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In situ Freezing of the Rat Urinary Bladder: DNA Adduct Formation in the Bladder Epithelium Demonstrated by ^{32}P-Postlabeling Assay

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***In situ* freezing of the urinary bladder has been demonstrated to exert tumor-initiating potential in two-stage urinary bladder carcinogenesis in the rat. In the present experiment, DNA modification was examined after *in situ* freezing of the whole urinary bladder performed by pinching with frozen forceps at −15°C or −30°C for 2 s. The ^{32}P-postlabeling analysis revealed at least 2 DNA adducts in the epithelial cells of the urinary bladder collected 3 days after freezing. Single-strand breaks of DNA were also found by means of the alkaline elution assay in the bladder epithelium collected 10 min after freezing. Thus, the previously demonstrated tumor-initiating activity of *in situ* freezing in urinary bladder carcinogenesis was revealed to be associated with substantial DNA damage and adduct formation.**

Key words: *In situ* freezing — Urinary bladder carcinogenesis — ^{32}P-Postlabeling — DNA adducts — Rat

In situ freezing of the rat urinary bladder performed without any carcinogen treatment has been demonstrated to initiate urinary bladder carcinogenesis in the two-stage model when followed by application of 5% sodium saccharin in the diet[1,2] or 2% sodium *o*-phenylphenate[3] as a promoter. A series of investigations, however, have revealed no mutagenic activity in urine collected immediately or during the reparative phase after freezing,[4] no increase in concanavalin A-mediated epithelial cell agglutinability,[5] which has been demonstrated to be an indicator to distinguish urinary bladder carcinogens and promoters in a short period,[6] and no difference in immunohistochemical pattern of keratin(s) in freezing-induced hyperplasia compared to lesions induced by urinary bladder carcinogens.[7] Thus, the freezing injury has been considered to exert its tumor-initiating activity through a different mechanism(s) from genotoxic carcinogens.[7] Furthermore, the activity is thought to be specific to the urinary bladder since no tumor initiating potential of *in situ* freezing was demonstrated in two-stage skin carcinogenesis in SENCAR mice.[8] A role for urine in freezing-induced urinary bladder tumor initiation has been discussed.[2,3,8]

The question of whether DNA modifications, generally considered to be involved in tumor initiation in many organ models, occur during freezing and/or subsequent reparative processes needs to be answered to understand the phenomenon. In the present experiment, possible DNA modifications were examined using a ^{32}P-postlabeling technique and an alkaline elution assay to analyze if the activity is associated with critical molecular changes in DNA of the urinary bladder epithelium.

MATERIALS AND METHODS

Animals Male F344 rats, 5 weeks old at the commencement of the experiment, were obtained from Shizuoka Laboratory Animal Center, Shizuoka. The rats were housed five per polycarbonate cage on soft-chip bedding in an air-conditioned room at a temperature of 22±2°C and a relative humidity of 55±5% with a 12-h light-dark daily cycle. A commercial pelleted diet (CRF-1; Charles River Japan, Inc., Atsugi) was used as the basal diet.

Freezing of the urinary bladder Freezing of the urinary bladder was performed with the rats under ether or pentobarbital (Nembutal) anesthesia by a modification of Shirai's original procedure.[9] An incision about 1 cm long was made in the center of the lower abdominal wall, and the urinary bladder was gently exposed using regular forceps. Instead of a focal freezing procedure as used in the previous studies,[1-3] the whole urinary bladder was tightly pinched once with large stainless-steel forceps, cut be 10 mm in width at the top, and precooled in dry ice in acetone. At each freezing treatment, the temperature of the acetone solution was checked using a digital thermometer (model LHT-100, Laytant Science Co., Tokyo) and, if necessary, adjusted by adding acetone at room

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temperature to increase the temperature or by adding small pieces of dry ice to decrease it. After the freezing treatment, the abdominal wall was immediately closed using surgical clips. As a sham operation, the urinary bladder was gently pinched with regular forceps.

Histological observations Groups of 3 rats were killed at 10 min and 3 and 7 days after freezing of the urinary bladder at −15°C or −30°C for 2 s, for histopathological examination of the urinary bladder. The urinary bladders were inflated with 10% phosphate-buffered formalin solution and cut into 8 longitudinal strips. The tissues were then routinely processed for hematoxylin and eosin (H-E) staining.

Tissue preparation For DNA adduct analysis by the ^{32}P-postlabeling method, *in situ* freezing of the urinary bladder was performed under ether anesthesia for 2 s once at −15°C or −30°C. In the preliminary study, the amounts of DNA collected 10 min after freezing were not enough for this analysis, and 3 days after freezing was selected as the time of sampling. The urinary bladder was removed and quickly everted. The epithelial cells were collected by scraping gently with the edge of a cover glass in cold 0.024 *M* EDTA-0.075 *M* NaCl (pH 7.5) solution. After a short centrifugation at 1000 rpm, the epithelial cell sediment was immediately placed in a freezer at −80°C. Samples of the urinary bladder wall were immediately frozen at −80°C.

For alkaline elution analysis, freezing of the urinary bladder was performed for 2 or 30 s at −20°C or −45°C with rats under Nembutal anesthesia. At 10 min after freezing, the urinary bladder was quickly removed and everted. The epithelial cells were sampled in the same way as above in cold 0.024 *M* EDTA-0.075 *M* NaCl (pH 7.5) solution. Throughout the procedure, the room light was kept dim.

DNA preparation for ^{32}P-postlabeling DNA was isolated from the epithelial cells and the wall by a modification of the published procedure.[10] Briefly, the tissue and/or cells were homogenized in 20 m*M* Tris-HCl (pH 7.5) containing 10 m*M* EDTA and the homogenate was incubated in a mixture of ribonuclease A (100 μg/ml), ribonuclease T1 (70 U/ml) and 1% sodium dodecyl sulfate for 1 h, and with proteinase K solution (0.5 mg/ml) for another 1 h at 37°C. Then DNA was extracted, precipitated with ethanol and dissolved in 0.01×SSC (1.5 m*M* NaCl/0.15 m*M* sodium citrate). DNA concentration was determined spectrophotometrically at 260 nm, and the solution was kept at −80°C until analysis of DNA adducts.[10]

^{32}P-Postlabeling analysis of adducts Formation of DNA adducts was individually analyzed in 3 rats for each treatment by a modification of the ^{32}P-postlabeling technique reported by Randerath *et al.*[11, 12] Conditions for DNA digestion and ^{32}P-labeling were as described previously.[13, 14] Briefly, DNA was digested with micrococcal nuclease and spleen phosphodiesterase (Worthington Biochemical Co., Freehold, NJ) to deoxyribonucleoside 3′-monophosphates. The digest containing 15 nmol of nucleotides (5 μg DNA equivalent) was ^{32}P-labeled by using T4-polynucleotide kinase in the presence of carrier-free [γ-^{32}P]ATP (200 μCi, 7000 Ci/mmol, ICN Biochemicals, Irvine, CA) under ATP-deficient conditions.[14] The labeled digest (15 μl) was applied to a C18 reversed-phase TLC plate (Whatman Inc., Clifton, NJ), and the plate was first developed in 0.4 *M* ammonium formate (pH 6.0) at 4°C for 15 h. Adducts at the origin area were transferred to polyethyleneimine-cellulose sheets (POLYGRAM CEL 300PEI, Macherey-Nagel, Dueren, FRG) by the contact-transfer method and resolved by two-dimensional PEI cellulose TLC as described,[11] with the following solvents: 1.4 *M* lithium formate, 2.6 *M* urea buffer (pH 3.5) for the first dimension (D1); 0.3 *M* lithium chloride, 0.15 *M* Tris-HCl, 2.6 *M* urea buffer (pH 8.0) at right angles to the previous development (D2) followed by 1.7 *M* sodium phosphate (pH 6.0) with a 1.5 cm paper wick in the same direction as D2. Adducts were visualized by autoradiography on Kodak XAR-5 film with intensifying screens (DuPont Lightning Plus) at −80°C. The detection limit of this method for specific DNA adducts is approximately one adduct in 10^8–10^9 normal nucleotides as determined by comparison of the results with those of a standard method and a method with heterocyclic amines as model compounds.

Alkaline elution method The collected epithelial cells from 2 animals were loaded together onto a 2 μm polycarbonate filter (Nucleopore Co.) in the dark within 10 min after removal of the urinary bladder. The alkaline elution assay was performed according to the method of Sina *et al.*[15] with minor modifications as described previously.[16] The cells were lysed on the filters with 1.5 ml of pH 9.6 lysis solution in the presence of proteinase K (0.5 mg/ml, Merck). Single-stranded DNA was eluted in the dark with 15 ml of an alkaline solution (0.2% sodium dodecyl sulfate in 20 m*M* EDTA, tetrapropylammonium hydroxide, pH 12.1) at a pump speed of 0.034 ml/min. A fraction collector (FRAC-100, Pharmacia) was set to collect a fraction every 60 min. DNA contents in the eluate and on the filter were assayed by the microfluorometric technique of Kissane and Robins.[17] The elution rate constant (ml^{-1}) of DNA was calculated from the plotted line between the 1st and the 5th eluted fractions.

RESULTS

Histopathological findings At 10 min after freezing at −15°C, condensation of nuclei and loss of the fine appearance of the cytoplasm in all layers of the whole

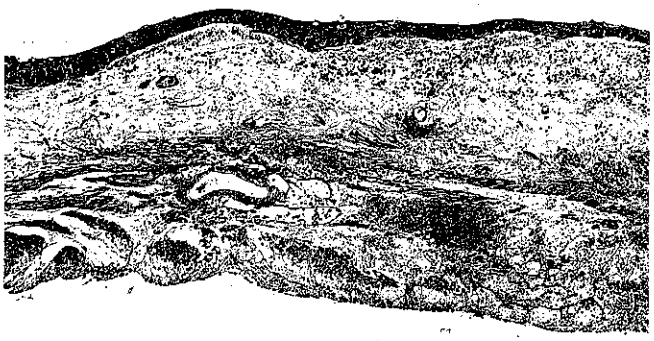

Fig. 1. Histological appearance of the rat urinary bladder 3 days after freezing at −15°C for 2 s. Freezing damage is widely observed in all the layers of the bladder. H-E, ×100.

Fig. 2. Regenerating epithelium observed at the edge of the ulcer induced by −15°C, 2 s freezing. H-E, ×250.

urinary bladder were microscopically observed. However, severe bleeding or apparent ulcer formation of the mucosa were not yet observed. Three days after freezing, necrosis of the epithelial and connective tissues associated with severe bleeding were found. Approximately one-third of the mucosal surface was denuded to form ulcers, and regeneration of the mucosa was obvious around the ulcerations (Figs. 1 and 2). Seven days after freezing, the regeneration of the epithelium was still occurring and the damage in the urinary bladder wall was almost the same as at day 3.

In the case of freezing at −30°C, the damage and the regenerative changes in the urinary bladder were almost same as those observed in samples frozen at −15°C.

DNA adduct formation Analysis of ^{32}P-adducts in the bladder epithelial DNA from control rats, in which the urinary bladder had been gently pinched with regular uncooled forceps, revealed the presence of at least 4 chromatographically distinct spots. Two of them were also weakly detected in the DNA extracted from the wall (Fig. 3).

When DNA collected after freezing was applied, 2 additional spots in the epithelial DNA and at least one minor spot in the wall DNA, which might be different from the epithelial ones, were observed (arrows). The profiles obtained in the experimental animals were reproducible and were seen in all the animals examined irrespective of the freezing temperature. The adduct levels for these additional spots observed after freezing were determined as about one per 10^8 nucleotides for both epithelial spots and about 2 to 3 per 10^9 nucleotides for the wall.

Alkaline elution assay As shown in Fig. 4, *in situ* freezing clearly caused DNA single-strand breaks. There was apparent temperature- and duration-dependent elution between −20°C and −45°C and between 2 and 30 s. Elution rates (ml^{-1}) were as follows: 0.159 for −45°C, 30 s; 0.069 for −20°C, 30 s; 0.036 for −45°C, 2 s; 0.007 for −20°C, 2 s; and 0.013 for untreated control. Although the effects of extensive cell death could not be eliminated since a rapid elution rate in the first fractions were observed, it was clear that DNA single-strand breaks were induced in the epithelial cells after freezing.

DISCUSSION

The ^{32}P-postlabeling method is thought at present to be the most sensitive technique to detect adducts in DNA, and has been widely used to analyze DNA adduct levels in relation to the possible carcinogenic potential of chemicals.[13, 14, 18] Using the ^{32}P-postlabeling method, we previously observed apparent DNA adduct formation in samples collected from the whole urinary bladder 3 days after *in situ* freezing performed by the same methods as the present study.[19] The present experiments confirmed this and clearly demonstrated that DNA adducts were predominantly formed in the urinary bladder epithelium rather than in the wall after *in situ* freezing of the urinary bladder.

The chemicals responsible for adduct formation should be very hydrophobic aromatic compounds because the conditions adopted here to separate adducts were much milder than those used in the case of heterocyclic amines.[18] The urine might indeed play a role in the generation of DNA adducts in the rat urinary bladder epithelium after freezing since it commonly contains significant levels of various metabolites including muta-

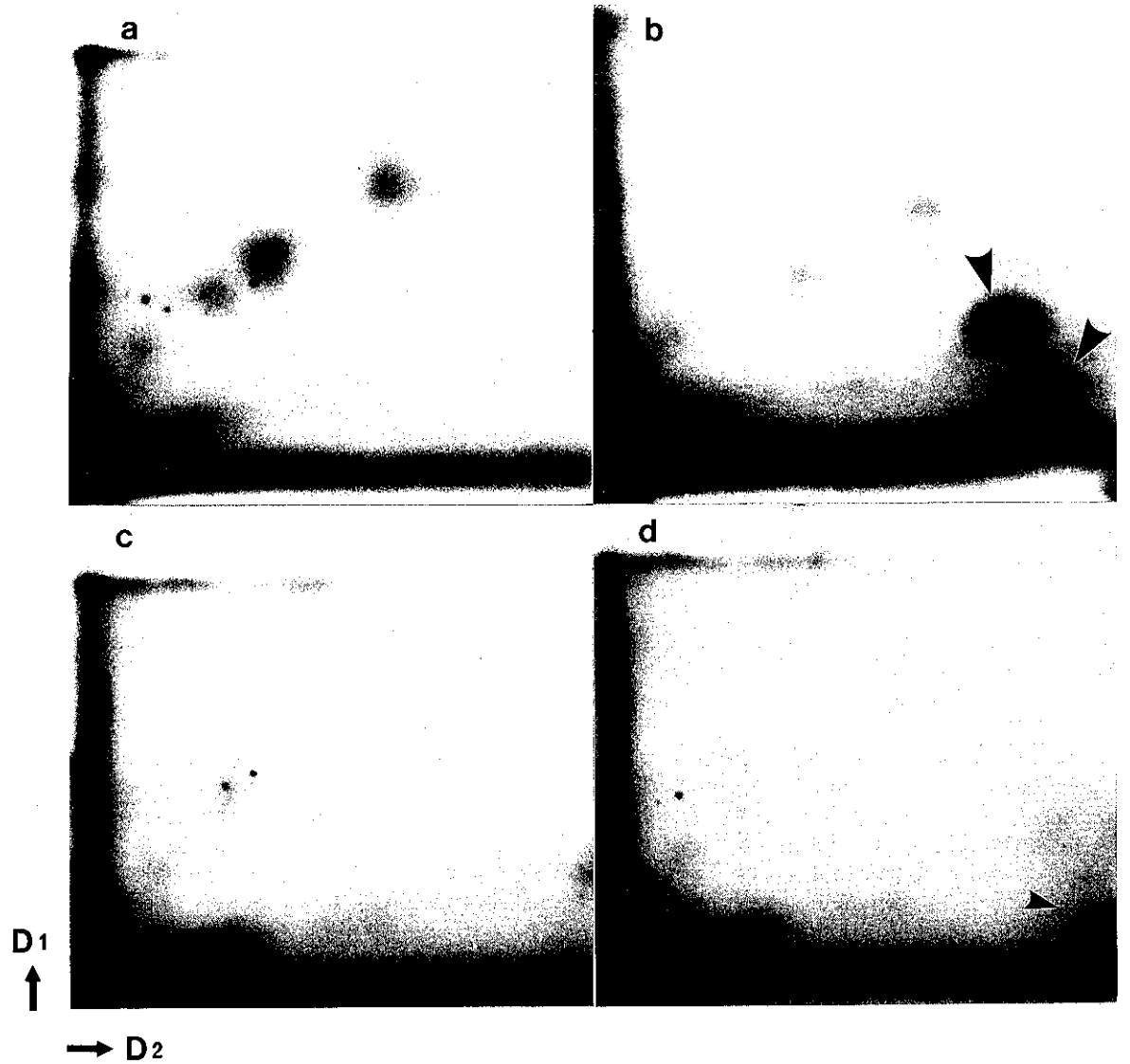

Fig. 3. ^{32}P-Postlabeling assay on DNA isolated separately from epithelium and wall of the bladder 3 days after *in situ* freezing of the bladder at −15°C for 2 s. (a) epithelium after sham operation, (b) epithelium after freezing, (c) wall after sham operation, and (d) wall after freezing.

genic substances[20–22] and it has been demonstrated that urinary bladder tumors will not develop in the urinary bladder without normal urine flow through the urinary tract.[23, 24] Furthermore, different modifying potential of *in situ* freezing in carcinogenesis has been observed between the rat urinary bladder[1–3] and the mouse skin.[8] This difference might be related to the anatomical differences as well as a critical role of the urine.[8] However, the blood still can not be neglected as a candidate for the source of DNA adduct formation.

Even in DNA samples obtained from the untreated control urinary bladder, several DNA adducts were observed. It is uncertain if the adducts detected in the untreated urinary bladder are different from the age-related adducts described in the other organs,[25, 26] or are formed during sample preparation in the presence of the urine. Tissue wounding and subsequent regeneration have been mainly discussed as a promoting factor in carcinogenesis of several organs,[27–33] including the urinary bladder.[34–36] Berenblum,[27] more than 50 years ago,

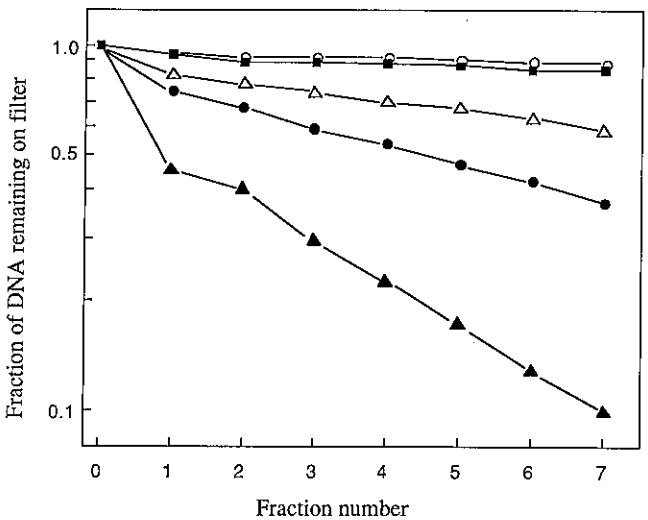

Fig. 4. Alkaline elution of the rat bladder epithelium collected 10 min after *in situ* freezing. ○: −20°C, 2 s; ●: −20°C, 30 s; △: −45°C, 2 s; ▲: −45°C, 30 s; ■: sham operation.

demonstrated that carbon dioxide snow painted on the backs of mice was weakly tumorigenic, but the effect was primarily related to the chronic irritating effect in promoting tumor formation initiated with coal tar.

It is generally recognized that cell proliferation is important in the carcinogenic process for fixation of altered gene information and for promotion of tumor development.[37] Cohen and his colleagues[38] proposed a mathematical model for the carcinogenic process based on their findings in the rat urinary bladder. In the model, variables include the population of cells, mitotic rates, hyperplasia and probabilities of cell initiation and transformation during replication. Based on this model, an increased incidence of tumors by initial freezing of the rat urinary bladder is thought to occur because of changes in the DNA that happen with a high rate of mitosis during the reparative phase. The formation of DNA adducts and DNA damage demonstrated by the alkaline elution method strongly supports this model.[1-3]

The alkaline elution assay, which was first demonstrated by Kohn *et al.*,[39] is also applicable to detect DNA damage of the rat urinary bladder mucosa.[16] In our previous work,[40] increased levels of DNA single-strand breaks were observed in epithelial samples from urinary bladder stored for 24 h at −20°C. Similar findings have been reported in irradiated frozen-stored bacteria.[41] Although DNA single-strand breaks demonstrated by the alkaline elution assay after *in situ* freezing might be partly the result of severe tissue damage relating to cell death, the fact that the freezing caused DNA breaks in the urinary bladder mucosa is meaningful, since regeneration of the mucosa begins around the ulcer where the mucosa was mildly to moderately frozen. It is also likely that DNA adducts were formed during DNA repair after DNA single-strand breaks, especially under the condition that the basal layer of the epithelium of the urinary bladder is exposed to the urine.

However, the development of an unusual type of papilloma,[3] or the high incidence of soft tissue tumors at skin wounds of the lower abdominal wall where the connective tissues may be somewhat exposed to frozen acetone solution during the freezing procedure,[2] suggests that freezing itself possesses specific effects related to tumor development besides DNA adduct formation. This is partly supported by the findings that freezing caused a variety of changes in the cells, including DNA damage,[41-43] and, also it is well known that spontaneous transformation will occur in stored frozen cells.[44]

It is possible that the reported tumor-initiating activity of *in situ* freezing in rat urinary bladder carcinogenesis[2,3] is associated with DNA modifications.

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