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Effects of seven chemicals on DNA damage in the rat urinary bladder: A comet assay study

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ABSTRACT

The *in vivo* comet assay has been used for the evaluation of DNA damage and repair in various tissues of rodents. However, it can give false-positive results due to non-specific DNA damage associated with cell death. In this study, we examined whether the *in vivo* comet assay can distinguish between genotoxic and non-genotoxic DNA damage in urinary bladder cells, by using the following seven chemicals related to urinary bladder carcinogenesis in rodents: *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), glycidol, 2,2-*bis*(bromomethyl)-1,3-propanediol (BMP), 2-nitroanisole (2-NA), benzyl isothiocyanate (BITC), uracil, and melamine. BBN, glycidol, BMP, and 2-NA are known to be Ames test-positive and they are expected to produce DNA damage in the absence of cytotoxicity. BITC, uracil, and melamine are Ames test-negative with metabolic activation but have the potential to induce non-specific DNA damage due to cytotoxicity. The test chemicals were administered orally to male Sprague-Dawley rats (five per group) for each of two consecutive days. Urinary bladders were sampled 3 h after the second administration and urothelial cells were analyzed by the comet assay and subjected to histopathological examination to evaluate cytotoxicity. In the urinary bladders of rats treated with BBN, glycidol, and BMP, DNA damage was detected. In contrast, 2-NA induced neither DNA damage nor cytotoxicity. The non-genotoxic chemicals (BITC, uracil, and melamine) did not induce DNA damage in the urinary bladders under conditions where some histopathological changes were observed. The results indicate that the comet assay could distinguish between genotoxic and non-genotoxic chemicals and that no false-positive responses were obtained.

1. Introduction

The comet assay was introduced by Singh et al. [1] as a simple method for detecting DNA damage at the individual cell level. The assay is relatively quick (requiring only a few days after administration of a substance) and can detect double-strand breaks, single-strand breaks, alkali-labile sites, incomplete excision repair sites, and DNA cross-linking with DNA or protein [2]. The comet assay has been used for genotoxicity studies in various tissues of experimental animals; however, a false-positive response can result from the effects of cytotoxicity, such as necrosis or apoptosis. Indeed, CCl_4 caused a false-positive response in the mouse liver, where histopathology showed centrilobular hepatocellular necrosis [3]. Therefore, histopathological examination is considered the "gold standard" for assessing necrosis and apoptosis, following positive results in an *in vivo* comet assay [4].

Data on the *in vivo* DNA damage associated with histopathological changes in the liver and stomach have been accumulated in an international validation study coordinated by the Japanese Centre for the Validation of Alternative Methods (JaCVAM). However, the *in vivo* comet assay has still to be validated for other organs and tissues.

Urinary bladder cancer is one of the most commonly diagnosed cancers among men in the USA [5]. Some chemicals are known to induce urinary bladder cancer in rats, and these have been classified according to their genotoxic or non-genotoxic mode of carcinogenicity [6]. The purpose of this study was to test whether the urinary bladder comet assay has the potential to distinguish between genotoxic and non-genotoxic responses, using urothelial cells collected from a whole urinary bladder *via* a simple mincing method [7] after short-term administration.

In this study, we performed the comet assay combined with histopathology on urothelial cells from rat urinary bladders treated with each of seven chemicals known to induce or promote urinary bladder carcinogenesis. Four of the chemicals,

Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; BMP, 2,2-*bis*(bromomethyl)-1,3-propanediol; 2-NA, 2-nitroanisole; BITC, benzyl isothiocyanate; MC, methylcellulose; % tail DNA, percentage of DNA in the tail.

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N-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) [8], glycidol [9], 2,2-*bis*(bromomethyl)-1,3-propanediol (BMP) [10], and 2-nitroanisole (2-NA) [11], are genotoxic and Ames test positive with metabolic activation [12–15], and are expected to show DNA damage in the absence of cytotoxicity. The remaining chemicals, benzyl isothiocyanate (BITC), uracil, and melamine, are classified as non-genotoxic promoters or carcinogens. BITC is a strong promoter of urinary bladder carcinogenesis [16]. Although BITC causes oxidative DNA damage [17], we categorized it as non-genotoxic for this study, based on reports that it is negative in the Ames test with S9 [18] and has some cancer-preventive activities [19]. Uracil and melamine are Ames test negative [20,21], but have cytotoxic effects on the urinary bladder, leading to the development of bladder cancer [22,23]. These chemicals were used to clarify assay performance in the presence of cytotoxicity-induced DNA damage. Although these chemicals are related to urinary bladder carcinogenesis, the liver was also evaluated, because it is the organ most often used for assessing the metabolic activation of chemicals in the comet assay [24].

2. Materials and methods

The study was conducted in accordance with the JaCVAM validation study protocol, version 14.2 [25], with minor modifications. The urinary bladder comet assay was performed using a simple method in which the bladder is minced with scissors and urothelial cells are collected preferentially [7].

2.1. Chemicals

The names, abbreviations, CAS numbers, suppliers, and vehicles used for the test chemicals are listed in Table 1. We used SYBR gold from Invitrogen Corp. (CA, USA), normal-melting-point agarose (GP-42) from Nacalai Tesque (Kyoto, Japan), and low-melting-point agarose (NuSieve GTG) from Lonza Group, Ltd. (Basel, Switzerland). Agarose was dissolved in Dulbecco's phosphate-buffered saline (without Ca^{2+}, Mg^{2+}, or phenol red) from Life Technologies Corp. (CA, USA).

2.2. Animals

The study was conducted in accordance with the Animal Care and Use Program at the Institute of Environmental Toxicology. Seven-week-old male Sprague-Dawley [Crl: CD (SD)] rats were used because, when BBN was administered, they showed higher DNA damage than did male or female F344 or Wistar rats (data not shown). The rats were purchased from Charles River Laboratories Japan, Inc. (Tsukuba, Japan), randomly assigned into groups of five animals each, and acclimated for seven days in the animal room (temperature, 22 ± 3 °C; humidity, 50 ± 20%; 12-h light/dark cycle). Standard laboratory pellet chow MF (Oriental Yeast Co., Ltd., Tokyo, Japan; in stainless steel feeding baskets) and tap water (in plastic bottles) were available *ad lib*.

2.3. Animal treatment

BBN, 2-NA, and BITC were dissolved in corn oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). BMP, uracil, and melamine were suspended in 0.5% methylcellulose (MC; Shin-Etsu Chemical Co., Ltd., Tokyo, Japan). Glycidol was dissolved in physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). The doses of each chemical that were used in previous sub-chronic and chronic studies of rats were as follows: BBN, 0.05% solution in drinking water [8]; glycidol, 37.5 and 75 mg/kg in distilled water, by gavage [9]; BMP, 2500 ppm–20,000 ppm in the diet [10]; 2-NA, 6000 and 18,000 ppm in the diet [11]; BITC, 0.1% in the diet [16]; uracil, 1% and 3% in the diet [22]; melamine, 4500 and 9000 ppm in the diet [23]. However, these doses were not used in the current study, because the comet assay can detect DNA damage after a single acute treatment [2]. Therefore, we administered the solvents and chemicals at the following doses. One dose (250 mg/kg) of BBN was used, based on the result of a preliminary comet assay, whereas two doses (high and low, the latter one-half the former) were chosen for the other six chemicals. The high doses of glycidol, 2-NA, and BITC were selected based on signs of toxicity, such that a higher dose would cause mortality or marked distress [2]. The limit dose of 2000 mg/kg [2] was used for the high uracil and melamine doses. The high and low doses of BMP were determined by the DNA damage induced in a dose range-finding study. The test chemicals and solvent controls were orally administered twice in a volume of 10 mL/kg body weight by gavage; the doses were administered 21 h apart. In this protocol, we were able to collect tissue/organ samples only once [24], which reduced the number of animals used. Clinical signs and body weight changes were recorded. The urine in the cage was examined for hematuria using urine test papers (Uriace®-Kc; Terumo Co., Tokyo, Japan). All animals were sacrificed 3 h after the second administration, and their urinary bladders and livers were removed for analysis.

2.4. Comet assay

The urinary bladder was cut in half, and one half was processed by the mincing method [7]. A portion of the left lateral lobe of the liver was cut out. The dissected tissues were washed in the cold mincing buffer (20 mM Na_2-EDTA and 10% DMSO in Hanks' balanced salt solution [Ca^{2+}- and Mg^{2+}-free], pH 7.5 for the liver, pH 9.0 for the urinary bladder) and minced with a pair of fine scissors for 20 s to release single cells. After centrifugation at 260 × *g* and 4 °C for 5 min, the cells were suspended in fresh buffer and placed on ice for several tens of seconds, to allow large clumps to settle; then, the supernatant was used to prepare comet slides. The remaining part of the urinary bladder and a portion of the left lateral lobe of the liver were fixed in 10% neutral buffered formalin for histopathology.

The comet assay was conducted under alkaline conditions. The cell suspension was mixed with 0.5% low-melting-point agarose at a ratio of 1:9. The mixture was dropped onto a bottom layer of 1.0% normal-melting-point agarose on glass slides (Matsunami Glass Ind. Ltd., Osaka, Japan). Two slides were prepared for each sample; they were coded, and the agarose was allowed to solidify at 4 °C for 10 min. Then, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, 1% Triton X-100, pH 10, and 10% DMSO) at 4 °C for 1 or 2 days. Next, the slides were rinsed with chilled pure water to remove residual detergent and salts and placed in electrophoresis solution (0.3 M NaOH and 1 mM Na_2-EDTA; pH > 13) for 20 min at 4 °C to allow the DNA to unwind. Electrophoresis was conducted in a horizontal electrophoresis platform in chilled electrophoresis solution for 20 min. The amperage was set to 300 mA and the voltage was 25 V (0.7 V/cm). The slides were neutralized with Tris–HCl buffer (pH 7.5) for 5 min, dehydrated by immersion in absolute ethanol for 5 min, and stained with SYBR gold. Cells (100 per tissue) with duplicate slides were analyzed with an Olympus fluorescence microscope equipped with an automatic digital analysis system (Komet 5.5; Andor Technology, Belfast, UK), and the percentage of DNA in the tail (% tail DNA), which is the most suitable measurement parameter of DNA-break frequency [26], was calculated. "Hedgehogs" that showed large diffuse tails containing 90% or more of the DNA were excluded from data collection; however, their frequency was measured as a reference for severe DNA damage per sample based on the visual scoring of 200 cells per tissue with duplicate slides.

2.5. Histopathology

Histopathology was performed on all bladder samples from rats treated with 0.5% MC, corn oil, and BBN. For the other six chemicals, histopathology was done only on samples treated with the higher doses. Similarly, histopathology was also performed on liver samples from rats treated with 0.5% MC, corn oil, and BBN as well as samples from rats treated with the high doses of glycidol, BMP, 2-NA, and BITC. The organs/tissues were embedded in paraffin wax and sectioned by a conventional method. Microscopic examinations were performed on sections stained with hematoxylin and eosin (H&E). Scores for severity were: normal/absent = 0; mild, focal, few or some = 1; mild/moderate, focal/diffuse, some or many = 2; moderate, diffuse, many = 3; severe, diffuse/almost all areas, many = 4. A final histopathology score was calculated by averaging the individual scores.

2.6. Statistics

To evaluate DNA damage, the mean % tail DNA in each treatment group was compared with the corresponding vehicle control group. Glycidol, which was dissolved in physiological saline, was compared to the 0.5% MC group. For the BBN treatment group, Student's *t*-test was used to compare between two groups ($P < 0.05$). For the other treatment groups, Dunnett's test was used to compare multiple groups ($P < 0.05$).

3. Results

3.1. Body weight gain or loss and clinical signs of toxicity

Body weight gain or loss and clinical signs of toxicity are shown in Table 2. Severe toxicities, such as emaciation and loss of spontaneous motor activity, were observed in animals treated with the high dose of 2-NA and in those treated with the low and high doses of BITC. Animals treated with glycidol, 2-NA, BITC, uracil, and melamine showed decreased body weight, whereas no decrease in body weight was observed in animals treated with BBN or BMP. Gross hematuria was detected in the urine of rats treated with the high dose of 2-NA, and the low and high doses of BITC, uracil, and melamine, as evidenced by an occult blood-positive test using urine test papers.

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