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Localization of zinc and metallothionein in the rat bladder epithelium during carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine

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Abstract This study investigated the presence of zinc and expression of metallothionein (MT) in different pathological changes of the rat bladder induced by administration of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN). Using the Timm staining method, the presence of zinc was observed in normal and benign hyperplastic epithelial cells of the rat bladder, particularly in the malignant bladder tumor, induced by the administration of BBN. Immunohistochemically, MT expression was detected only in noninvasive (30%) and invasive transitional cell carcinoma (80%) of the rat bladder where the tumor cells were rich in zinc. Our data suggest that: (1) growth and development of the rat bladder tumor, especially malignant tumors, may have a high requirement for zinc and (2) MT synthesis may be induced by a high zinc concentration in rat bladder tumor cells.

Key words Zinc · Metallothionein · Carcinogenesis · Rat bladder · Transitional cell carcinoma

Introduction

Zinc is an essential participant in a number of DNA and RNA polymerases and serves as a structural feature of the zinc finger domains in at least 300 DNA-binding proteins [22]. Zinc has been found in all organs, fluids and cells of the human body, and may play a role in normal growth, in keratinization of the skin, in spermatogenesis and in the process of tumor cell proliferation. It has been reported that dietary zinc deficiency can increase the incidence of some tumors while decreasing the incidence of others [2]. Serum and tissue zinc levels have also been reported to be lower in cancer patients than in controls, but epidemiological studies have suggested a positive association of zinc intake and certain cancers [18].

Metallothioneins (MTs) are a group of intercellular metalloproteins of low molecular weight with a high content of cysteinyl residues. The biological functions of MTs have not been clearly resolved. Since their discovery, MTs have been reported to have a physiological role in the absorption, transport and metabolism of the important trace metals zinc and copper, as well as a role in heavy metal detoxification [9]. Recently, detection of MT has been reported in some human cancers including bladder cancer, and association of MT with pathological parameters has also been discussed [1, 5, 13, 17].

Although research concerning the relationships of zinc intake and carcinogenesis, and of MT expression and bladder tumor, has been undertaken, any correlation between zinc existence and MT detection in different bladder pathological changes has not been investigated. In this study, we investigated the presence of zinc histochemically and the expression of MT immunohistochemically in the rat bladder epithelium during 28 weeks of bladder carcinogenesis induced by administration of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN).

Materials and methods

Sixty-five male Wistar rats (Kurea Co., Osaka), 7 weeks of age at the time of the start of the experiment, were divided into a control group consisting of 15 rats and a BBN group consisting of 50 rats. BBN (Tokyo Kasei Industry Co., Tokyo) was administered at a dosage of 0.05% in the drinking water for 28 weeks. The commercial stock diet (Oriental MF, Osaka) was supplied to the rats in the two groups. Three rats of the control group and ten rats of the BBN group were sacrificed at 4, 8, 12, 20 and 28 weeks from the beginning of the

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experiment. The bladders obtained were cut into two parts and each was processed for either histochemical or histological and immunohistochemical analysis. Classification of different pathological lesions of the rat bladder induced by BBN administration, including three types of hyperplasia, papilloma and TCC, was based on previous reports [6, 7, 23]. Histological findings of the tumor were described according to the classification system of the International Union Against Cancer [10].

Histochemical staining

For histochemical examination, the Timm staining method [19] was used. The rat bladder tissues were fixed for 48 h in 100% ethanol saturated with hydrogen sulfide and embedded in paraffin according to routine techniques. Deparaffinized 4-μm-thick sections were developed for 60 min at 26 °C in the dark. The composition of the developer was as follows: 60 ml filtered gum arabic solution, 10 ml sodium citrate buffer and 0.85 g hydroquinone dissolved in 15 ml distilled water. Immediately before use, 0.121 g silver lactate in 15 ml distilled water was added and the solution was mixed thoroughly. After development, sections were rinsed in water and then incubated in a solution of 5% sodium thiosulfate for 10 min followed by a rinse in water. Finally the sections were dehydrated and counterstained with hematoxylin. Timm staining results were scored as negative reaction (no staining), mild reaction (less than 50% staining cells) and strong reaction (more than 50% staining cells).

Immunohistochemical staining

The specimens were placed in 10% formalin solution and processed for paraffin embedding. Each paraffin block was step-sectioned and stained with hematoxylin and eosin for histopathological examination. Two slides were selected from each paraffin block and these were then deparaffinized. Mouse monoclonal antiserum to MT (Dako Co.) (dilution 1:50) was used.

Endogenous peroxidase was blocked with 2.5% hydrogen peroxide (H_2O_2) in methanol. The sections were washed with TRIS-buffered saline (TBS) and incubated with 5% normal goat serum in TBS for 20 min. After being washed with TBS, the sections were incubated with the above antibody for 4 h at room temperature. After further washing, anti-goat IgG was diluted to 1:200 in TBS and incubated on the sections for 30 min. The sections were then washed and incubated with avidin-biotin peroxidase complex for 30 min. Peroxidase was demonstrated by the addition of diaminobenzidine tetrahydrochloride (DAB). All slides were counterstained with hematoxylin, dehydrated and mounted with Permount.

MT immunostaining results were scored as follows: 0 = no staining cells, 1 + = less than 50% staining cells, and 2 + = more than 50% staining cells. The bladder tissues with 2 + staining intensity were classified as showing MT expression.

Results

Histopathological findings

The rat bladder epithelium showed normal in all 15 rats within the period of 28 weeks in the control group. In the BBN group, all ten rats showed mild hyperplasia at 4 weeks and moderate hyperplasia at 8 weeks. At 12 weeks, severe hyperplasia was found in eight rats, and papilloma was found in two rats. At 20 weeks, noninvasive (stage Ta) and grade 1 transitional cell carcinoma (TCC) of the rat bladder was observed in all ten rats. Invasive (stage T2) and grade 2 TCC of the rat bladder occurred in all ten rats at 28 weeks.

Histochemical findings

Using the Timm staining method, a mild reaction showing the local presence of zinc on the membrane and top of the luminal cells was observed both in normal epithelium of the rat bladder in the control group and in the benign hyperplastic epithelium of the rat bladder in the BBN group from 4 to 12 weeks (Fig. 1). A strong reaction was observed and the presence of zinc was well distributed in the tumor cells of the rat bladder with noninvasive TCC at 20 weeks and with invasive TCC at 28 weeks as well as in the interstitial stroma (Fig. 2).

Immunohistochemical findings

MT immunostaining was not detected in all 15 rats in the control group. Following BBN administration, MT expression was detected in three of the ten rats with noninvasive TCC at 20 weeks (30%) and in eight of the ten rats with invasive TCC at 28 weeks (80%). Both cytoplasmic and nuclear staining patterns were observed. At 20 weeks, cytoplasmic staining was observed in one rat, nuclear staining in one rat and both in one rat. At 28 weeks, cytoplasmic staining was observed in four rats, nuclear staining in rats and both in two rats (Fig. 3). Table 1 shows the relationship between the histopathological findings and the histochemical, immunohistochemical staining results.

Discussion

The Timm staining method is considered a highly sensitive histochemical procedure for the localization of heavy metals, mainly for zinc in biological tissues [19]. Using this method, we observed the presence of zinc in normal and benign hyperplastic epithelial cells of the rat bladder, particularly in the malignant bladder tumor, induced by the administration of BBN.

Zinc has long been considered to be one of the least toxic metals in the environment, and many reports have shown that exposure to zinc presents no risk [3, 14]. In contrast, excessive intake of zinc has been reported to be related to cancer of the oesophagus and stomach [16]. Recently, some investigators pointed to a role for zinc in contributing to spontaneous mutagenesis in Chinese hamster V79 cultured cells [8]. On the other hand, it was found that ingestion of zinc inhibited the development of tumors [20]. An inhibitory effect of zinc on nickel subsulfide, a potent muscle carcinogen,

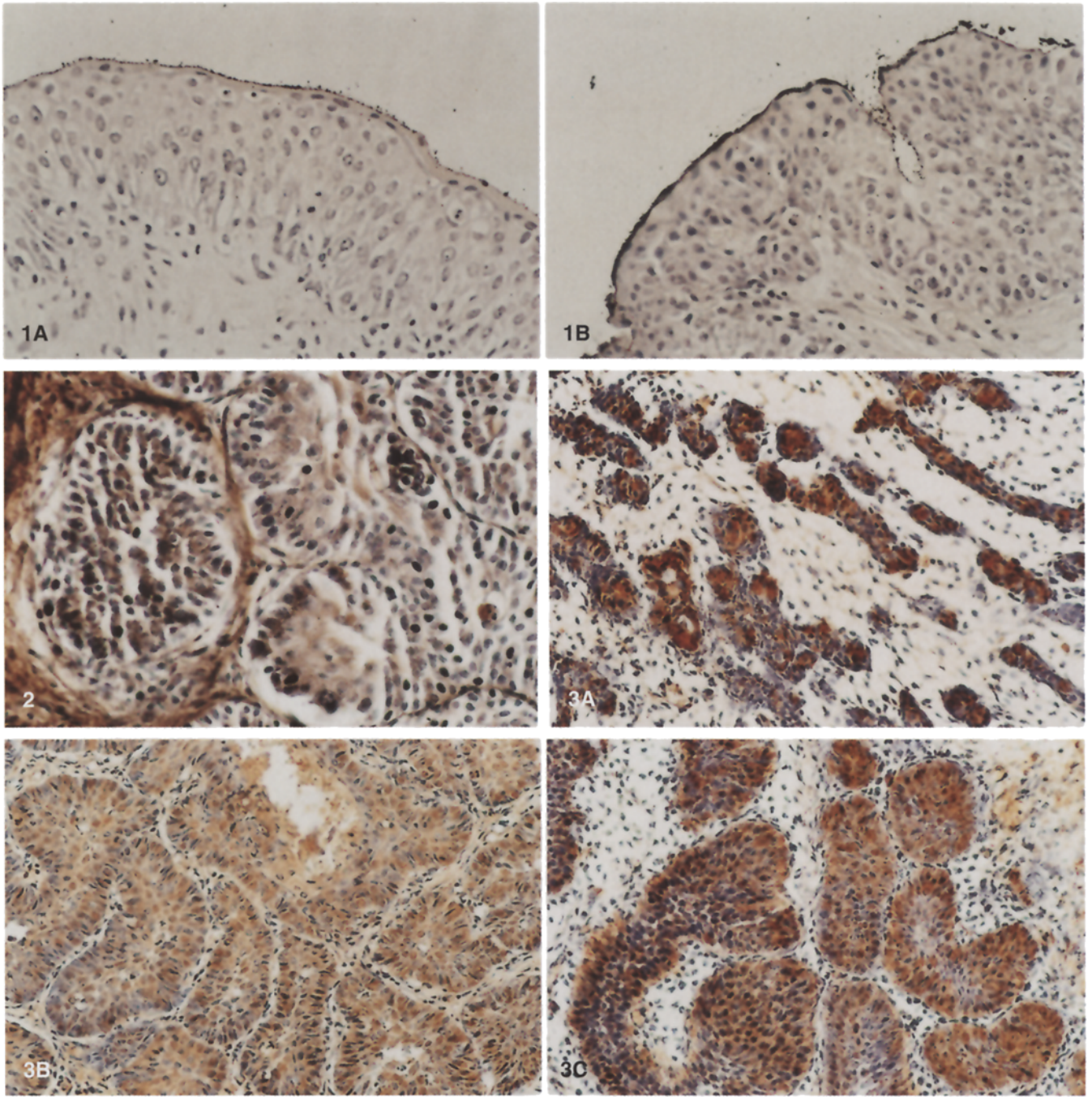

Fig. 1A, B Mild reaction for Timm staining in rat bladder epithelium. **A** BBN group, 4 weeks, ×100. **B** BBN group, 12 weeks, ×76

Fig. 2 Strong reaction for Timm staining in invasive TCC of the rat bladder in the BBN group, 28 weeks, ×115

Fig. 3A–C Expression of MT in noninvasive and invasive TCCs of the rat bladder. **A** Cytoplasmic staining pattern in invasive TCC of the rat bladder in the BBN group, at 28 weeks, ×60. **B** Nuclear staining pattern in noninvasive TCC of the rat bladder in the BBN group, at 20 weeks, ×76. **C** Both cytoplasmic and nuclear staining patterns in invasive TCC of the rat bladder in the BBN group, at 28 weeks, ×76

Table 1 Relationship between histopathological findings and histochemical, immunohistochemical staining results (*HP* hyperplasia)

		Control group	BBN group				
		4–28 weeks	4 weeks	8 weeks	12 weeks	20 weeks	28 weeks
No. of rats		15	10	10	10	10	10
Histopathological findings	Normal	15					
	Mild HP		10				
	Moderate HP			10			
	Severe HP				8		
	Papilloma				2		
	Ta and G1 TCC					10	
	T2 and G2 TCC						10
Histochemical (Timm) staining	Negative reaction						
	Mild reaction	15	10	10	10		
	Strong reaction					10	10
Immunohistochemical (MT) staining	0 score	9	7	8	5	4	1
	1+ score	6	3	2	5	3	1
	2+ score					3	8

was also observed in Fischer rats [12]. Until now, however, there has been no definite suggestion of a relationship between zinc and carcinogenesis.

In our study, although the existence of zinc has been observed either in normal or in the proliferative epithelial cells of the rat bladder induced by BBN, a large difference has been found in the Timm staining reaction and the distribution between benign and malignant bladder lesions, while no difference between normal and benign hyperplastic tissues has been found. According to these findings, it is suggested that growth and development of the bladder tumor, especially malignant tumors, may have a high requirement for zinc.

MT is synthesized in most body tissues, and the highest concentrations are found in the liver and kidney. Recently, MT has been found to be localized in a few kinds of human cancer tissues. A high positivity for MT expression has been demonstrated in testicular embryonal carcinoma (100%), thyroid tumors (91%), breast carcinoma (47%) and bladder cancer (100%) [1, 5, 13, 17]. In addition, studies of breast carcinoma have shown a close correlation between MT expression and invasive breast carcinoma with a poor prognosis [5], whereas other investigators found MT expression was most intense in superficial uroepithelial cells in areas of dysplasia and/or carcinoma in situ and less consistent in the invasive portions of the human bladder TCC [1]. In the present study, MT expression was detected in 30% of noninvasive TCCs and in 80% of invasive TCCs of the rat bladder while MT has not been detected in the benign hyperplastic lesions of the rat bladder. This result also reveals a correlation between MT expression and invasive TCC of the rat bladder induced by administration of BBN.

Another striking result of this study is that MT expression was only detected in the noninvasive and invasive TCC of the rat bladder, where the tumor cells are rich in zinc, during the period of bladder carcinogenesis. It has been suggested for many years that MT may function in the regulation of zinc metabolism, i.e., its storage, transport and distribution. On the other hand, MT synthesis can easily be induced by physiological heavy metals such as zinc [11]. It is believed to be important to maintain a normal zinc balance in the cells, not only to provide zinc when it is needed but also to protect DNA from its genotoxicity, since a sudden increase in the intercellular zinc concentration might lead to DNA damage. Our present results indicate that the bladder tumor cells not only need zinc for their growth and development but also need MT to protect themselves. Based on these findings, it is suggested that MT synthesis in the rat bladder tumor may be induced by a high zinc concentration in the rat bladder tumor cells.

However, in addition to the heavy metals, MT synthesis can be induced by a wide variety of compounds, including the glucocorticoids [15], estrogens and interleukin 1 [4]. Furthermore, a correlation between MT synthesis and amplification of oncogenes such as *ras* has also been reported [21]. In this study, a strong reaction for Timm staining was observed in all ten rats at both 20 and 28 weeks while the positive rate for MT expression was 30% and 80% at 20 and 28 weeks, respectively. In addition, comparing the Timm staining reaction and distribution in the TCC tissues with positive immunostaining for MT to that with negative immunoreaction, no difference was found between them. This result shows that MT synthesis may also be modulated by other factors which may be involved in the tumor growth and progression; thus there is a need for further investigation on this point.

References

1. Bahnson BR, Banner BF, Ernstoff MS, Lazop JS, Cherin MG, Banerjee D, Chin JL (1991) Immunohistochemical localization of metallothionein in transitional cell carcinoma of the bladder. J Urol 146:1518
2. Barch DH, Iannaccone PM (1986) Role of zinc deficiency in carcinogenesis. Adv Exp Med Biol 206:517
3. Bauchinger ME, Schmid HJ, Dresp J (1976) Chromosome aberrations in lymphocytes after occupational exposure to lead and cadmium. Mutat Res 40:57
4. Cousins RJ, Leinart AS (1988) Tissue-specific regulation of zinc metabolism and metallothionein genes by interkeukin 1. FASEB J 2:2884
5. Fresno M, Wu W, Rodriguez JM, Nadji M (1993) Localization of metallothionein in breast carcinomas. An immunohistochemical study. Virchows Arch [A] 423:215
6. Fukushima S, Hirose M, Tsuda H, Sjirai T, Hirao K, Ito N (1976) Histological classification of urinary bladder cancers in rats induced by *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. Gann 67:81
7. Fukushima S, Marusaki G, Hirose M, Nakanishi K, Hasegawa R, Ito N (1982) Histopathological analysis of preneoplastic changes during *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine induced urinary bladder carcinogenesis in rats. Acta Pathol Jpn 32:243
8. Goncharova EI, Rossman TG (1994) A role for metallothionein and zinc in spontaneous mutagenesis. Cancer Res 54:5318
9. Hamer DH (1986) Metallothionein. Ann Rev Biochem 55:913
10. International Union Against Cancer (1987) TNM classification of malignant tumors, 4th edn. Springer, New York, p 133
11. Kagi JHR, Schaffer A (1988) Biochemistry of metallothionein. Biochemistry 27:8509
12. Kasprzak KS, Kovatch RM, Poirier LA (1988) Inhibitory effect of zinc on nickel subsulfide carcinogenesis in Fischer rats. Toxicology 52:253
13. Kontozoglou TE, Banerjee D, Cherian G (1987) Immunohistochemical localization of metallothionein in human testicular embryonal carcinoma cells. Virchows Arch [A] 415:545
14. Loenard A, Geser GB, Loenard F (1986) Mutagenicity, carcinogenicity and teratogenicity of zinc. Mutat Res 168:343
15. Mayo KE, Palmiter RD (1981) Glucocorticoid regulation of metallothionein-I mRNA in cultured mouse cells. J Biol Chem 256:2621
16. McGlashan ND (1972) Zinc and oesophageal cancer. Lancet 1:578
17. Nartey N, Cherian MJ, Banerjee D (1987) Immunohistochemical localization of metallothionein in human thyroid tumors. Am J Pathol 129:177
18. Palmer S (1985) Diet, nutrition and cancer. Prog Food Nutr Sci 9:283
19. Perez-Clausell J, Danscher G (1985) Intravesicular localization of zinc in rat telencephalic boutons. A histochemical study. Brain Res 337:91
20. Poswillo DE, Cohen B (1971) Inhibition of carcinogenesis by dietary zinc. Nature 231:447
21. Schmidt CJ, Hamer DH (1986) Cell specificity and effect of *ras* on human metallothionein gene expression. Proc Natl Acad Sci USA 83:3346
22. Vallee BC, Falchuk KM (1993) The biological basis of zinc physiology. Physiol Rev 73:79
23. Zhang XH, Takenaka I (1995) Morphological changes of microvilli on different surfaces of epithelial cells in the rat bladder treated with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. Urol Res 23:371