Loss of Heterozygosity in (LewisxF344)F1 Rat Urinary Bladder Tumors Induced with N-Butyl-N-(4-hydroxybutyl)nitrosamine Followed by Dimethylarsinic Acid or Sodium L-Ascorbate

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Dimethylarsinic acid (DMA), a main metabolite of arsenicals which are carcinogenic in man, exerts tumor-promoting activity on rat urinary bladder carcinogenesis initiated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN). Sodium L-ascorbate (Na-AsA) is also a strong tumor promoter in this animal model. In this study, we used (LewisxF344)F1 rats to compare molecular alterations in urinary bladder tumors caused by BBN followed by DMA or Na-AsA. Male, 6-week-old rats were given 0.05% BBN in their drinking water for 4 weeks, and then the rats in group 1 were maintained with no further treatment for 40 weeks. The animals of groups 2 and 3 were administered 0.01% DMA in their drinking water (group 2) or 5% Na-AsA in the powder diet (group 3) after the BBN treatment. Group 4 rats were given 0.05% BBN continuously for 36 weeks. At weeks 12, 20, 36 and 44, subgroups of rats were killed. Histopathological examination revealed promoting activity for DMA and, to a greater extent, Na-AsA on urinary bladder carcinogenesis. Loss of heterozygosity (LOH), detected with the polymerase chain reaction using 36 microsatellite markers, was found to be present in 2 of 9 (22%) urinary bladder tumors after treatment with DMA and 3 of 22 (14%) induced by continuous administration with BBN. No LOH was, however, detected in urinary bladder tumors after treatment with Na-AsA. The results thus suggest that the mechanisms of action of these two promoters, DMA and Na-AsA, may differ in rat urinary bladder carcinogenesis.

Key words: F1 rat — Urinary bladder tumor — LOH analysis — N-butyl-N-(4-hydroxybutyl)nitrosamine — Dimethylarsinic acid—Sodium L-ascorbate

Epidemiological studies indicate that arsenicals are carcinogenic for the lung and skin in man.1, 2) Dimethylarsinic acid (DMA) is a main metabolite of inorganic arsenicals in most mammals.3) Studies on the fate of DMA administered to mice and hamsters have shown about 80% of the inorganic arsenicals are excreted as DMA, eliminated through the kidneys and excreted in the urine.3) DMA is also found in natural water and bird eggsells,4) and in fact was used as a general herbicide or pesticide for many years.5) Recent in vitro studies indicate that DMA is a potent clastogenic agent and can induce chromosome aberrations, such as tetraploid formation.6, 7) Our laboratory has shown that DMA exerts promoting effects on F344 rat urinary bladder carcinogenesis.8, 9) There are, however, no reports on the in vivo carcinogenicity of DMA. Sodium L-ascorbate (Na-AsA) is well known to be itself a non-genotoxic strong promoter of rat urinary bladder carcinogenesis.10, 11) No p53 mutations were detected in urinary bladder tumors due to long-term treatment with Na-AsA after initiation with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN).12)

Molecular analysis has established that accumulated alterations of oncogenes and tumor suppressor genes lead to carcinogenesis.13) Allelic loss of specific chromosomal loci is one important mechanism involved in the development of human tumors, and loss of heterozygosity (LOH) of specific genetic markers suggests inactivation of tumor suppressor genes.14) LOH analysis is therefore used to determine the locations of potential tumor suppressor genes, for example in chromosomes 3, 4, 8, 9, 11, 13 and 17 in human urinary bladder tumors.15, 16) In the present study, LOH was investigated in urinary bladder tumors of (LewisxF344)F1 rats induced by DMA or Na-AsA administration after BBN treatment in a two-stage carcinogenesis model or continuous BBN treatment.

MATERIALS AND METHODS

Animals and experimental design A total of 120, 6-week-old (LewisxF344)F1 male rats were purchased from Charles River, Inc., Hino, Shiga. All animals were randomly divided into 4 groups of 30 animals each. Animals in group 1 were given 0.05% BBN (obtained from Tokyo Kasei Co., Osaka) in their drinking water for 4 weeks and...
then drinking water with no chemicals ad libitum for 40 weeks. Animals in groups 2 and 3 were similarly given 0.05% BBN and then 0.01% dimethylarsinic acid (DMA, Wako Pure Chemical Ind., Osaka) in drinking water, and 5% Na-AsA (Wako Pure Chemical Ind.) in powder diet, respectively, for 40 weeks. Group 4 rats were continuously given 0.05% BBN for 36 weeks. For groups 1 to 3, subgroups of 10 rats were killed to check the incidence of bladder lesions at week 12, 5 rats at weeks 20 and 36, and 10 rats at 44 week. In group 4, because of the toxic effects of BBN, 15 animals were killed at week 36. Single F1, Lewis and F344 rats (44 weeks old) were killed as controls for LOH analysis without treatment. All of the animals were killed under ether anesthesia. Urinary bladders were fixed in 10% buffered formalin at 4°C for 3 days and then embedded in paraffin for HE staining and light microscopic examination. Urinary bladder lesions were histopathologically classified into simple hyperplasia, papillary or nodular (PN) hyperplasia, and tumors, including papillomas and carcinomas, as previously described.\(^{17,18}\)

**LOH analysis** A microdissection technique was used to extract DNA from paraffin sections.\(^{19}\) Seven urinary bladders were randomly selected from group 2 (5 at 44 weeks, and 2 at 36 weeks), and all the tumors, 2 carcinomas and 7 papillomas, were analyzed. Five urinary bladders were selected from group 3 at 44 weeks, and all of the 15 carcinomas were examined. In group 4, because there were many tumors in the urinary bladders, we randomly selected 22 carcinomas. Papillomas in groups 3 and 4 were not examined. Single F1, Lewis and F344 rat urinary bladders were used as normal controls. Specimens were sectioned at 7 mm, and one sample was collected from 5 to 7 serial sections in each tumor and placed in a 1.5 ml tube with 80 µl of distilled water. Special care was taken to avoid mixtures of stromal or lymphatic tissue with tumor cells. After 150 µl of InstaGene (Bio-Rad Laboratories, Hercules, CA) was added to each tube, the mixtures were vortexed for 10 s, and then incubated at 56°C for 2 h and at 100°C for 10 min before storage at −20°C until examined. The primers (Table I) used for LOH analysis were randomly chosen from chromosome x and 1 to 20 (chromosomes 8 and 19 excised). The differences in base pairs between Lewis and Fischer 344 strains were from 8 to 54 and could be detected directly by 3% TAE agarose gel electrophoresis. Template DNA (1 µl) was mixed with primers (0.15 µl, 6 mM), dNTP (0.4 µl, 2.5 mM), 10× buffer (0.8 µl, 1.5 mM MgCl\(_2\) included), 25 mM MgCl\(_2\) (0.1 to 0.5 µl), Ampli Taq Gold (0.1 µl, 5 units/µl) and 5 µl of distilled water. Initial heating was done at 96°C for 10 min, and then 30 or 35 cycles of 95°C for 30s, 54–60°C for 30 s, and 72°C for 45 s were performed. Polymerase chain reaction (PCR) products were electrophoresed through 3% agarose gels in 1× TAE buffer to detect LOH. With the control DNAs, the Lewis and F344 inbred rats exhibited only single bands while the (Lewis×F344)F\(_1\) rats had both bands. For informative cases, allelic loss was scored if the amount of one allele was at least 70% reduced in the tumor DNA as compared with normal F\(_1\) allele. All LOH were checked in duplicate or triplicate.

**Statistical analysis** For the assessment of differences in lesion incidence, the Fisher exact probability test (StatView-J 4.02) was used. Student’s t test (StatView-J 4.02) was applied for analyses of other parameters.

**RESULTS**

The average body weights in group 4 decreased from week 20 (data not shown), and the animals were killed at week 36. Experimental termination for the other groups was at week 44. The average urinary bladder weights in every group were dependent on the tumor size and multiplicity, and there were significant intergroup differences (P<0.05). These were apparent at the gross pathology level, as shown in Fig. 1. The incidences of urinary bladder lesions, PN hyperplasia and tumors (papillomas and/or carcinomas) in this experiment are shown in Table II. At weeks 12 and 20, no bladder lesions were observed in groups 1 and 2. In group 3, 20% PN hyperplasia occurred at week 12 and all rat urinary bladders had PN hyperplasia at week 20. In group 4, all rats already had PN hyperplasia at week 12 and papillomas occurred at week 20 (60%). At 36 or 44 weeks, the incidences of papillomas and/or carcinomas were 7% in group 1, 47% in group 2, and 100% in groups 3 and 4. Data for numbers of urinary bladder

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Table I. Markers Used for LOH Analysis

| Chromosomes | Loci         |
|-------------|-------------|
| 1           | D1Mgh2, D1Mit13 |
| 2           | D2Mgh7, D2Mit12, D2Mit14 |
| 3           | D3Mgh2, D3Mgh3, D3Mgh10 |
| 4           | ENO2 |
| 5           | D5Mgh5, D5Mit4, D5Rjr1 |
| 6           | D6Mit6, D6Mgh5 |
| 7           | D7Mgh9 |
| 9           | D9Bro1 |
| 10          | D10Mgh7, D10Mit6, D10Mit9 |
| 11          | D11Mgh1, D11Mgh4, D11Mit1 |
| 12          | D12Mit2, D12Mit4 |
| 13          | D13Mgh7, D13Mit4 |
| 14          | D14Mit1, D14Mit2 |
| 15          | D15Mgh8 |
| 16          | D16Mit2 |
| 17          | D17Mgh5, D17Mit4 |
| 18          | D18Mit1 |
| 20          | D20Mgh1 |
| x           | DxMgh4, DxMit5 |
lesions are shown in Table III. In group 2, the number of tumors (including papillomas and carcinomas) was significantly increased as compared to group 1 ($P<0.05$). In group 3, PN hyperplasia and tumors were significantly increased in comparison to groups 1 and 2.

LOH in the urinary bladder tumors were detected in 2 tumors (papillomas, 22%) in group 2 and 3 tumors (carcinomas, 14%) in group 4. Thus the same frequencies of LOH were detected in groups 2 and 4. No LOH was detected in group 3. Details of the LOH are presented in Table IV. LOH were randomly distributed on chromosomes 2, 9, 11, 14, 18 and 20 and there was no hot-spot. From 1 to 3 markers were used for each chromosome to check allelic deletion. For example, three markers, D2Mgh7, D2Mit12 and D2Mit14, in different regions of chromosome 2 were used for the carcinoma (BT7) in group 4. As a result, only one LOH was detected at marker D2Mgh7. In a carcinoma in group 4 (BT6), there is only one deletion detected in 2 or 3 markers at one chromosome 11 or 14. The deletions were not thought to

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**Table II. Incidences of Urinary Bladder Lesions in Rats**

| Group | Treatment | Week 12 | Week 20 | Weeks 36 and 44 |
|-------|-----------|---------|---------|-----------------|
|       |           | PN hyperplasia | PN hyperplasia | Tumor | PN hyperplasia | Tumor |
| 1     | BBN→No treatment | 0 | 0 | 9/15 (60) | 1/15 (7) |
| 2     | BBN→DMA   | 0 | 0 | 8/15 (53) | 7/15 (47) |
| 3     | BBN→Na-AsA | 2/10 (20) | 5/5 (100) | 15/15 (100) | 15/15 (100)$^{a,b}$ |
| 4     | BBN continuously | 10/10 (100)$^{a}$ | 5/5 (100) | 3/5 (60) | 15/15 (100) | 15/15 (100)$^{a,b}$ |

Parenthesized numbers are percent incidences.

- $a$) $P<0.05$ vs. groups 1 and 2.
- $b$) $P<0.05$ vs. group 1.

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Fig. 1. Bladder lesions in groups 1 to 3 at week 44 and group 4 at week 36. In group 1 (A), there was only 1 small lesion in the bladder. In group 2 (B), the number of lesions was less than in group 3 (C) and the lesion size was also smaller. In group 4 (D), there were many large lesions in each bladder.
be due to monosomy and might only have been partial allelic losses. In one papilloma in group 2 (DT8), LOH was detected at marker D18Mit1 (at chromosome 18) in the Lewis rat allele (Fig. 2).

**DISCUSSION**

Both DMA and Na-AsA exerted promoting activity in rat two-stage urinary bladder carcinogenesis in the present study, as described previously. Na-AsA demonstrated stronger promotion potential at the doses tested, but was not associated with LOH. Na-AsA is well known to be a non-genotoxic promoter, leading to epithelial hyperplasia. We reported earlier a lack of genetic alterations (p53 and H-ras mutations) in F344 rat urinary bladder tumors induced with 4 weeks of BBN followed by 32 weeks of Na-AsA. In addition, we recently found that microsatellite instability, which reflects defective mismatch repair, was lacking in urinary bladder tumors induced by the same experimental protocol using Na-AsA. Therefore, the lack of genetic alterations in Na-AsA-induced urinary bladder tumors may suggest an epigenetic action of this promoter. However, since promoters may exert effects on DNA in other ways, further analyses are required to assess the underlying mechanisms. Cyclin D1 seems to be an important factor in promotion of rat two-stage urinary bladder carcinogenesis by Na-AsA, and cyclin D1 gene amplification is also a feature of some human urinary bladder carcinomas (unpublished data).

Arsenic is well known to be a carcinogen which causes cancers with genetic alterations in man. DMA is a metabolite of inorganic arsenicals, producing DNA damage in the lungs of the mouse and rat. In recent

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Table III. Number of Urinary Bladder Lesions per Rat

| Group | Treatment                  | Week 12 | Week 20 | Weeks 36 and 44 |
|-------|----------------------------|---------|---------|-----------------|
|       |                            | PN hyperplasia | PN hyperplasia | Tumor          | PN hyperplasia | Tumor          |
| 1     | BBN→No treatment           | 0       | 0       | 0               | 0.9±0.8        | 0.1±0.26       |
| 2     | BBN→DMA                    | 0       | 0       | 0               | 1.2±1.9        | 0.5±0.7*a*    |
| 3     | BBN→Na-AsA                 | 0.8±3.7 | 11.8±5.8[b] | 0              | 13.6±4.81[b]  | 3.5±1.2[b]    |
| 4     | BBN continuously            | 33.7±13.7[c] | 54.8±52.7[c] | 2.2±4.2        | 22.7±6.1[c]   | 18.3±4.2[c]   |

*a* P<0.05 vs. group 1.

*b* P<0.05 vs. groups 1 and 2.

*c* P<0.05 vs. groups 1, 2 and 3.

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Table IV. LOH Analysis in Urinary Bladder Tumors of (Lewis×F344)F1 Rats

| Tumors | Histology | Loci | PCR products (bp) | Deleted allele |
|--------|-----------|------|------------------|---------------|
|        |           |      | Lewis | F344              |
| BBNDMA |           |      |       |                  |
| DT1    | Papilloma | D9Bro1 | 160   | 150 | F344 |
|        |           | D14Mit2 | 193   | 178 | Lewis |
| DT8    | Papilloma | D11Mgh1 | 140   | 156 | F344 |
|        |           | D18Mit1 | 309   | 255 | Lewis |
| BBN continuously | | D20Mgh1 | 236   | 204 | Lewis |
| BT6    | Carcinoma | D11Mgh1 | 140   | 156 | F344 |
|        |           | D14Mit2 | 193   | 178 | Lewis |
| BT7    | Carcinoma | D2Mgh7 | 220   | 236 | Lewis |

Fig. 2. LOH analysis by the PCR method. Note the single bands for the Lewis and F344 rat cases, whereas both bands are seen with the F1 rat. In case 1 (DT8, a papilloma in group 2) LOH was detected at marker D18Mit1 in Lewis rat allele.

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D18Mit1

1 2 3 Lewis F344 F1
studies, it was found that DMA exerts promoting activity on rat two-stage urinary bladder carcinogenesis, as confirmed in the present study. The question of whether DMA is a carcinogen or a promoter, has not been answered, but the appearance of LOH in DMA-treated rat urinary bladder, at a similar incidence to that caused by continuous BBN treatment is indicative. The sensitivity of the urinary bladder in F1 rats is low and we obtained only 9 tumors in the DMA-treated group, though we found LOH in these tumors. The results suggest a difference in the mechanism of promoting activities between DMA and Na-AsA, although both could affect cell proliferation.

No LOH was detected in chromosome 10 on which rat p53 is located, despite the fact that recent findings and those in literature, we speculate that DMA may be carcinogenic to the rat urinary bladder and indeed, we recently found that F344 male rats treated with 0.01% or 0.02% DMA for 2 years without any initiating pretreatment, had urinary bladder tumors (Carcinogenesis, in press).

In summary, we conclude that DMA enhances urinary bladder carcinogenesis in (Lewis×F344)F1 rats and that this might be associated with genetic alteration, in contrast to the case with the promoter Na-AsA. Based on our recent findings and those in literature, we speculate that DMA may be a complete carcinogen for the rat urinary bladder.

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