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Site-specific DNA Methylation and Apoptosis: Induction by Diabetogenic Streptozotocin

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ABSTRACT. Streptozotocin (STZ) is known to induce insulin-dependent diabetes mellitus via DNA damage in experimental animals. The mechanism of induction of DNA damage by STZ was investigated *in vitro*, using a human cell line and ^{32}P-labeled DNA fragments isolated from human genes. STZ induced cellular DNA damage and apoptosis, and frequently initiated DNA modification at guanines, especially at the middle guanine in runs of three and at the guanine at the 3′-end of runs of two guanines, similar to *N*-methyl-*N*-nitrosourea, a typical methylating agent. Scavengers for reactive oxygen species or nitric oxide did not inhibit the induction of DNA damage by STZ. On the other hand, damage induction was inhibited by sodium acetate and sodium chloride, which can reduce the reactivity of methylating agents to DNA via the sodium cation. These results suggest that STZ induces DNA damage by methylation of guanines via methyl cations. This alkylation may be responsible for triggering apoptosis, and subsequently diabetes. BIOCHEM PHARMACOL **57**;8:881–887, 1999.

KEY WORDS. streptozotocin; DNA damage; methylation of guanine; methyl cation; apoptosis; diabetogenesis

STZ, an antibiotic produced by a strain of *Streptomyces achromogens*, is used for induction of diabetes mellitus in experimental animals and for the treatment of pancreatic neoplasms [1]. STZ is a 2-deoxy-D-glucose derivative of the carcinogen MNU which selectively destroys pancreatic β-cells, because its glucose carrier facilitates uptake of the cytotoxic group, MNU, to islets [2]. With reference to a mechanism of STZ-induced diabetes, the following observations have drawn much interest: STZ may damage DNA, which activates poly(ADP-ribose) synthetase, leading to depletion of NAD^+, and in turn a decrease in proinsulin synthesis [3–5]. Several mechanisms by which STZ damages pancreatic β-cells have been proposed. The first (Fig. 1[I]) is that STZ is a strong alkylating agent, causing direct alkylation of DNA by $\bullet CH_3$ [5] or CH_3^+ [6, 7] via the decomposition of STZ. The second (Fig. 1[II]) is that the generation of reactive oxygen species or NO from STZ [8–11] participates in STZ toxicity in diabetogenesis. Some reports have hypothesized the production of intracellular free oxygen radicals related to STZ, since the diabetogenic effect was inhibited by SOD [12, 13]. Furthermore, low doses of STZ can elicit non-specific islet inflammation with infiltration by mononuclear cells and macrophage-derived NO and superoxide, i.e. can also produce cytotoxic effects indirectly through inflammation [14–16]. Thus, these two possible mechanisms of direct DNA damage by STZ and the indirect action of STZ through islet inflammation are proposed in Fig. 1.

To clarify which is the major mechanism through which STZ damages DNA, we investigated DNA damage induction by STZ *in vitro*, using human cultured cells and ^{32}P-5′-end-labeled DNA fragments obtained from human genes.

MATERIALS AND METHODS

Materials

Restriction enzymes *Sma* I, *Eco* RI, *Apa* I, and *Sty* I were purchased from Boehringer Mannheim GmbH. Restriction enzymes *Hind* III, *Ava* I, and *Xba* I and T_4 polynucleotide kinase were purchased from New England Biolabs. A human *p53* amplimer panel was from Clontech Labs. The primers designed for use in the PCR process for the amplification of *p53* are contained in this product (kit). [γ-^{32}P]-ATP (222 TBq/mmol) was from New England Nuclear. STZ and MNU were from Nacalai Tesque, Co. DTPA and bathocuproinedisulfonic acid were from Dojin Chemicals Co. SOD (3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver)

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§ *Abbreviations:* STZ, streptozotocin; MNU, *N*-methyl-*N*-nitrosourea; $\bullet CH_3$, methyl radical; CH_3^+, methyl cation; NO, nitric oxide; SOD, superoxide dismutase; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; PTIO, 2-phenyl-4,4,5,5-tetramethyl-imidazoline-3-oxide-1-oxyl; HL 60, human myelogenous leukemic cell line; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine (also known as 8-hydroxy-2′-deoxyguanosine); HPLC-ECD, an electrochemical detector coupled to a high performance liquid chromatograph; DAN, 2,3-diaminonaphthalene; MEP, molecular electrostatic potential; and PCR, polymerase chain reaction.

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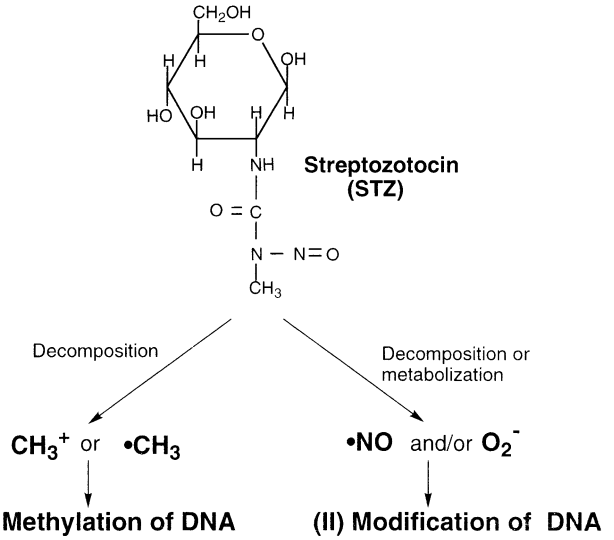

FIG. 1. Proposed mechanisms of toxicity induced by streptozotocin. Two direct toxic mechanisms: (I) methylation of DNA induced by CH_3+ or $\bullet CH_3$ and (II) modification of DNA induced by reactive oxygen radicals or NO from STZ. O_2^-, superoxide.

were from Sigma Chemical Co. PTIO was from Tokyo Kasei Kogyo Co. Spermine NONOate was from Cayman Chemical Co. DMSO was from Wako Co.

Detection of Cellular DNA Damage by Pulsed-field Gel Electrophoresis

HL60 cells were grown in RPMI 1640 (GIBCO BRL, Life Technologies, Inc.) supplemented with 6% fetal bovine serum (Whittaker Bioproducts) at 37° under 5% CO_2 in a humidified atmosphere. HL60 cells (1×10^6 cells/mL) were incubated with STZ in 2 mL of RPMI 1640 containing 6% fetal bovine serum for 24 hr at 37°. After the incubation, the medium was removed and the cells were washed twice with PBS(−) and resuspended in 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 described previously [17]. For the detection of DNA single-strand breakage and alkali-labile sites, the DNA in agarose plug was treated in 1 mL of 25 mM NaOH/2 mM EDTA (pH 12.5) at 4° for 60 min in the dark, and neutralized. Thereafter, the agarose plugs were electrophoresed on 1% agarose gel in Tris–borate EDTA buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.0]) by a CHEF-DRII pulsed-field electrophoresis system (Bio-Rad) at 6 V/cm at 14°. Switch time was 60 sec for 15 hr followed by a 90-sec switch time for 9 hr. The DNA in the gel was visualized in ethidium bromide.

Detection of Apoptotic Cells and DNA Ladder Formation Induced by STZ

HL60 cells (1×10^6 cells/mL) were incubated with STZ in 2 mL of RPMI 1640 supplemented with 6% fetal bovine serum for 24 hr at 37°. Apoptotic cells were determined by fluorescence microscopy after staining with 0.02 mg/mL acridine orange. For the detection of DNA ladder formation, the medium was removed and the cells were washed twice with PBS(−). The cells were suspended in 1 mL of cytoplasm extraction buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM $MgCl_2$ in 0.5% Triton X) and centrifuged. The pellet was treated with lysis buffer (10 mM Tris, pH 7.5, 400 mM NaCl and 1 mM EDTA in 1% Triton X) for 10 min and centrifuged at 4°. The supernatant was treated with 0.2 mg/mL RNase overnight at room temperature and subsequently with 0.1 mg/mL proteinase K for 2 hr at 37°. The DNA was extracted with phenol/chloroform and then with water-saturated ether, and precipitated with ethanol for 30 min at −80°. The pellet was dissolved in 40 μL of TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA). The DNA was electrophoresed on 1.4% agarose gel containing 0.375 μg/mL ethidium bromide in 0.5 × TBE buffer.

Preparation of ^{32}P-5′-End-Labeled DNA Fragments

DNA fragment was obtained from the human *p*53 tumor suppressor gene [18]. The fragments from the *p*53 gene containing exons were amplified by the PCR method using an Omnigene Temperature Cycling System. The PCR products were digested with *Sma* I and ligated into *Sma* I-cleaved pUC 18 plasmid, and then transferred to *Escherichia coli* JM 109. The plasmid pUC 18 was digested with *Eco* RI and *Hind* III, and the resulting DNA fragments were fractionated by electrophoresis on 2% agarose gels. The 5′-end-labeled 460-bp fragments (*Hind* III*13038–*Eco* RI*13507) were obtained by dephosphorylation with bovine intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The 460-bp fragment was further digested with *Sty* I to obtain a singly labeled 348-bp fragment (*Sty* I 13160–*Eco* RI*13507), according to the method described previously [19]. The asterisk indicates ^{32}P labeling. DNA fragment was also obtained from human c-Ha-*ras*-1 proto-oncogene [20]. DNA fragment was prepared from plasmid pbcNI, which carries a 6.6-kb *Bam* HI chromosomal DNA segment containing c-Ha-*ras*-1 gene; a singly labeled 341-bp fragment (*Xba* I 1906–*Ava* I*2246), a 261-bp fragment (*Ava* I* 1645–*Xba* I 1905), and a 337-bp fragment (*Pst* I 2345–*Ava* I*2681) were obtained according to the method described previously [21]. Nucleotide numbering starts with the *Bam* HI site [20].

Detection of ^{32}P-5′-End-Labeled DNA Damage by STZ

The standard reaction mixture (in a microtube; 1.5 mL) contained STZ, ^{32}P-5′-end labeled DNA fragments, and sonicated calf thymus DNA (10 μM/base) in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. DTPA was added to remove any metal ions which may be contained in phosphate buffer. After incubation at 37° for the indicated duration, the DNA fragments were

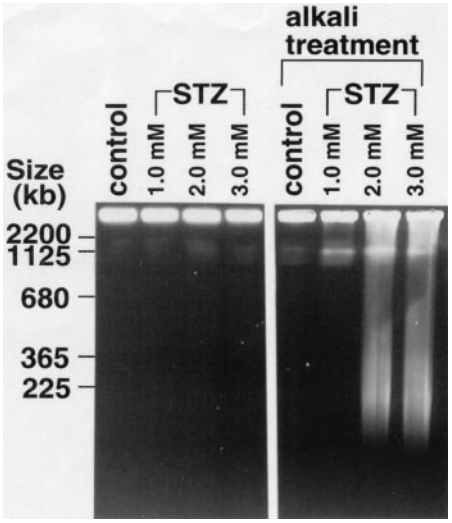

FIG. 2. Detection of cellular DNA damage by pulsed-field gel electrophoresis in cells treated with STZ. HL60 cells were treated with STZ for 24 hr at 37°. The cells were prepared into agarose plugs, lysed, and treated with alkali solution where indicated. Thereafter, the plugs were subjected to pulsed-field gel electrophoresis through 1% agarose gel, as described in Materials and Methods. The gel was stained in ethidium bromide.

heated at 90° in 1 M piperidine for 20 min where indicated and treated as described previously [21].

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 [22] using a DNA-sequencing system (LKB 2010 Macrophor, Pharmacia Biotech). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech).

Analysis of 8-oxo-dG Formation in Calf Thymus DNA by STZ

The amount of 8-oxo-dG (also known as 8-hydroxy-2′-deoxyguanosine) was measured by a modified method of Kasai *et al.* [23]. The standard reaction mixture (in a microtube; 2.0 mL; Eppendorf) contained STZ and calf thymus DNA (100 μM/base) in 400 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation with STZ for 1 hr at 37°, DNA fragments were precipitated with ethanol, and DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by the HPLC-ECD, as previously described [24].

Measurements of NO from STZ by Fluorometric Assay

To measure NO from STZ, fluorometric assay with DAN [25] was used. NO reacts with molecular oxygen and water to form nitrite and nitrate, while DAN reacts with nitrite under acidic conditions to form 1-(H)-naphthotriazole, a fluorescent product. One hundred microliters of sample was first brought to volume (100 μL) with double-deionized water. To this, 10 μL of freshly prepared DAN (0.05 mg/mL in 0.62 M HCl) was added and mixed immediately. The DAN reagent was protected from light. After a 10-min incubation at 20°, the reaction was terminated with 5 μL of 2.8 N NaOH. The intensity of the fluorescent signal produced by the product is maximized by the addition of base. Formation of the 2,3-diaminonaphthotriazole was measured using an RF-1500 spectrofluorometer (Shimadzu Co.) with excitation at 365 nm and emission read at 450 nm with a gain setting at 100%.

RESULTS

DNA Damage, DNA Ladder Formation, and Apoptotic Changes in Cultured Cells Treated with STZ

Figure 2 shows cellular DNA damage detected by pulsed-field gel electrophoresis. Without alkali treatment, little or no DNA cleavage was observed in cells treated with STZ. After alkali treatment, DNA fragments corresponding to 1000–2000 kb were observed in the cells treated with 1 mM STZ, and smaller DNA fragments corresponding to 200–300 kb with 2–3 mM STZ. Figure 3A shows apoptotic cells observed with acridine orange staining by fluorescence microscopy. Apoptotic cells, identified by chromatin condensation, nuclear fragmentation, and cytoplasmic budding, were observed frequently in cells treated with 2 mM STZ for 24 hr. Figure 3B shows DNA ladder formation in cells treated with STZ. The electrophoresis of DNA isolated from apoptotic cells reveals characteristic fragmentation by endonuclease, i.e. 'DNA ladder' [26]. DNA ladder formation was not observed at 0.5–1 mM STZ, but apparent fragmentation was detected at 2 mM STZ. NaCl suppressed apoptosis by STZ. On the other hand, addition of PTIO, an NO scavenger, did not suppress apoptosis by STZ, but did suppress apoptosis by spermine NONOate, an NO donor as positive control for exogenous NO-induced apoptosis. These results indicated that the mechanism of induction of apoptosis by STZ was different from that induced by NO from spermine NONOate.

Damage of ^{32}P-Labeled DNA Fragments by STZ and MNU

Figure 4 shows an autoradiogram of DNA fragments treated with STZ and MNU. Oligonucleotides were detected as a result of DNA cleavage. DNA cleavage was caused by STZ, even in the absence of metal ions, and the amount of DNA damage increased with STZ concentration and incubation time (data not shown). The DNA damage was markedly enhanced with piperidine treatment, compared with no treatment (data not shown), indicating that STZ induced strong base modification with slight strand breakage. Damage to native DNA was greater than that to denatured DNA. Similar results were obtained in the case of MNU.

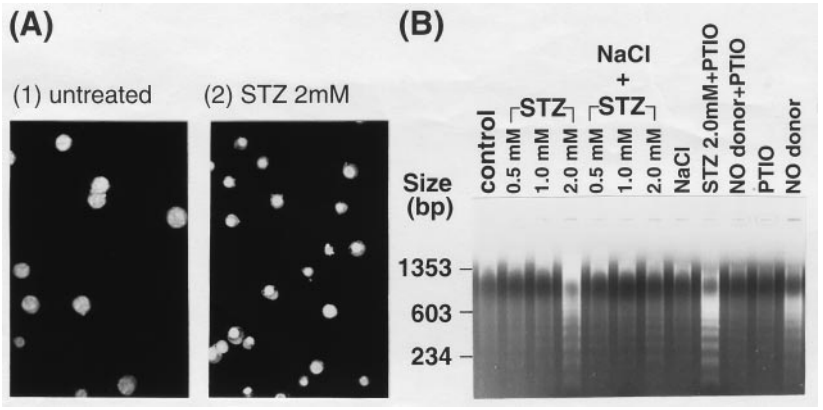

FIG. 3. Detection of apoptotic cells and internucleosomal DNA fragmentation in HL60 cells treated with STZ. (A) The morphology of cells, stained with acridine orange from (1) untreated cultures and (2) cultures exposed to 2 mM STZ for 24 hr at 37°. (B) Internucleosomal DNA fragmentation in cultured cells treated with STZ. HL60 cells were treated with either STZ, STZ+NaCl, STZ+PTIO, spermine NONOate, or spermine NONOate+PTIO, at the indicated concentration for 24 hr at 37°. The cells were lysed and DNA was extracted as described in Materials and Methods. The DNA was electrophoresed in a 1.4% agarose gel containing 0.375 μg/mL ethidium bromide. The concentration of the chemicals was as follows; STZ 0.5, 1.0, and 2.0 mM, NaCl 50 mM, PTIO 50 μM, and spermine NONOate 100 μM.

Effects of Scavengers on DNA Damage Induced by STZ

The effects of scavengers of reactive oxygen species on DNA damage induced by STZ was examined (Fig. 5A). Sodium formate markedly reduced DNA damage, whereas other typical free hydroxyl radical scavengers, such as ethanol, mannitol, and DMSO, showed little or no inhibitory effect on the induction of DNA damage by STZ. Catalase and SOD also showed no inhibitory effect on the induction of DNA damage. Figure 5B shows the effect of inhibitors of methylating agents. Sodium chloride (NaCl), sodium formate, and sodium acetate markedly reduced DNA damage by STZ, as did sodium propionate and sodium butylate (data not shown). These results suggest that sodium cations can reduce STZ-induced DNA damage.

Site Specificity of DNA Modification by STZ

To examine the DNA modification sites, ^{32}P-5′-end-labeled DNA fragments treated with STZ and subsequently with piperidine were electrophoresed. The autoradiogram was scanned with a laser densitometer to measure the relative intensities of the bands, as shown in Fig. 6. STZ frequently generated piperidine-labile sites at guanine residues, especially at the middle guanine in runs of three or at the 3′ guanine at sites of two neighboring guanines. On the other hand, single guanines were somewhat less susceptible. Damage to native DNA was greater than that to denatured DNA (Fig. 6B). Similar DNA modification patterns were also observed after measurement with MNU (data not shown).

Formation of 8-Oxo-dG in Calf Thymus DNA by STZ

Using HPLC-ECD, we measured 8-oxo-dG content in calf thymus DNA treated with STZ. STZ treatment did not produce significant amounts of 8-oxo-dG.

Measurement of NO Generation from STZ

One mM STZ generated 5.27 μM NO and 3.38 μM NO at pH 7.4 and pH 5.0, respectively. This indicates that STZ

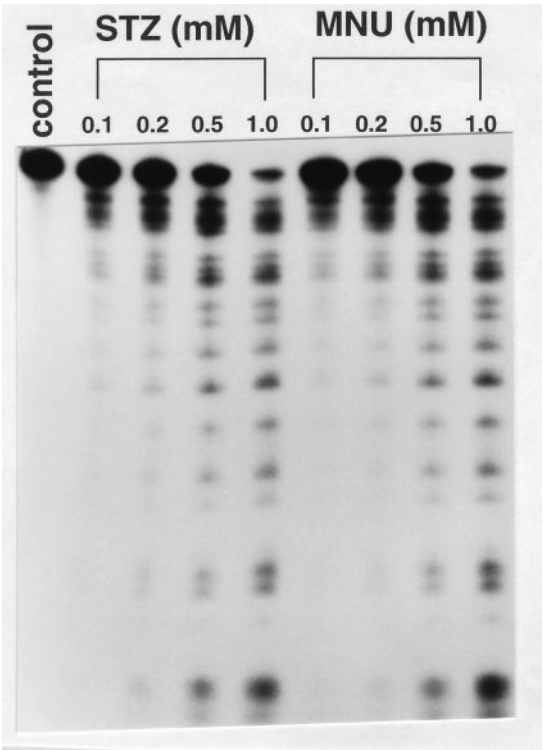

FIG. 4. Autoradiogram of ^{32}P-labeled DNA fragments incubated with STZ and MNU. The reaction mixture contained ^{32}P-5′-end-labeled 341-bp DNA fragments, 10 μM per base of sonicated calf thymus DNA, and indicated concentrations of STZ and MNU in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37° for 2 hr, followed by piperidine treatment. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel, and the autoradiogram was obtained by exposing X-ray film to the gel.

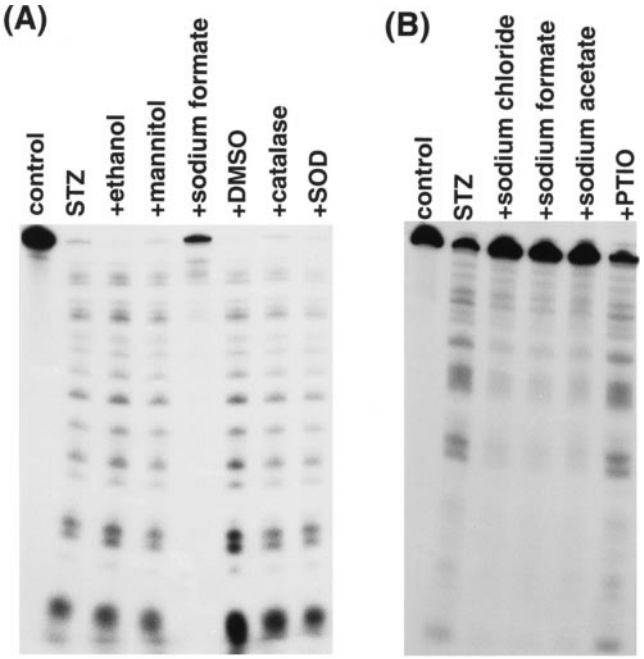

FIG. 5. Effects of scavengers on DNA damage induced by STZ. The reaction mixture contained ^{32}P-5′-end-labeled 341-bp (A) and 337-bp (B) DNA fragments, 10 μM per base of sonicated calf thymus DNA, and 0.5 mM of STZ (A and B) in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37° for 1 hr, followed by piperidine treatment unless otherwise noted. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel, and the autoradiogram was obtained by exposing X-ray film to the gel. (A) Scavenger for oxygen radical was added to the reaction mixture of STZ as follows: 5% (v/v) ethanol; 0.1 M mannitol; 0.1 M sodium formate; 10% (v/v) DMSO; 30 units of catalase; and 30 units of SOD. (B) Inhibitor for methylating agent or NO was added to the reaction mixture of STZ as follows: 0.1 M NaCl; 0.1 M sodium formate; 0.1 M sodium acetate; and 0.1 M PTIO.

had only a weak ability to generate NO under the condition used.

DISCUSSION

Pulsed-field gel electrophoresis is generally used for detection of cellular DNA double-strand breaks, and combined with alkali treatment, it can be employed for detection of DNA single-strand breaks plus alkali-labile sites [17]. In the present study, pulsed-field gel electrophoresis showed that STZ induced high molecular weight DNA breakage in HL-60 cells after alkali treatment. DNA ladder formation was also observed with STZ. These results strongly suggest that STZ causes alkali-labile DNA modification, as detected by DNA fragments of 1000–2000 kb and 200–300 kb with alkali treatment. The DNA modification may induce or trigger apoptosis.

The experiment with ^{32}P-labeled DNA showed that STZ-induced site-specific DNA damage was inhibited by NaCl, sodium formate, and sodium acetate. It was reported that methylation of DNA by MNU was dose dependently

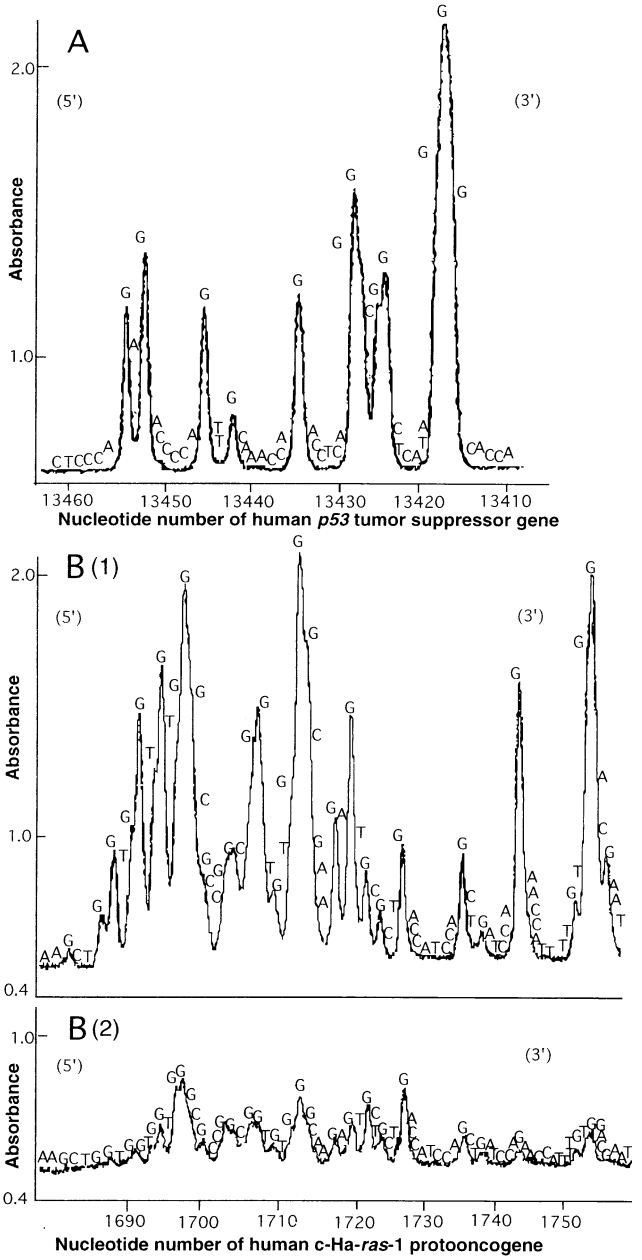

FIG. 6. Site specificity of DNA cleavage induced by STZ or MNU. The ^{32}P-5′-end-labeled 348-bp fragment (*Sty* I 13160–*Eco* RI*13507) of *p53* gene (A), or 261-bp fragment (*Ava* I* 1645-*Xba* I 1905) of c-Ha-*ras*-1 gene (B), 10 μM per base of sonicated bovine thymus DNA, and 0.5 mM STZ (A and B[1, 2]) in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA (*, ^{32}P-labeling). DNA fragments were denatured before the incubation (B[2]). Reaction mixtures were incubated at 37° for 2 hr. After the peperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 3. The relative amounts of oligonucleotide were measured by scanning the autoradiogram with a laser densitometer (LKB 222 UltroScan XL). The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene [18] or c-Ha-*ras*-1 protooncogene starting with the *Bam*HI site [20].

inhibited by NaCl [27]. Inhibition of DNA methylation by MNU can be explained in terms of the screening of the DNA electrostatic potential by salt. The strong electro-

static attraction between an intermediate for methylation and the polyanionic DNA backbone at low salt would be inhibited by the presence of salt [27]. Highly electrophilic species such as CH_3^+ would be expected to attack the most nucleophilic guanine sites on DNA. Such a reaction would be governed to some extent by the MEP of the attacked site, and the potential of DNA bases is known to be shielded by sodium cation [28], resulting in a decrease in reactivity of methylating agents to DNA, as seen in scavenger experiments using ^{32}P-labeled DNA. It was observed that the absolute value of MEP progressively increases with the increasing complexity of DNA structure, i.e. single bases<nucleotides<single helices<double helices [28]. A decrease in the intensity of STZ-induced damage by DNA denaturation may show a good relationship with the MEP value. Furthermore, the accessibility of the N-7 of a central guanine in GGG may be greater than in other representative triplets whose value has been computed [29]. It has been reported that methylating agents like MNU preferentially alkylated the middle guanines in runs of three or more [30]. As expected, STZ as well as MNU caused frequent DNA modification at the middle guanine in runs of three.

It has been reported that $\bullet CH_3$ has the ability to alkylate the C-8 of guanine residues [31], however, we previously showed that *N*,*N*′-dimethylhydrazine frequently caused Cu(II)-mediated DNA damage at thymine residues but not at guanine residues, although we did detect both $\bullet CH_3$ and oxygen radicals generated from *N*,*N*′-dimethylhydrazine [31]. This difference may be explained by assuming that the C-8 adduct of guanine residues induced by $\bullet CH_3$ is not so labile to the piperidine treatment as is the N-7 adduct. The present study showed that STZ and MNU frequently caused DNA damage at guanine, even in the absence of metal ions, although it remains possible that base damage might be over- or under-represented, depending on their sensitivity to piperidine treatment. We then considered whether the mechanism of DNA damage by STZ and MNU was completely different from that by $\bullet CH_3$ from *N*,*N*′-dimethylhydrazine. These results and previous reports [27–30, 32] led us to the notion that DNA damage by STZ was intermediated by CH_3^+ rather than $\bullet CH_3$. This idea is also supported by the inhibitory effect of NaCl on DNA damage.

Several papers have suggested that the generation of reactive oxygen radicals or NO from STZ [8–11] may play a role in STZ toxicity in diabetogenesis. However, we have shown here that DNA fragmentation in HL60 cells treated with STZ was not inhibited by PTIO, the NO scavenger, whereas DNA fragmentation by NO generated from spermine NONOate was inhibited by PTIO. PTIO also had no inhibitory effect on DNA damage induction by STZ, using ^{32}P-labeled DNA. These results suggest that DNA fragmentation and induction of apoptosis by STZ is not primarily caused via NO. In addition, the formation of 8-oxo-dG, a relevant indicator of oxidative base damage, was not observed in calf thymus DNA treated with STZ. Damage of ^{32}P-labeled DNA by STZ was not inhibited by SOD, catalase, or hydroxyl radical scavengers. Together, these results confirmed that DNA damage by STZ was not mediated by reactive oxygen species or NO. We demonstrated earlier that another diabetogenic agent, alloxan, induced DNA damage via reactive oxygen species [33].

When nicotinamide, a poly(ADP-ribose) synthetase inhibitor, was applied at the same time, proinsulin synthesis of β-cells was not affected; rather, islet cell tumors were found in the STZ-treated rats after 14–18 months [34]. It is reasonable to assume that poly(ADP-ribose) synthetase inhibitors inhibit repair of STZ-induced DNA damage and hence lead to carcinogenesis. Otherwise, the DNA damage would activate nuclear poly(ADP-ribose)synthetase for repair of DNA strand breaks, thereby depleting intracellular NAD levels which may lead to β-cell destruction [3–5]. Recent evidence suggests that apoptosis is the mode of β-cell death responsible for the development of autoimmune insulin-dependent diabetes mellitus in the non-obese diabetic mouse [35]. Apoptosis can also be induced by DNA damage [36–38]. Therefore, we suggest that methylation of guanines by STZ observed here may also induce apoptosis in β-cells, possibly explaining how β-cell destruction and diabetes are triggered by this compound.

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