Urol Res (1996) 24:177–181

ORIGINAL PAPER

X. H. Zhang · I. Takenaka

Interstitial stroma and carcinogenesis: ultrastructural observations in the rat bladder treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine

Received: 4 July 1995 / Accepted: 6 December 1995

Abstract This study describes ultrastructural alterations of the interstitial stroma in the rat bladder epithelium during *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)-induced bladder carcinogenesis using scanning (SEM) and transmission (TEM) electron microscopy with NaOH treatment. The results obtained with SEM demonstrated the occurrence and development of stroma protrusions which exhibited pipe-like structures in the rat bladder epithelium following administration of BBN. Number and size of blood vessel sections also gradually increased both in the stroma and within the layer of the proliferated epithelial cells as examined by light microscopy (LM) and TEM. In this study stroma alterations were not only observed in malignant lesions of rat bladder, but hyperplastic lesions were also accompanied by stroma alterations. It is suggested that: (1) the interstitial stroma of the rat bladder epithelium may exhibit pathological changes in structure and these changes may correlate with the progression of epithelial cell proliferation following administration of BBN and (2) one of the most important alterations in the stroma is the occurrence of neovascularization, which may induce structural modification of the stroma in the processes of bladder tumor growth and development.

Key words Interstitial stroma · Carcinogenesis · Bladder epithelium · Neovascularization · Electron microscopy

Introduction

The extracellular matrix (ECM) consists mainly of basement membrane and interstitial stroma, which normally play an important role in the support and nutrition of epithelial cells [11, 12]. The ECM changes in malignant lesions that have been described so far are basement membrane alterations and occurrence of desmoplasia as demonstrated by transmission electron microscopy (TEM) [2, 4, 6] and stromal neovascularization as demonstrated by scanning electron microscopy (SEM) [3]. In our previous studies, the correlation between the basement membrane and either bladder carcinogenesis or tumor invasion was also investigated with the aid of TEM in the rat bladder treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) [19, 20]. BBN has been proved to be an effective organ-specific carcinogen on the urinary bladder and is widely used in the study of bladder tumors in animal models [9]. In the study described here, we investigated ultrastructural alterations of the interstitial stroma in the rat bladder epithelium during BBN-induced bladder carcinogenesis using SEM and TEM with NaOH treatment.

Materials and methods

Fifty male Wistar rats (Clea, Osaka), 7 weeks of age at the start of the experiment, were divided into a control group consisting of 15 rats and a BBN group consisting of 35 rats. Rats were housed three to a polyCarbonate cage, and placed in an environmentally controlled room illuminated for 12 h/day. BBN (Tokyo Kasei Industry) was administered at a dosage of 0.05% in drinking water for 28 weeks. A commercial stock diet (Oriental MF, Oriental Yeast, Tokyo) was provided to both groups of rats. Three rats from the control group and seven rats from the BBN group were killed at 4, 8, 12, 20 and 28 weeks after the start of the experiment. The bladders were inflated with 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.4), and then cut into three parts, each of which was studied using LM, SEM and TEM, respectively.

For LM, 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. Multiple sections of each bladder were examined.

For electron microscopy, the specimens were cut into 4 × 4 × 4-mm blocks and placed in fixation fluid (2.5% glutaraldehyde in

X. H. Zhang (✉) · I. Takenaka
Department of Urology, Kagawa Medical School, 1750-1, Miki-cho, Kita-gun, Kagawa 761-07, Japan

0.1 M phosphate buffer solution at pH 7.4) for 4 h at room temperature. In order to remove the cellular elements of the rat bladder epithelium, the specimens were immersed in 1 N sodium hydroxide solution (NaOH) for 2–3 days at 25 °C, and the NaOH solution was changed twice a day. The specimens were then rinsed in distilled water for 1–2 days at 25°C, after which the specimens were post-fixed in 1% osmic acid for 2 h, stained with 2% tannic acid for 1 h and dehydrated with graded ethanol solutions. For SEM, the specimens were dried in a Hitachi Hcp-2 critical point evaporator and examined under an Hitachi-900 SEM. For TEM, the specimens were embedded in Epon 812. Ultrathin sections were then cut on an LKB ultramicrotome with a diamond knife, double-stained with uranyl acetate and lead citrate and examined with a hitachi JEM-1200EX TEM.

In addition, the hyperplastic lesions were divided into three types: mild, moderate and severe hyperplasia. The bladder epithelium is three to five cell layers thick in mild hyperplasia, six to eight cell layers thick in moderate hyperplasia, and over nine cell layers thick in severe hyperplasia.

Results

LM findings

The rat bladder epithelium appeared normal in all 15 rats within the 28-week periods in the control group. In the BBN group, all seven rats showed mild hyperplasia at 4 weeks and moderate hyperplasia at 8 weeks. At 12 weeks, severe hyperplasia was found in five rats, and papilloma was found in two rats. At 20 weeks, noninvasive transitional cell carcioma (TCC) of the rat bladder was observed in all seven rats. Invasive TCC of the rat bladder was observed in all seven rats at 28 weeks, and the bladder tumor had focally invaded the muscle layers. On the other hand, number and size of blood vessel sections, both in the stroma and within the layer of proliferated epithelial cells, increased gradually following administration of BBN (Figs. 1, 2).

SEM findings

In the control group, the stroma surface of the rat bladder epithelium displayed flat networks of collagen fibrils (Fig. 3). In the BBN group, mild protrusions were frequently observed in the stroma of the rat bladder epithelium at 4 and 8 weeks (Fig. 4). At 12 and 20 weeks, moderate protrusions occurred, which became much more diffuse and connected extensively (Fig. 5). At 28 weeks severe protrusions were observed, which displayed three-dimensional meshwork structures resembling hornet's nests in appearance (Fig. 6). On examination at higher magnification, the protrusions showed pipe-like structures which were believed to comprise collagen fibrils and blood vessels.

TEM findings

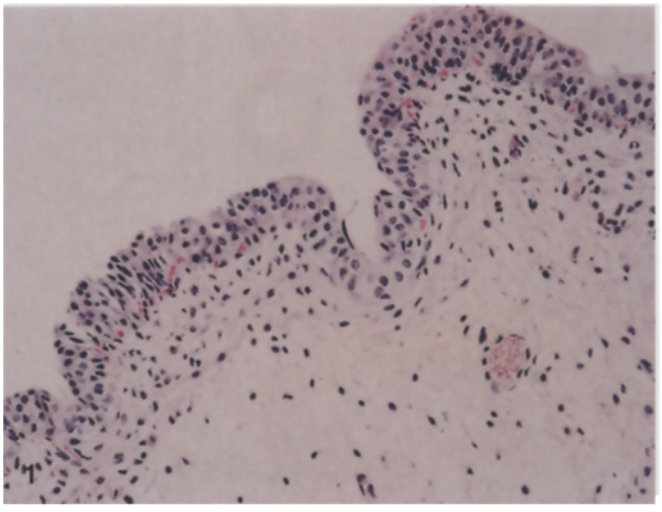
In the control group, the collagen fibrils exhibited a flat-layer structure in the stroma (Fig. 7). Following administration of BBN, round sections of pipe-like structures appeared and developed in number and size either on the stroma surface or within the layer of the collagen fibrils, and they were surrounded by the collagen fibrils (Figs. 8–9). The round-section structures have been regarded as sections of blood vessels because of the residue of blood components within them. Table 1 shows the pathological changes of the epithelial mucosa and of the interstitial stroma in the rat bladder during the 28 weeks of BBN administration.

Discussion

Although the superficial changes in the bladder epithelium in experimental situations and the pathological changes have been well observed by SEM [1, 10], demonstration of the interstitial stroma changes by SEM observation have been hindered by the presence of interfering cellular elements. Recently, SEM observations of the stroma in some normal tissues such as pancreas [14], skin, intestine [15] and ureter [5] have been described in the literature, whereas the urinary bladder has received less attention both under normal conditions and in pathological situations. The present results showed that with NaOH treatment the cellular elements of the rat bladder epithelium were effectively removed, and the stroma alterations in the rat bladder epithelium could be ultrastructurally observed during the carcinogenesis.

To investigate the correlation between the interstitial stroma and bladder carcinogenesis, the major problem is whether the stroma structure is altered and what changes are present in the stroma during the carcinogenesis. The present observations showed that pathological changes in the rat bladder epithelium occurred both in the epithelial mucosa and in the stroma following administration of BBN. The former exhibited benign and malignant proliferation of the epithelial cells, and the latter displayed occurrence of the stroma protrusions as observed by SEM and the occurrence of neovascularization as observed by LM and TEM. This demonstrated that the stroma alterations were not only present in the malignant lesions of the rat bladder, but also in the hyperplastic lesions; the stroma structure had merely become much more diffuse in the malignant lesions.

It is believed that the processes of the bladder tumor growth and development may be the result of an interaction between proliferative cells of the epithelium and the extracellular matrix components, including the basement membrane and stroma. Following administration of BBN, the proliferative cells of the rat bladder epithelium may require a greater supply of blood and greater tissue support from the stroma than under normal conditions. Such requirements may induce structural modification of the stroma due to the



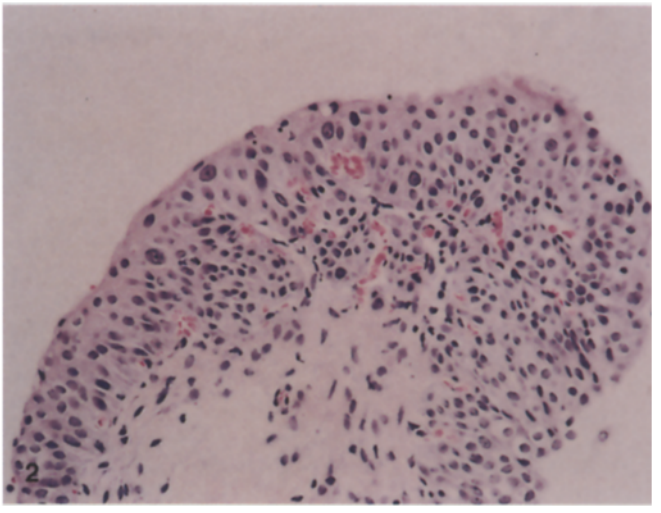


Fig. 1 Mild hyperplasia of the rat bladder epithelium. Some blood vessel sections can be seen both in the stroma and within the layer of the proliferated epithelial cells. BBN group, 4 weeks, H&E, ×60

Fig. 2 Severe hyperplasia of the rat bladder epithelium. Many blood vessel sections can be seen both in the stroma and within the layer of the proliferated epithelial cells. BBN group, 12 weeks, H&E, ×80

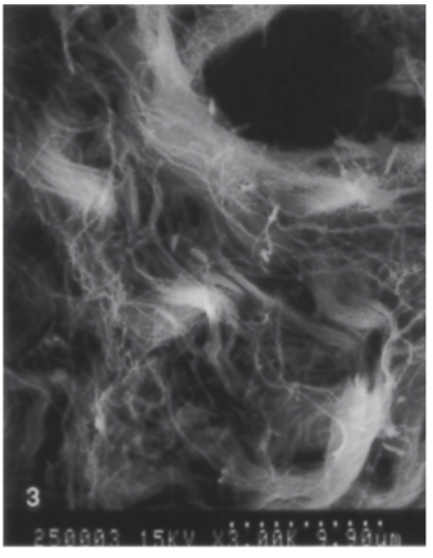


Fig. 3 Observation of the stromal surface in the rat bladder in the control group, displaying flat networks of collagen fibrils and fenestrae. Control group, 20 weeks, SEM

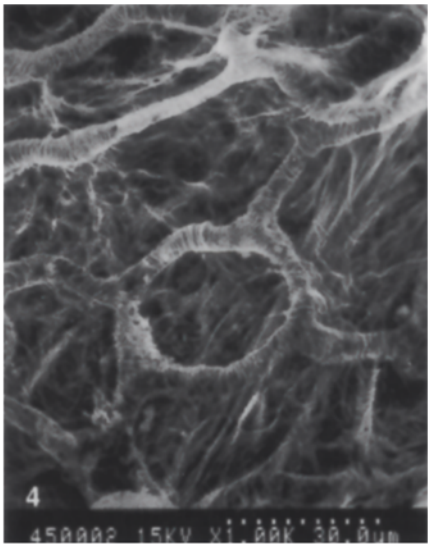


Fig. 4 Mild protrusions are observed in the stroma of the rat bladder. BBN group, 4 weeks, SEM

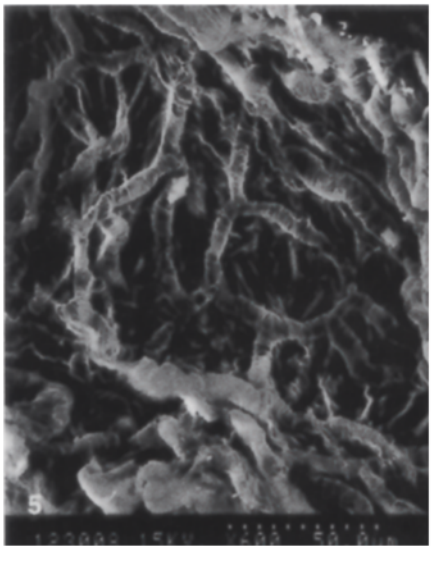


Fig. 5 Moderate protrusions are observed in the stroma of the rat bladder. BBN group, 8 weeks, SEM

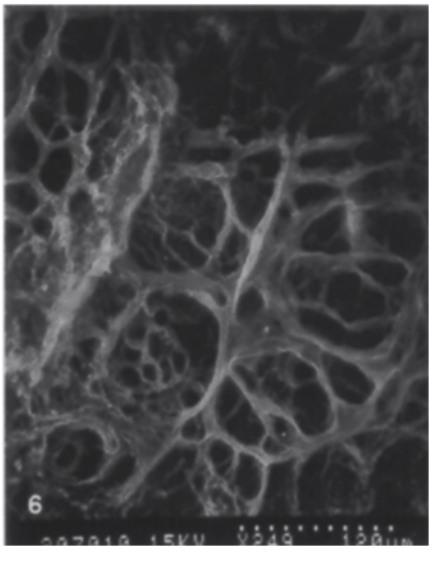


Fig. 6 Severe protrusions are observed in the stroma of the rat bladder, displaying three-dimensional meshwork structures which resemble hornet's nests in appearance. BBN group, 28 weeks, SEM

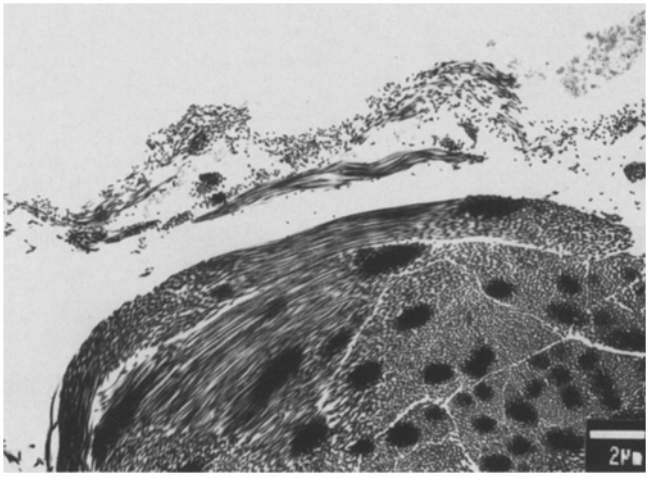


Fig. 7 Observation of the stromal ultrastructure in the rat bladder in the control group. The collagen fibrils are the main components of the stroma. Control group, 20 weeks, TEM, ×2500

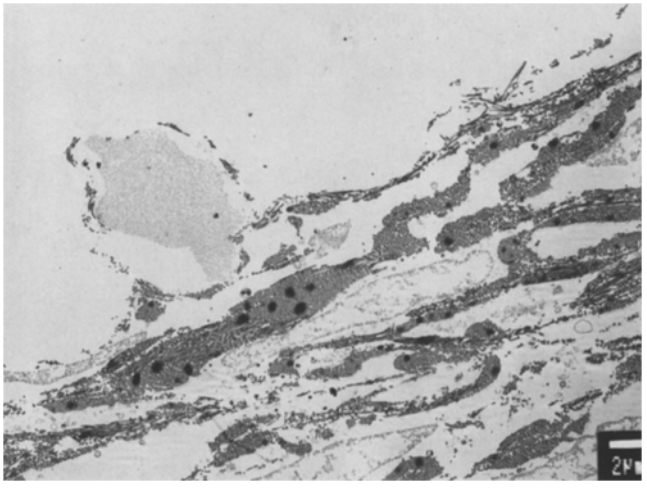


Fig. 8 A round section of a pipe-like structure is observed in the stroma of the rat bladder in the BBN group. Eight weeks, TEM, ×2500

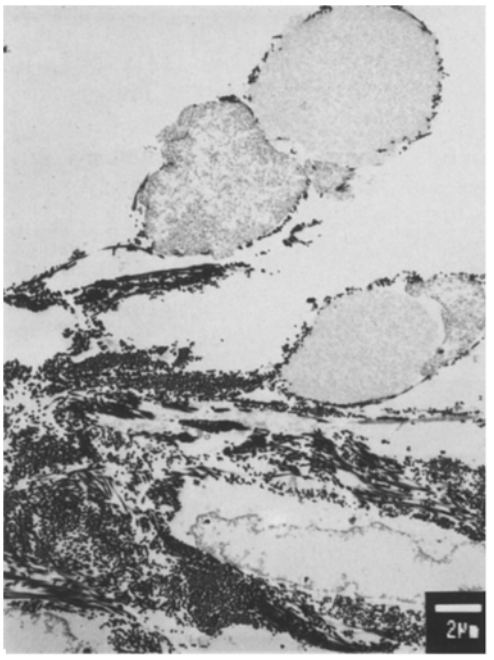


Fig. 9 Many round sections of pipe-like structures are observed in the stroma of the rat bladder in the BBN group. These structures have been regarded as sections of blood vessels because residue of blood components can be found within them. BBN group, 20 weeks, TEM, ×2000

occurrence of neovascularization in the rat bladder epithelium. Compared with the histopathological findings, it can be seen that the alterations in stromal structure developed with the progression of epithelial cell proliferation within the period of bladder carcinogenesis. Based on these findings it is suggested that the interstitial stroma may also exhibit pathological changes in structure, and that these changes may correlate with the progression of the epithelial lesions in the processes of bladder tumor growth and development.

It has long been known that tumors need the formation of new blood vessels for their growth, and that

Table 1 Pathological changes of epithelial mucosa and of the stroma in the rat bladder (Number of rats in parantheses, *HP* hyperplasia, *SP* stroma protrusions, *NEOV* neovascularization)

	Control group (4–28 weeks)	BBN group				
		4 weeks	8 weeks	12 weeks	20 weeks	28 weeks
LM	Normal appearance (15)	Mild HP (7)	Moderate HP (7)	Severe HP (5) Papilloma (2)	Noninvasive TCC (7)	Invasive TCC (7)
SEM	Flat networks of collagen fibrils (15)	Mild SP (7)	Mild SP (7)	Moderate SP (7)	Moderate SP (7)	Severe SP (7)
TEM	Layer structure of collagen fibrils (15)	Mild NEOV (7)	Mild NEOV (7)	Moderate NEOV (7)	Moderate NEOV (7)	Severe NEOV (7)

tumor cells can release tumor angiogenesis factor to promote the stromal angiogenesis and to increase the production of matrix components by stimulating fibroblasts [7, 16]. In previous studies, stromal neovascularization induced by bladder carcinogenesis in rats [3] and the occurrence of neovascularization in the processes of breast tumor growth and invasion have been reported [13, 17]. Recently, the occurrence of neovascularization and increased vascular density have been shown to correlate with a higher incidence of metastases and worse prognosis in tumors of the breast and prostate [8, 18]. With the aid of light and electron microscopic observations, the present study reveals that proliferation of the epithelial cells may closely correlate with the stroma alterations, especially with the occurrence of neovascularization in the stroma of the rat bladder epithelium. Comparing the SEM results with the LM and TEM findings, it can be seen that both the stroma protrusions showing pipe-like structures and neovascularization may occur and develop simultaneously in the stroma of the rat bladder epithelium following BBN treatment. Therefore, these pipe-like structures observed by SEM can be regarded as blood vascular structures in the interstitial stroma of the rat bladder. In the light of these results, it is suggested that one of the most important alterations in the stroma is the occurrence of neovascularization, which may induce structural modification of the stroma in the processes of bladder tumor growth and development.

References

1. Bryan GT, Cohen SM (1983) The pathology of bladder cancer, vol II. CRC Press, Florida
2. Chomette G, Auriol M, Tranbaloc P, Blondon J (1990) Stromal changes in early invasive breast carcinoma: an immunohistochemical, histoenzymological and ultrastructural study. Pathol Res Pract 186:70
3. Cohen SM, Tatematsu M, Shinohara Y, Nakanishi K, Ito N (1980) Neovascularization in rats during bladder carcinogenesis induced by *N*-{4-(5-nitro-2-furyl)-2-thiazolyl} formamide. J Natl Cancer Inst 65:145
4. Dingemans KP (1988) What's new in the ultrastructure of tumor invasion in vivo. Pathol Res Pract 183:792
5. Dorrell P, Wilkinson R, Gorham SD, Aitchison M, Scott R (1993) Collagen arrangements in ureter. Urol Res 21:325
6. Fisseler-Echoff A, Prebeg M, Voss B, Muller KM (1990) Extracellular matrix in preneoplastic lesions and early cancer of the lung. Pathol Res Pract 186:95
7. Folkman J (1974) Tumor angiogenesis. Adv Cancer Res 19:331
8. Horak ER, Leek R, Klen N, Le Jeune S, Smith K, Stuart N, Greenall MA, Harris AL (1992) Angiogenesis assessed by platelet/endothelial cell adhesion molecule antibodies, as an indicator of node metastasis and survival in breast cancer. Lancet 340:1120
9. Ito N, Hiasa Y, Okajima E, Kitamura H (1969) Histogenesis of urinary bladder tumors induced by *N*-butyl-*N*(4-hydroxybutyl) nitrosamine. Gann 60:401
10. Jacobs JB, Arai M, Cohen SM, Friedell GH (1976) Early lesions in experimental bladder cancer: scanning electron microscopy of cell surface markers. Cancer Res 36:2512
11. Liotta LA, Rao CN, Barsky SH (1983) Tumor invasion and the extracellular matrix. Lab Invest 49:636
12. Martinez-Hernandez A, Amenta PS (1983) The basement membrane in pathology. Lab Invest 48:656
13. Monschke F, Muller WU, Winkler U, Streffer C (1991) Cell proliferation and vascularization in human breast carcinomas. Int J Cancer 49:812
14. Ohtani O (1987) Three-dimensional organization of the connective tissue fibers of the human pancreas: a scanning electron microscopy study of NaOH treated tissues. Arch Histol Jpn 50:557
15. Ohtani O, Ushiki T, Taguchi T, Kikuta A (1988) Collagen fibrillar networks as skeletal frameworks: a demonstration by cell-maceration/scanning electron microscopy method. Arch Histo Cytol 51:249
16. Simpson JG, Fraser RA, Thompson WD (1983) Angiogenesis and angiogenesis factors. Progr Appl Microcirc 1:17
17. Verhoeven D, Van March E (1993) Proliferation, basement membrane changes, metastasis and vascularization patterns in human breast cancer. Pathol Res Pract 189:851
18. Vesalainen S, Lipponen P, Talja M, Alhava E, Syrjanen K (1994) Tumor vascularity and basement membrane structures as prognostic factors in T12M0 prostatic adenocarcinoma. Anticancer Res 14:709
19. Zhang XH, Takenaka I (1994) Basement membrane and tumor invasion: ultrastructural observations in the basement membrane of rat bladder with invasive transitional cell carcinoma induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. Urol Res 22:317
20. Zhang XH, Matsuoka N, Sugimoto M, Takenaka I (1994) Basement membrane and carcinogenesis: ultrastructural observations in the basement membrane of the bladder epithelium in rats treated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN). Int J Urol 1:129