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Involvement of preferential formation of apurinic/apyrimidinic sites in dimethylarsenic-induced DNA strand breaks and DNA-protein crosslinks in cultured alveolar epithelial cells

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We previously found that lung-specific DNA damage induced by administration of dimethylarsinic acid (DMAA), a main metabolite of inorganic arsenics in mammals, in mice might be due to dimethylarsenic peroxyl radical [$(CH_3)_2AsOO\bullet$] produced in the further metabolic processing of DMAA. Further analysis of DNA damage was performed in the present study using a human embryonic cell line of alveolar epithelial (L-132) cells. Alkali-labile sites in DNA were produced prior to DNA single-strand breaks (SSB) and DNA-protein crosslinks (PC) in L-132 cells by exposure to 10mM DMAA. An experiment using methoxyamine (MA), an agent reacting with the aldehyde group of apurinic/apyrimidinic (AP) sites in DNA, indicated that, of the alkali-labile sites formed by exposure to DMAA, major ones were AP sites. These findings suggest that SSB and PC induced by exposure of L-132 cells to DMAA occurred via the formation of AP sites in DNA. That is, SSB were produced by a β-elimination reaction on AP sites in the DNA and PC by a Schiff-base reaction between amino groups of nuclear proteins and aldehyde groups of AP sites.

Although inorganic arsenics, *e.g.*, arsenite and arsenate, have been epidemiologically established as carcinogens for human lung and skin, there is no satisfactory evidence for the carcinogenicity in experimental animals (1). As regards their genotoxicity, several reports have demonstrated the induction of chromosomal abnormality (2, 3), but little has known about the genetic damage such as gene mutation and DNA damage (4). To solve this apparent paradox, several trials have been made; a concept proposed, though not fully probed, is that inorganic arsenics inhibit the repair process for DNA damage caused by other carcinogens and mutagens (3, 5-9). On the other hand, we have focused on the genotoxic action of metabolically methylated arsenics, since inorganic arsenics are known to be readily metabolized to methylarsenics by a pathway thought to be an

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Abbreviations used: DMAA, dimethylarsinic acid; MEM, minimal essential medium; MA, methoxyamine; SSB, DNA single-strand breaks; PC, DNA-protein crosslinks; AP, apurinic/apyrimidinic; CMF-PBS, Ca-, Mg-free phosphate buffered saline; SOD, superoxide dismutase.

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important detoxication process for the acute toxicity of inorganic arsenics (10). In the course of our investigations, we found that administration of dimethylarsinic acid (DMAA), a main metabolite of inorganic arsenics, to mice induced lung-specific DNA damage, *e.g.*, DNA single-strand breaks (SSB) (11-14) and DNA-protein crosslinks (PC) (15). One of the major ultimate substances to cause DNA damage was assumed to be dimethylarsenic peroxyl radicals produced in the further metabolic processing of DMAA (13, 14, 16).

This article describes the mechanism of genetic damage induced by exposure of human alveolar-epithelial (L-132) cells (17) to DMAA. We found that apurinic/apyrimidinic (AP) sites in DNA were formed in the early stage of DNA damage and that might lead to the formation of SSB and PC.

MATERIALS AND METHODS

Materials

Sodium salt of DMAA was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). [Methyl-^{3}H]thymidine (3.1 TBq/mmol) was obtained from NEN Research Products (Boston, USA). L-132 cells, an established human embryonic cell line of alveolar cultured cells (20), were purchased from ICN Biochemicals Inc. (Costa Mesa, CA).

Cell Culture

L-132 cells were cultured in Eagle's MEM (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10 % heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS). Having a doubling time of *ca.* 20 hr, the cells were grown in 9-cm^2 petri dishes to monolayers at 37°C in an atmosphere of 5 % CO_2.

DNA-^{3}H Labeling

The cells (1 x 10^6 cells) were plated in 9-cm^2 petri dishes and allowed to grow overnight. Then, [methyl-^{3}H]thymidine (37 kBq/ml) was added to the medium of the cultures, and 24 hr later the culture were washed twice with CMF-PBS (Ca-, Mg-free phosphate-buffered saline), and subsequently incubated at 37°C for 3-12 hr in the complete medium (2.5 ml) containing 10mM DMAA. The viability of the cells assayed by the dye-exclusion method was more than 90 % in all of the experiments.

Alkaline Elution

SSB were determined by the alkaline elution method (12) based on the method of Kohn *et al.*(18). With regard to the reaction of DNA prepared on the filter and methoxyamine (MA) (Wako Pure Chemical Industries, Osaka, Japan), 5mM MA (0.5 ml) was added to the filter-mounted funnel and allowed to stand for 30 min at 20°C in the dark.

Alkaline Sucrose Gradient

The length of single-stranded DNA was measured by the alkaline sucrose gradient sedimentation based on the method of Wassermann *et al.* (19). [Methyl-^{3}H]thymidine-labeled DNA was prepared by phenol extraction, digested with a restriction enzyme (Kpn I, Takara Biomedicals, Kyoto, Japan), and dissolved in 10mM phosphate buffer (pH 7.5). Then, 5mM MA was added to the DNA solution and the mixture was allowed to stand for 30 min at 37°C. The sample (*ca.* 1.5μg DNA) on the 5-20 % alkaline sucrose gradient (4 ml) was centrifuged in a PRS56T rotor (Hitachi Co., Tokyo, Japan) at 32,000 rpm, 4°C for 2.5 hr. Each fraction (6 drops) was soaked into a 3MM paper. The papers were dried with air, placed in each vial with 5 ml of scintillation cocktail (Aquasol-2, NEN Research Products, Boston, USA). The radioactivity was counted in a liquid scintillation counter.

RESULTS

First, to further confirm our previous finding that exposure of cultured cells to DMAA induced SSB (20), the time course of SSB in L-132 cells exposed to DMAA was determined. As shown in Figure 1(a), the induction of SSB was dependent on the duration of incubation with 10mM DMAA. The SSB were not detected up to 6 hr of incubation but appeared remarkably at 9 and 12 hr of incubation.

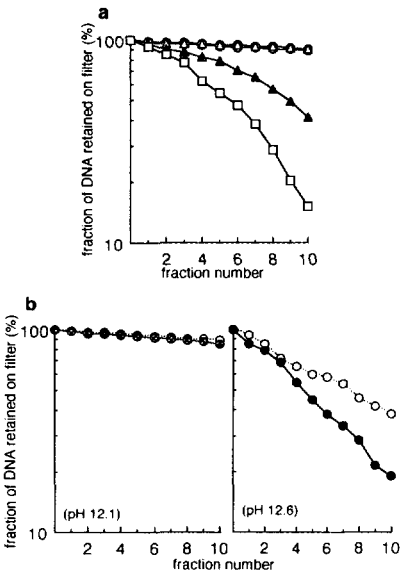

Figure 1. Alkaline elution profiles of DNA from L-132 cells exposed to DMAA. (a) The cells were exposed to 10 mM DMAA for 0 hr (○), 3 hr (●), 6 hr (△), 9 hr (▲), or 12 hr (□). (The former three symbols locate almost the same position at the top of graph.) (b) The cells were exposed to 10 mM DMAA for 0 hr (○), 6 hr (●), and alkaline elution was performed at pH 12.1 (left panel) or 12.6 (right panel).

To reveal DNA damage, which was not detected as SSB, induced at the early stage after exposure to DMAA, we assayed alkali-sensitivity of DNA in L-132 cells exposed to DMAA for 6 hr (Figure 1[b]). Significant differences in the alkaline elution profiles of the DNA between pH 12.1 and pH 12.6 were observed; at pH 12.1 the pattern of DNA in DMAA-exposed cells was not significantly different from that of control DNA as shown in Figure 1(a), but at pH 12.6 the pattern of DNA showed an apparent difference between DMAA-exposed and control cells. DNA in the cells exposed to DMAA for 6 hr was eluted more alkali-dependently than control DNA, similarly to that in cells exposed to alkylating agents (18). These results indicate that formation of alkali-labile sites in DNA occurred prior to the induction of SSB and PC (15, 20). Most probable one of such alkali-labile DNA damages induced by exposure to DMAA may be the formation of AP sites, which are important DNA lesions resulting from cleavage of the N-glycosyl bond between the deoxyribose moieties and nucleic bases. In fact, when DNA from the cells exposed to DMAA for 6 hr was treated with exonuclease III (derived from *Escherichia coli*), having an AP endonuclease activity in the presence of Ca^{2+} (21), the length of single-stranded DNA became shorter than that of control DNA (unpublished data). This also suggests the formation of AP sites in the DNA.

Liuzzi and Talpaert-Borle (22) reported that methoxyamine reacted quantitatively with the aldehyde residues of AP sites without cleaving the polynucleotide and that DNA including the MA-bound AP sites was more resistant to hydrolysis than non-bound AP sites under alkaline condition. In fact, the elution pattern at pH 12.6 of DNA in the cells exposed to DMAA for 6 hr showed alkali-resistant one by pretreatment with MA (Figure 2). This result suggests that AP sites were formed in the DNA by exposure of the cells to DMAA.

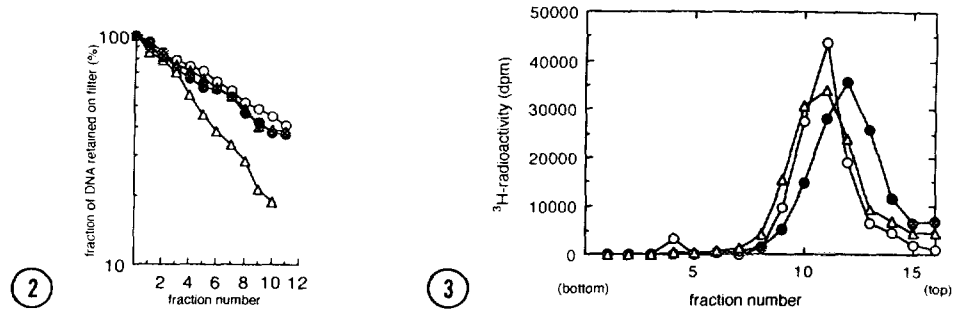

Figure 2. Effect of methoxyamine (MA) treatment on DNA single-strand breaks of L-132 cells induced by exposure to DMAA. The cells were exposed to 10 mM DMAA for 0 or 6 hr. DNA prepared from the cells was pretreated with 5 mM MA for 30 min and then eluted at pH 12.6. Symbols are 0 hr (○), 0 hr + MA (●), 6 hr (△), and 6 hr + MA (▲).

Figure 3. Alkaline sucrose density gradient centrifugation profiles of DNA from L-132 cells exposed to DMAA. The cells were exposed to 10 mM DMAA for 0 or 6 hr. The DNA isolated from the cells was pretreated with 5 mM MA for 30 min and then analyzed by 5-20 % alkaline sucrose sedimentation assay. Symbols are 0 hr (○), 6 hr (●), and 6 hr +MA (△).

To further clarify this, alkaline sucrose gradient centrifugation analysis was applied to estimate the length of the single-stranded DNA. As shown in Figure 3, when assayed L-132 cells exposed to DMAA for 6 hr, the MA-treatment made the length of the single-stranded DNA longer than that of MA-nontreated, which was shorter than of control DNA. These results support that exposure of L-132 cells to DMAA induced AP sites in DNA prior to the induction of SSB and PC observed in our previous studies (15, 20).

DISCUSSION

It has been reported that inhalation exposure of smelter workers to inorganic arsenics induced chromosomal aberrations in the peripheral lymphocytes (23) and that *in vitro* exposure of cells to inorganic arsenics also induced chromosomal aberrations (24, 25). However, there is no conclusive evidence that inorganic arsenics cause mutation in any *in vitro* system (4, 26). These findings support the view that inorganic arsenics are not mutagenic, but clastogenic. Li and Rossman, however, have indicated that arsenite caused DNA repair inhibition after the incision step in Chinese hamster V79 cells (8) and that the activities of DNA ligase II and DNA polymerases were inhibited by the treatment with arsenite (8, 27), proposing that arsenite might have a co-mutagenic activity. On the other hand, a recent report (28) represented that superoxide dismutase (SOD) and catalase protect against arsenic toxicity and reduce chromosomal aberrations in cultured human lymphocytes, suggesting that arsenic-induced genotoxicity may be related to the production of active oxygen species. However, no evidence was given that inorganic arsenics caused DNA damage such as SSB and PC. Concerning the relationship between arsenic genotoxicity and free radical production, we previously reported that administration of DMAA, which is a main metabolite of inorganic arsenics, to mice induced lung-specific DNA damage (11-14), possibly via the production of dimethylarsenic peroxyl radicals in the further metabolic processing of DMAA (14, 16).

As schematically shown in Figure 4, we postulate a possible pathway of DNA damage produced by the arsenic peroxyl radical based upon our present and previous studies. We assume that

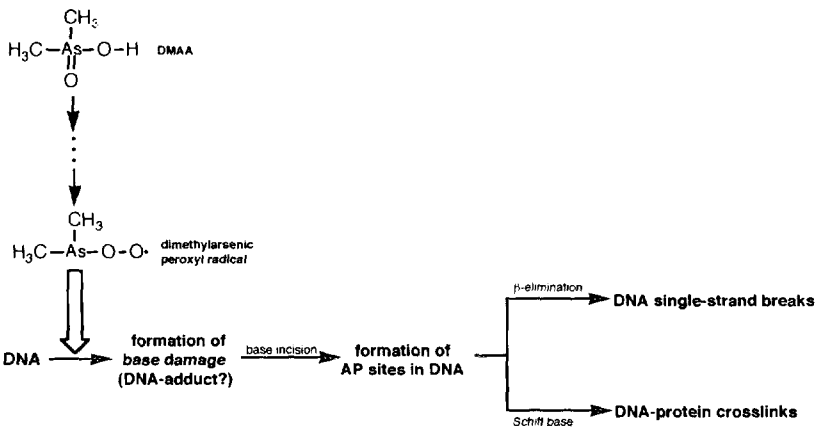

Figure 4. Proposed mechanism of DNA damage by free radical species produced in the metabolic processing of DMAA.

SSB and PC induced by the arsenic peroxyl radical may occur via the production of AP sites in DNA. This arsenic peroxyl radical was earlier thought to directly induce SSB, similarly to the hydroxyl radical. However, it now becomes very likely that SSB occur after a β-elimination reaction at the aldehyde group of deoxyribose moieties of AP sites in DNA and, further, that PC occur by a Schiff-base reaction between their aldehyde groups and amino groups of nuclear proteins.

We have postulated a possibility of DNA-adduct formation with radical species such as the arsenic peroxyl radical (20). We recently observed the induction of the repair system by exposure of L-132 cells to DMAA at low concentrations; the exposure to DMAA at 100 μM or lower activated the DNA repair system involving DNA polymerase α in L-132 cells, at an earlier stage after the start of DMAA exposure, at least prior to the formation of AP sites in the DNA (unpublished data). Some of the base damage induced by exposure to mutagens such as alkylating agents is known to be repaired through the excision of the damaged base by the action of DNA glycosylase, which hydrolyzes the N-glycosyl bond to form AP sites in DNA, and this action is thought to be an important part of the mechanism that produces AP sites in DNA (29). Furthermore, the formation of AP sites is considered to be one of the important events of excision repair of not only alkylated bases but also a variety of DNA adducts. We therefore assume that the formation of AP sites demonstrated here reflect the DNA-adduct formation.

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