Metallothionein modulates the carcinogenicity of N-butyl-N-(4-hydroxybutyl)nitrosoamine in mice

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We examined the carcinogenicity of N-butyl-N-(4-hydroxybutyl)nitrosoamine (BBN) in transgenic mice deficient in the metallothionein (MT) I and II genes and in control (129/Sv) mice. Both strains of mice were given BBN for 8 weeks with or without Zn treatment. All mice were killed at 12 weeks after the cessation of BBN administration. BBN induced bladder tumors in 75% of MT null mice and in 43% of 129/Sv mice. The average number of bladder tumors per mouse was significantly higher in MT null mice (1.18 ± 0.27) than in 129/Sv mice (0.43 ± 0.20). Zn treatment suppressed the carcinogenicity of BBN in 129/Sv mice but not in MT null mice. Histopathological examination of the tumors revealed that the malignant potential of bladder tumors in 129/Sv mice was greater than that in MT null mice. These results indicate that MT is an important modulator of carcinogenicity of BBN in the bladder of mice.

Almost 30 years ago, it was reported that dietary Zn inhibited chemical carcinogenesis, but little information on the role of Zn to explain this observation was available at that time (1). We now recognize that many proteins, including transcription factors, require Zn for their functionality. Zn homeostasis may be regulated, in part, by an intracellular Zn-binding protein, metallothionein (MT). MT is a low molecular weight, thiol-rich protein constitutively expressed in a wide variety of organisms from yeast to human. MT strongly binds to heavy metal ions such as Zn and Cu, essential trace elements, as well as Cd and Hg, potentially toxic elements. The roles of metal ions such as Zn and Cu, essential trace elements, as well as Cd and Hg, potentially toxic elements. The roles of metal ions such as Zn and Cu, essential trace elements, as well as Cd and Hg, potentially toxic elements. The roles of MT include metal detoxification and homeostasis, but the full biological functions of this protein have not yet been resolved. MT is readily inducible by metals, hormones and cytokines. The promoter region of the MT gene contains not only a specific metal-responsive element but also a 12-O-tetradecanoylphorbol-13-acetate-responsive element; the latter is known to be involved in tumor progression (2). Several studies suggested that the expression of MT confers protection against the cytotoxicity of not only heavy metals but also electrophilic mutagens such as N-methyl-N′-nitrosoguanidine and N-nitro-N-methylurea as well as anticancer agents such as chlorambucil, melphalan and cisplatin (3,4). In addition, MT is reported to reduce the carcinogenic effects of Cd (5).

Some anticancer agents, such as etoposide, cisplatin and melphalan, are able to induce secondary carcinogenesis in chemotherapy (6). In a previous study, we reported that the MT induced by Bi or Zn efficiently reduced the carcinogenicity of anticancer agents such as cisplatin and melphalan in A/J mice (7). However, whether the reduced carcinogenicity was due to induced MT or due to other effects of the metals has not yet been clarified. In general, the utilization of MT inducers for the study of MT function has a limitation in that the applied inducers have multiple actions in addition to MT induction. The generation of mice in which the genes for the major isoforms of MT (I and II) are disrupted has provided a model to examine the biological attributes of MT (8,9). In this study, we examined the carcinogenicity of N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in transgenic mice deficient in the MT I and II genes (MT null mice) with or without Zn treatment. MT null mice developed by Masters et al. (9) as well as the control mice (129/Sv) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at Kitasato University (Tokyo, Japan). Five mice (22–24 g) were housed per cage under specific-pathogen-free conditions with free access to water and diet. Forty-six MT null mice and 25 129/Sv mice were divided into four groups and treated with BBN and/or ZnCl2. Mice were given drinking water containing 0.05% BBN (Tokyo Chemical Industry, Tokyo, Japan) and 0.01% Tween 20 or tap water ad libitum for 8 weeks. ZnCl2 (100 μmol/kg) or saline was administered s.c. to mice twice a week for the 8 weeks of BBN administration. All mice were killed at 12 weeks after the cessation of BBN administration.

For histopathological studies, tissue samples were fixed in 10% neutral formalin at room temperature and then embedded in paraffin at 56°C. The sections (4 µm thick) obtained from the corresponding tissue blocks were stained with hematoxylin and eosin. The MT levels in the liver, kidney and bladder were determined by Hg binding assay (7).

BBN is one of the nitrosamines that induces tumors specifically in the bladder. As shown in Table I, BBN treatment induced bladder tumors in both strains of mice. To our knowledge, this is the first observation of tumors in which no MT was expressed. The percent of tumor-bearing MT null mice (75.0%) was higher than that for 129/Sv mice (42.9%), though the difference was not statistically significant. The average number of bladder tumors in MT null mice (1.18 ± 0.27) was significantly greater than that in 129/Sv mice (0.43 ± 0.20). These results suggest that MT has a protective role against the generation of bladder cancer by BBN. In support of our observation, Zhang et al. recently reported that treatment with 7,12-dimethylbenz[a]anthracene (DMBA) induced skin tumors in MT null mice only, while no change was observed in the control mice given the same doses of DMBA (10).

In a previous study (7), we reported that treatment of A/J

Abbreviations: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; DMBA, 7,12-dimethylbenz[a]anthracene; MT, metallothionein.
mice with Zn or Bi reduced the carcinogenicity induced by cisplatin and melphalan, but other effects of Zn or Bi than the induction of MT could not be excluded. As shown in Table I, the administration of Zn in MT null mice did not reduce the frequency of tumor-bearing mice nor the average number of tumors, while a decrease in both indicators were observed in 129/Sv mice. These data suggest that the induction of MT may be the major cause of Zn-induced suppression of tumor formation.

Tissue MT levels determined at the time of death (12 weeks after the cessation of BBN and Zn administration) were about the same in all groups and as low as the basal level (data not shown). Further, an elevation in MT in the liver and kidney due to tumor formation was not observed. To examine the extent of MT induction by ZnCl₂, five mice were injected with a single dose of ZnCl₂ (100 μmol/kg i.p.) and killed at 24, 48 and 72 h after administration. MT levels in the liver markedly increased at 24 h after the administration of ZnCl₂ and then decreased with time (Table II). MT was also induced in the kidney but the extent of induction was much lower than that in the liver. In the bladder, MT levels of untreated mice were not less than that of the liver and kidney. However, induction of bladder MT was only 1.6-fold of the control mice at 24 and 48 h after administration of ZnCl₂. MT levels in the liver, kidney and bladder of MT null mice were around the detection limit even after ZnCl₂ treatment (data not shown). These data suggested that MT levels in 129/Sv mice were kept high during the period of the treatment with BBN and Zn. Furthermore, tissue MT levels in mice which received BBN for a week were examined to determine whether BBN itself induces MT or not. The MT levels in the liver, kidney and bladder were not increased by the administration of BBN (data not shown).

A protective role of MT against carcinogenesis has been observed in a few studies (7,10), but the underlying mechanism remains unclear. As an antioxidant (11), MT may delay the promotion step of carcinogenesis. Enhanced cytotoxicity by DNA damaging agents such as UVC and cisplatin was observed in primary embryonic cells lacking the MT I and II genes (4). An antimutagenic effect of MT has also been reported (3). The preferential localization of MT in the nucleus in transformed cells (12–14) may be beneficial for protection of DNA. On the other hand, Zaia et al. demonstrated that incubation of MT with chlorambucil yielded a covalent adduct and suggested a role of MT in the direct sequestration of chemicals (15). However, there has been no evidence that MT can form a covalent adduct with BBN. Further studies are required to clarify the mechanism of how MT reduces the carcinogenicity of BBN.

Histopathological examination revealed that all tumor samples showed morphological changes characteristic of transitional cell carcinoma in both MT null and 129/Sv mice (Figure 1). However, the major bladder tumors in 129/Sv mice displayed the features of high grade (grade III), including adenomatous changes, whereas none of the tumors in MT null mice were designated as grade III. Invasion of the muscle layer by tumor cells was observed only in 129/Sv mice. In addition, the bladder tumors in 129/Sv mice were larger than

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**Table I. Induction of bladder tumors by BBN in 129/Sv and MT null mice with or without ZnCl₂ treatment**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Zn treatment</th>
<th>No. tumor-bearing mice</th>
<th>No. tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>129/Sv</td>
<td>-</td>
<td>3/7 (42.9)</td>
<td>0.43 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1/5 (20.0)</td>
<td>0.20 ± 0.20</td>
</tr>
<tr>
<td>MT null</td>
<td>-</td>
<td>9/12 (75.0)</td>
<td>1.18 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>8/11 (72.7)</td>
<td>0.91 ± 0.21</td>
</tr>
</tbody>
</table>

No bladder tumor was detected in 129/Sv and MT null mice receiving tap water.

*No. of mice with tumors/no. of total mice.

*Percent of mice having bladder tumors.

*Values are mean ± SE.

*Significantly different from 129/Sv mice without Zn treatment (P < 0.05, Student’s t-test).

*Significantly different from 129/Sv mice with Zn treatment (P < 0.05, Student’s t-test).

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**Table II. Levels of MT in the liver, kidney and bladder of 129/Sv mice given a single dose of ZnCl₂ (100 μmol/kg) treatment**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time after injection (h)</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>5.06 ± 0.38</td>
<td>259.7 ± 20.7</td>
<td>33.1 ± 3.4</td>
<td>40.3 ± 11.0</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>1.99 ± 0.45</td>
<td>12.9 ± 3.4</td>
<td>5.73 ± 0.52</td>
<td>7.43 ± 0.53</td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>6.62 ± 1.38</td>
<td>10.6 ± 1.45</td>
<td>10.8 ± 3.55</td>
<td>7.75 ± 1.08</td>
<td></td>
</tr>
</tbody>
</table>

The values are mean ± SE for four mice. Concentrations of MT were expressed as nmol Hg bound/g tissue.

*Significantly different from the control (0 h) (P < 0.01, Student’s t-test).

*Significantly different from the control (0 h) (P < 0.05, Student’s t-test).

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**Fig. 1.** Histopathological study of bladder tumor in 129/Sv mice (A) and MT null mice (B). Hematoxylin and eosin stain. (A) ×40; (B) ×200.
those in MT null mice. Thus, the bladder tumors in 129/Sv mice exhibited more malignant potential than those in MT null mice.

The relationship between tumor grade and MT expression has so far been documented by several studies. The studies on human cancers reported that cancer tissues containing higher amounts of MT tended to show a higher grade and a poorer prognosis (13,14). In rats treated with BBN, MT was induced only in transitional cell carcinomas but not in benign hyperplastic epithelial cells of the bladder (16). However, the causal relationship between MT expression and tumor grade has not yet been clarified. Tumor cells are known to release various kinds of cytokines which may induce MT in the tumor cells in an autocrine or paracrine mode. Our data have shown more malignant potential of bladder tumors in the control mice than in MT null mice, suggesting that MT expression may affect the malignancy of tumors.

In the present study, we have demonstrated that MT may be able to suppress chemical carcinogenesis, but once the cancer tissue begins to grow, MT appears to be involved in cancer progression. Establishment of cancer cell lines with or without MT expression from MT null and control mice may be useful for the elucidation of the relationship between MT expression and cancer progression.

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