (w/v) formamide, 20 mM EDTA, 0.05% (w/v) Bromophenol Blue, and 0.05% (w/v) xylene cyanol] and the samples were boiled for 2 min. DNA fragments were electrophoresed on 5% polyacrylamide gel containing 50% (w/v) urea. The gels were dried and exposed to X-ray film (Kodak, XAR) at −80 °C overnight. The total amount of single-stranded and double-stranded forms of DNA fragment was evaluated by densitometry to determine the percentage of remaining DNA. (The total amount of both DNA forms in control was regarded as 100% level of remaining DNA.)

Cell lines and culture conditions

K562 cells, human myelogenous leukaemia cells, and HIT cells, hamster pancreatic β-cells, were grown in RPMI 1640 medium (Nikken Biomedical Laboratories) supplemented with 10% (v/v) foetal calf serum (Whittaker M. A. Bioproducts), 100 i.u./ml penicillin, and 0.1 mg/ml streptomycin sulphate in a humidified atmosphere of 5% CO_2 at 37 °C. Exponentially growing cells were used throughout the experiments at a concentration of 5×10^5 cells/ml.

Isolation of nuclei

The K562 cells and HIT cells were collected by centrifugation at 120 *g* for 5 min and washed twice with PBS (0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The cells were suspended in lysis buffer [10 mM Tris/HCl (pH 7.4) 10 mM NaCl/3 mM $MgCl_2$/0.5% Nonidet P-40] and incubated on ice for 10 min. This process was carried out twice. The resulting nuclei were washed in PBS twice and then pelleted by centrifugation at 120 *g* for 5 min.

Incubation of nuclei

The standard reaction mixture contained 20 μM $CuCl_2$ or 1 mM H_2O_2 in 500 μl of PBS and 0.025% NaN_3. After incubation of nuclei at 37 °C for 1 h, nuclear DNA was extracted and examined. In the experiments involving Cu,Zn-SOD and glucose, nuclei were incubated at 37 °C for 3 h in a solution that had been pre-incubated with 1 mg/ml Cu,Zn-SOD and/or 100 mM glucose in 500 μl of PBS and 0.025% NaN_3 for 10 days. According to our previous studies, after 10 days incubation of 1 mg/ml Cu,Zn-SOD with 100 mM glucose, random fragmentation of Cu,Zn-SOD occurs, presumably caused by the production of hydroxy radical [9].

Analysis of nuclear DNA cleavage

After incubation, nuclei were lysed by addition of a nuclei lysis buffer [0.2 M Tris/HCl (pH 8.0) 0.1 M Na_2EDTA/1% SDS/100 μg/ml proteinase K] and incubated for 4 h at 55 °C. The nuclear lysates were extracted twice with phenol and then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol). DNA was precipitated with 0.05 vol. of 5 M NaCl and 2.5 vol. of absolute ethanol overnight at −35 °C, and sedimented at 500 *g* for 10 min at 4 °C. The DNA pellet was dried and dissolved in 10 mM Tris/HCl (pH 7.4)/1 mM EDTA, containing 1 mg/ml RNAase A and incubated for 1 h at 37 °C. The DNA was finally extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). DNA samples were analysed by electrophoresis on 1.5% agarose gels, and the results were visualized by staining with 1 μg/ml ethidium bromide. *Pst* I-digested λ DNA was used as molecular-mass standards.

Analysis of 8-OH-dG in DNA formed by glycation of Cu,Zn-SOD

Bluescript II plasmid containing Mn-SOD genomic DNA (20 pmol) was incubated with 1 mg/ml Cu,Zn-SOD plus 100 mM glucose in 50 mM potassium phosphate buffer and 0.025% NaN_3, pH 7.4, for 3–10 days. After incubation, the DNA was separated by ethanol precipitation, digested to deoxynucleosides by treatment with nuclease P1 and alkaline phosphatase, and analysed by an h.p.l.c. electrochemical detector (e.c.d.) system. Digestion conditions, the h.p.l.c. analysis and the method of 8-OH-dG quantification were the same as previously reported [28].

RESULTS

Cleavage of a ^{32}P-labelled DNA fragment induced by Cu,Zn-SOD plus glucose

The extent of DNA damage induced by incubation with sugars and proteins was estimated by gel electrophoretic analysis. Figure 1 shows the results of incubating a labelled double-stranded DNA fragment (0.6 pmol) with glucose, BSA, Cu,Zn-SOD, BSA plus glucose, or Cu,Zn-SOD plus glucose for 5 days at 37 °C. The upper band and lower band in the control (lane 1) show double-stranded and single-stranded forms of the DNA fragment respectively. Substantial DNA cleavage was observed only after incubation with Cu,Zn-SOD plus glucose; glucose, BSA, or

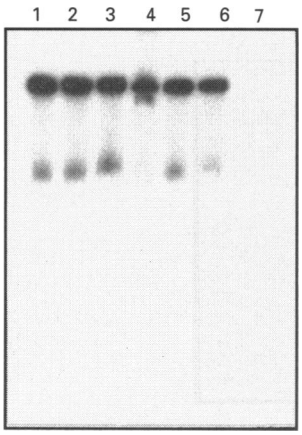

Figure 1 Autoradiogram of ^{32}P-labelled DNA fragments incubated with glucose, BSA, or Cu,Zn-SOD

The reaction mixture contained the ^{32}P-5′-end-labelled 743 bp DNA fragment (0.6 pmol) in 20 μl of 50 mM potassium phosphate buffer, pH 7.4. After incubation with the reagents described below at 37 °C for 5 days, the DNA fragments were electrophoresed on a 5% polyacrylamide, 50% urea gel, and an autoradiogram was obtained by exposing X-ray film to the dried gel. Lane 1, DNA kept at −20 °C; lane 2, DNA alone; lane 3, 100 mM glucose; lane 4, 1 mg/ml BSA; lane 5, 1 mg/ml Cu,Zn-SOD; lane 6, 1 mg/ml BSA plus 100 mM glucose; lane 7, 1 mg/ml Cu,Zn-SOD plus 100 mM glucose.

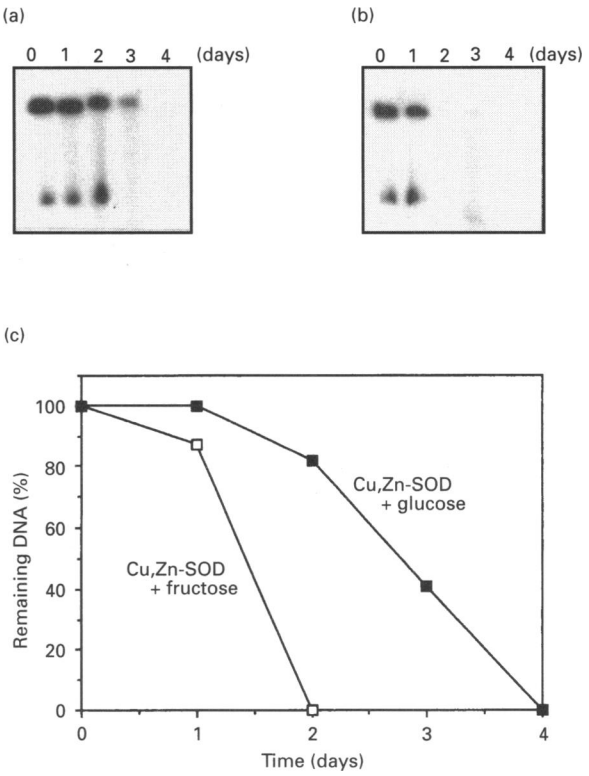

Figure 2 Time course of DNA cleavage induced by glycation of Cu,Zn-SOD by glucose (a) or fructose (b)

The ^{32}P-5′-end-labelled 570 bp DNA fragment (0.6 pmol) was incubated with 1 mg/ml Cu,Zn-SOD plus 100 mM glucose (**a**) or 100 mM fructose (**b**) at 37 °C for the indicated durations, and analysed by the method described in the legend to Figure 1. The extent of DNA damage was evaluated by densitometry (**c**). The total amount of single-stranded and double-stranded forms of DNA fragment was evaluated by densitometry to determine the percentage of remaining DNA. (The total amount of both DNA forms in control was regarded as 100% level of remaining DNA.)

Cu,Zn-SOD alone had no effect. The cleavage occurred without alkali treatment, suggesting breakage at the deoxyribose–phosphate backbone. The extent of DNA cleavage increased with time (Figures 2a and 2c) and with increasing concentrations of Cu,Zn-SOD (Table 1) and glucose (Table 2). As fructose has a much greater reducing capacity than glucose in the Maillard reaction, the effect of fructose on DNA cleavage was also studied. Incubation with Cu,Zn-SOD and fructose instead of glucose resulted in more rapid and marked DNA cleavage (Figures 2b and 2c). All these data are consistent with the idea that DNA cleavage was induced by glycation of Cu,Zn-SOD.

DNA damage induced by Cu,Zn-SOD plus various kinds of sugars

Figures 3(a) and 3(b) show a comparison of DNA cleavage induced by Cu,Zn-SOD plus various sugars. Incubation with any reducing sugar alone did not result in notable DNA damage; but DNA was undetectable after 3 days of incubation in medium containing Cu,Zn-SOD and F-6-P or fructose, which have strong reducing capacities. Other sugar phosphates, G-6-P and F-1,6-P_2, caused substantial degradation of the DNA. Incubation with the non-reducing sugar, sorbitol, however, had no effect. These results indicated that the DNA cleavage was dependent on the reducing capacity of the sugar. DNA cleavage induced by

Table 1 Effects of Cu,Zn-SOD concentration on DNA cleavage

The ^{32}P-5′-end-labelled 570 bp DNA fragment (0.6 pmol) was incubated at 37 °C for 5 days with 100 mM glucose plus different concentrations of Cu,Zn-SOD and analysed by the method described in the legend to Figure 1. The extent of DNA damage was evaluated by densitometry. The percentage of remaining DNA was determined as described in the legend to Figure 2.

Cu,Zn-SOD (mg/ml)	Remaining DNA (%)
0	100
0.05	60
0.2	46
1	0

Table 2 Effects of glucose concentration on DNA cleavage

The ^{32}P-5′-end-labelled 570 bp DNA fragment (0.6 pmol) was incubated at 37 °C for 5 days with 1 mg/ml Cu,Zn-SOD plus different concentrations of glucose, and analysed by the method described in the legend to Figure 1. The extent of DNA damage was evaluated by densitometry. The percentage of remaining DNA was determined as described in the legend to Figure 2.

Glucose (mM)	Remaining DNA (%)
0	100
5	94
20	55
100	0

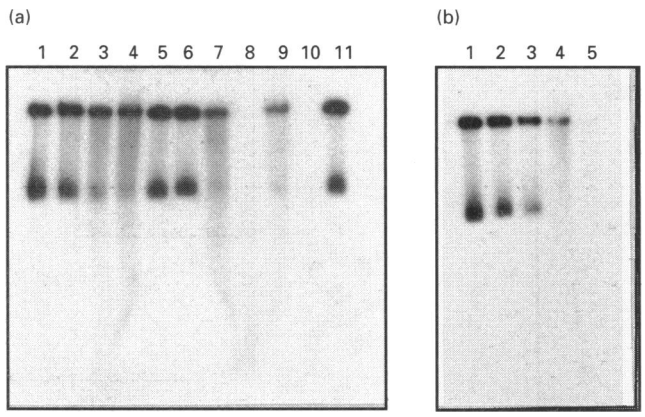

Figure 3 DNA cleavage induced by Cu,Zn-SOD plus various sugars

(**a**) the ^{32}P-5′-end-labelled 743 bp DNA fragment (0.6 pmol) was incubated with 1 mg/ml Cu,Zn-SOD plus 100 mM sugar as described below at 37 °C for 3 days and analysed by the method described in the legend to Figure 1. Lane 1, control; lane 2, 100 mM G-6-P; lane 3, 100 mM F-6-P; lane 4, 100 mM F-1,6-P_2; lane 5, 100 mM fructose; lane 6, 100 mM sorbitol; lane 7, 1 mg/ml Cu,Zn-SOD plus 100 mM G-6-P; lane 8, 1 mg/ml Cu,Zn-SOD plus 100 mM F-6-P; lane 9, 1 mg/ml Cu,Zn-SOD plus 100 mM F-1,6-P_2; lane 10, 1 mg/ml Cu,Zn-SOD plus 100 mM fructose; lane 11, 1 mg/ml Cu,Zn-SOD plus 100 mM sorbitol. (**b**) The same DNA fragment as used in (**a**) was incubated with ribose or ADP-ribose as described below at 37 °C for 1 h and analysed by the method described in the legend to Figure 1. Lane 1, control; lane 2, 100 mM ribose; lane 3, 1 mg/ml Cu,Zn-SOD plus 100 mM ribose; lane 4, 100 mM ADP-ribose; lane 5, 1 mg/ml Cu,Zn-SOD plus 100 mM ADP-ribose.

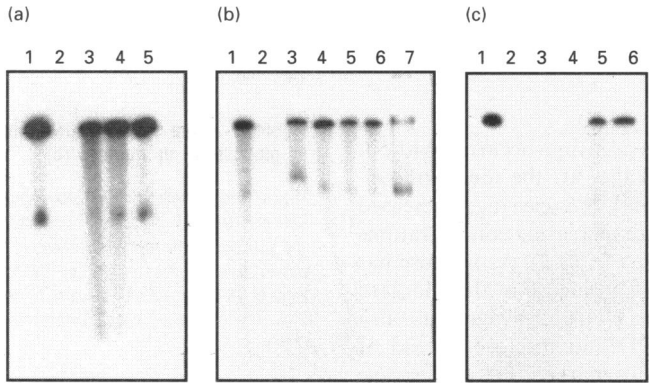

Figure 4 Inhibitory effect of catalase, chelating reagents (a), hydroxy radical scavengers (b) and aminoguanidine (c) on DNA cleavage induced by glycation of Cu,Zn-SOD

The ^{32}P-5′-end-labelled 743 bp DNA fragment (0.6 pmol) was incubated with reagents as described below at 37 °C for 5 days and analysed by the method described in the legend to Figure 1. (**a**) Lane 1, control; lane 2, 1 mg/ml Cu,Zn-SOD plus 100 mM glucose; lane 3, lane 2 plus 0.1 mg/ml catalase; lane 4, lane 2 plus 10 mM EDTA; lane 5, lane 2 plus 50 μM bathocuproine. (**b**) Lane 1, control; lane 2, 1 mg/ml Cu,Zn-SOD plus 100 mM glucose; lane 3, lane 2 plus 0.6 M mannitol; lane 4, lane 2 plus 2.0 M ethanol; lane 5, lane 2 plus 2.0 M DMSO; lane 6, lane 2 plus 0.1 M methionine; lane 7, lane 2 plus 1.0 M sodium formate. (**c**) Lane 1, control; lane 2, 1 mg/ml Cu,Zn-SOD plus 100 mM glucose; lanes 3 to 6 were treated with 1 mg/ml Cu,Zn-SOD, 100 mM glucose, and different concentrations of aminoguanidine; lane 3, 5 mM; lane 4, 20 mM; lane 5, 50 mM; lane 6, 100 mM, respectively.

incubation with ADP-ribose or ribose was also investigated (Figure 3b). DNA cleavage was marked after only 1 h incubation with Cu,Zn-SOD plus ribose or ADP-ribose. DNA cleavage was also observed after incubation with ADP-ribose alone, probably because of reactive oxygen species produced by the autoglycation of ADP-ribose, as reported by Wolff and co-workers [29,30].

Effects of catalase, chelating reagents, hydroxy-radical scavengers, and aminoguanidine on DNA damage induced by glycation of Cu,Zn-SOD

To investigate the involvement of metal ions and H_2O_2 in this DNA damage, metal-chelating reagents, EDTA and bathocuproine, and catalase were employed (Figure 4a). Addition of 0.1 mg/ml catalase to the incubation medium suppressed DNA cleavage. EDTA (10 mM) or bathocuproine (50 μM), a Cu^+-specific chelating reagent, also inhibited DNA damage. These results indicated that H_2O_2, Cu^{2+}, and Cu^+ would participate in DNA cleavage induced by glycation of Cu,Zn-SOD. Because hydroxy radical, which could be produced from H_2O_2 and Cu^{2+}/Cu^+ by a Fenton reaction, was a likely agent for DNA cleavage induced by glycation of Cu,Zn-SOD, the effect of various scavengers of hydroxy radical on DNA damage was examined (Figure 4b). All reagents examined, 0.6 M mannitol, 2.0 M ethanol, 2.0 M DMSO, 0.1 M methionine, and 1.0 M sodium formate, inhibited DNA cleavage. Incubation of Cu,Zn-SOD with 100 mM glucose at 37 °C for 3 days generated enough hydroxy radical for detection by e.s.r. as a 5,5-dimethyl-1-pyrroline *N*-oxide–OH adduct [10]. This signal could not be detected after addition of EDTA or catalase [10]. Taken together,

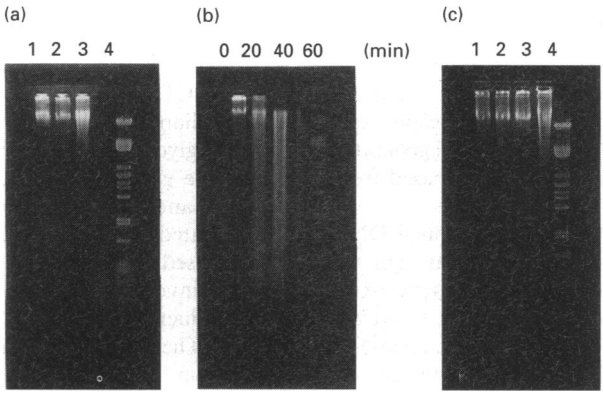

Figure 5 Electrophoretic analysis of nuclear DNA cleavage induced by $CuCl_2$ plus H_2O_2 (a, b) or glycation of Cu,Zn-SOD (c)

Nuclei of K562 cells were incubated with $CuCl_2$ and/or H_2O_2 as described below in PBS at 37 °C for 1 h. The DNA was then isolated and analysed on a 1.5% agarose gel. *Pst* I-digested λDNA was used as molecular-mass markers. (**a**) Lane 1, control; lane 2, 20 μM $CuCl_2$; lane 3, 1 mM H_2O_2; lane 4, 20 μM $CuCl_2$ plus 1 mM H_2O_2; lane 5, molecular-mass markers. (**b**) Time course of nuclear DNA cleavage induced by 20 μM $CuCl_2$ plus 1 mM H_2O_2. (**c**) Nuclei were incubated with Cu,Zn-SOD and/or glucose as described below in PBS at 37 °C for 3 h. The DNA was then isolated and analysed on a 1.5% agarose gel. Lane 1, control; lane 2, 1 mg/ml Cu,Zn-SOD; lane 3, 100 mM glucose; lane 4, 1 mg/ml Cu,Zn-SOD plus 100 mM glucose; lane 5, molecular-mass markers.

these results indicated that hydroxy radical is one of the reactive oxygen species responsible for the DNA damage resulting from glycation of Cu,Zn-SOD. The effects of aminoguanidine, an inhibitor of glycation, on DNA damage induced by glycation of Cu,Zn-SOD was also investigated (Figure 4c). DNA damage was inhibited by addition of more than 50 mM aminoguanidine to the reaction solution.

Nuclear DNA damage induced by $CuCl_2$ plus H_2O_2

Because nuclear DNA is bound to histones and is protected from chemical and physical damage in eukaryotic cells, we investigated the effect of hydroxy radical produced by a Fenton reaction on nuclear DNA in isolated nuclei. To examine the effects of $CuCl_2$ and H_2O_2 on nuclear DNA, isolated nuclei of K562 cells were treated with 20 μM $CuCl_2$ and/or 1 mM H_2O_2 for 1 h at 37 °C. DNA was isolated and analysed by agarose gel electrophoresis (Figure 5a). While $CuCl_2$ alone caused no nuclear DNA damage, slight DNA damage was observed with H_2O_2 alone. There was almost no trace of DNA fragment in the lane for nuclei incubated with $CuCl_2$ plus H_2O_2. The extent of DNA cleavage increased with time (Figure 5b). The nuclear DNA of HIT cells, pancreatic β-cells, was also damaged to a similar extent (results not shown).

Nuclear DNA damage induced by glycation of Cu,Zn-SOD

We examined whether glycation of Cu,Zn-SOD actually damaged DNA in isolated nuclei. Nuclei of K562 cells were treated with 1 mg/ml Cu,Zn-SOD pre-incubated with 100 mM glucose in 500 μl of PBS for 10 days, and the nuclear DNA was extracted and analysed by agarose gel electrophoresis (Figure 5c). Cleavage of nuclear DNA was observed only when nuclei were incubated with Cu,Zn-SOD that had been incubated with glucose. The nuclear DNA cleavage was also inhibited by catalase or metal-chelating reagents (results not shown). The nuclear DNA of HIT cells was also damaged to a similar extent (results not shown). This clearly indicated that reactive oxygen species, probably hydroxy radical, were produced from glycated Cu,Zn-SOD and participated in cleavage of nuclear DNA in isolated nuclei.

Table 3 8-OH-dG formation in DNA incubated with Cu,Zn-SOD and glucose

Bluescript II plasmid containing Mn-SOD genomic DNA (20 pmol) was incubated with 1 mg/ml Cu,Zn-SOD and 100 mM glucose for the indicated durations. After ethanol precipitation, the DNA was subjected to enzymic digestion and then analysed by h.p.l.c.-e.c.d. Data represent means ± S.E. ($n = 3$).

Time (days)	No additions	Cu,Zn-SOD + glucose
0	1.4 ± 0.1	1.6 ± 0.1
3	1.6 ± 0.1	1.9 ± 0.1
5	1.5 ± 0.1	11.3 ± 1.5
10	1.6 ± 0	8.3 ± 1.8

Formation of 8-OH-dG in DNA induced by glycation of Cu,Zn-SOD

By using h.p.l.c.-e.c.d., we measured the 8-OH-dG content in DNA (20 pmol) which had been incubated with 1 mg/ml Cu,Zn-SOD plus 100 mM glucose in 50 mM potassium phosphate buffer, pH 7.4 (Table 3). Although a small amount of 8-OH-dG (1.5 ± 0.1 residue/10^5 guanosine residues) was detected in control DNA as previously reported [28], it was not increased at all after incubation without Cu,Zn-SOD or glucose. However, with addition of Cu,Zn-SOD plus glucose, the amount of 8-OH-dG was substantially increased up to 11.3 ± 1.5 residues/10^5 guanosine residues after 5 days of incubation, whereas it was not changed after 3 days of incubation. Figure 2 shows that DNA cleavage induced by glycation of Cu,Zn-SOD proceeds remarkably after 3 days' incubation. The time course of DNA cleavage and formation of 8-OH-dG clearly indicates that the cleavage of deoxyribose-phosphate backbone precedes base modification.

DISCUSSION

Cu,Zn-SOD undergoes the glycation reaction at specific lysine residues, Lys^{122} and Lys^{128}, upon incubation with glucose [9]. The glycation reaction is followed by site-specific cleavage between Pro^{62} and His^{63}, followed by random fragmentation

[10]. Cu^{2+} of Cu,Zn-SOD has catalytic activity in the generation of hydroxy radical from H_2O_2 [31,32]. The generation of superoxide anion in the glycation reaction and the subsequent generation of H_2O_2 or hydroxy radical are thought to play an important role in the site-specific fragmentation reaction. The second step of fragmentation is believed to involve hydroxy radical generated from H_2O_2 through a Fenton reaction with Cu^{2+} released from the damaged enzyme molecules.

In the present study, we have shown that both cloned DNA and nuclear DNA in isolated nuclei are cleaved through the glycation of Cu,Zn-SOD. H_2O_2 is generated from superoxide anion which is produced by the glycation of protein [4]. However, glycation of Cu,Zn-SOD results in a time-dependent release of Cu^{2+} from the Cu,Zn-SOD molecule [10]. These two compounds, H_2O_2 and Cu^{2+}, are essential components of the DNA cleavage mechanism. The inhibitory effects of catalase, EDTA, bathocuproine, and several kinds of hydroxy-radical scavengers (Figure 4) suggest that H_2O_2, Cu^{2+}, Cu^{+} and hydroxy radical all have important roles in the DNA cleavage induced by glycation of Cu,Zn-SOD. Several reports [11] have suggested that hydroxy radical is the principal agent in the DNA-strand breakage. Other researchers [12] have disputed this hypothesis and suggested that the main active species causing DNA damage by Cu^{2+} and H_2O_2 are more likely copper–peroxide complexes. All of the hydroxy-radical scavengers used here totally inhibit DNA cleavage induced by glycation of Cu,Zn-SOD (Figure 4b), suggesting that hydroxy radical itself is responsible for the DNA damage. The production of hydroxy radical by glycation of Cu,Zn-SOD is demonstrated by e.s.r. [10]. Taken together, the following reactions could be suggested; i.e. incubation of proteins with glucose leads to glycation with formation of ϵ-amino fructosyl lysine residues. These autoxidize with production of superoxide anion and H_2O_2. This autoxidation is due to tautomerism to the enediol form [4]. The protein being glycated releases Cu^{2+}, and Cu^{2+} is reduced to Cu^{+} by superoxide anion or the enediol, and then hydroxy radical is produced by reduction of H_2O_2 by Cu^{+} [4,10].

In addition to the cloned DNA fragment, DNA in isolated nuclei was cleaved by $CuCl_2$ plus H_2O_2 (Figures 5a and 5b). In this mechanism, H_2O_2 and Cu^{2+} probably enter the nucleus and produce hydroxy radical just beside the DNA. The hydroxy radical would then attack the nuclear DNA in the nucleosome. Hydroxy radical produced outside the nucleus would not reach the nucleosome, because the life span of hydroxy radical is very short (10^{-6}–10^{-8} s). Cleavage of nuclear DNA by glycation of Cu,Zn-SOD probably occurs through the same mechanism (Figure 5c).

Hydroxy radical produced by glycation of Cu,Zn-SOD is thought to induce the formation of 8-OH-dG (Table 3). The time course of DNA cleavage and formation of 8-OH-dG suggests that the deoxyribose-phosphate backbone is more sensitive than the base to attack by hydroxy radical. We think that after breakage of the deoxyribose-phosphate backbone, the reactivity of base to hydroxy radical increases and the formation of 8-OH-dG is facilitated. Recently, 8-OH-dG has been used as an early marker of DNA damage induced by reactive oxygen species. But, considering the present results, it is possible that the deoxyribose-phosphate backbone has already been damaged when 8-OH-dG is formed *in vivo*.

In vivo, as the blood glucose level increases, glucose enters into some cells such as pancreas and kidney via glucose transporters. Glucose changes into G-6-P, F-6-P and F-1,6-P_2 during glycolysis. Glucose also changes into sorbitol and then fructose through the Polyol pathway. As shown in Figure 3, most of these sugar compounds have strong capacities for glycation. In addition to pentoses and hexoses, several trioses such as glyceraldehyde [33] and methylglyoxal [34] are suspected to be cytotoxic due to their capacity for the glycation reaction. Cu,Zn-SOD is richly and widely distributed in the cytoplasm of mammalian cells. Thus it is possible that cytoplasmic Cu,Zn-SOD is glycated by these reducing sugars and their derivatives. After the glycation of Cu,Zn-SOD, Cu^{2+} is released from the enzyme, and the dismutation of superoxide anion produced from the glycated Cu,Zn-SOD results in production of H_2O_2. Some of the released Cu^{2+} (or Cu^{+}) and H_2O_2 would enter the nucleus and may be involved in induction of nuclear DNA damage through the production of hydroxy radical in proximity to the nucleosomes. There are many kinds of metal-containing proteins in cytoplasm, and such proteins may also induce DNA breakage through the glycation reaction. Moreover, Cu,Zn-SOD has recently been found to be widely distributed both in the cell cytosol and in the nucleus [35]. Glycation of Cu,Zn-SOD in the nucleus would have a particularly high potential for damaging nuclear DNA. The level of free ADP-ribose *in vivo* is not known. In the nucleus, however, ADP-ribose polymers are synthesized and are rapidly converted into free ADP-ribose. Thus, the nuclear molecules are likely targets for glycation by ADP-ribose.

The level of glycated Cu,Zn-SOD increases in patients with diabetes mellitus [7,8]. Our results suggest that under hyperglycaemic conditions, nuclear DNA cleavage may be induced through the glycation of metal-containing proteins, such as Cu,Zn-SOD, and may cause the irreversible deterioration seen in diabetes and diabetic complications.

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