Jpn. J. Cancer Res. 82, 650–656, June 1991

Time- and Dose-dependent Induction of Invasive Urinary Bladder Cancers by N-Ethyl-N-(4-hydroxybutyl)nitrosamine in B6C3F$_1$ Mice

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A sequential investigation of N-ethyl-N-(4-hydroxybutyl)nitrosamine (EHBN) bladder carcinogenesis was performed in male B6C3F$_1$ mice maintained *ad libitum* on tap water containing 0.025% EHBN for 4, 12, 20, 28 and 36 weeks. A total of 81 invasive tumors, comprising 55 squamous cell carcinomas (SCCs) (68%), 25 transitional cell carcinomas (TCCs) (31%) and 1 adenocarcinoma (1%) were found. Of these, 23 (22 SCCs and 1 TCC) demonstrated invasion to the prostate, 3 metastasized to the lung, and 2 spread by peritoneal seeding. The anaplastic grade and extent of invasion of the SCCs significantly exceeded those of the TCCs. The results suggested a histogenetic pathway from simple dysplasia through papillary or nodular dysplasia and/or carcinoma *in situ* to eventual development of invasive carcinomas.

Key words: Bladder carcinoma — N-Ethyl-N-(4-hydroxybutyl)nitrosamine — B6C3F$_1$ mouse — Metastasis

Experimental bladder carcinogenesis induced by nitroso compounds has been reported in various species.[1–9] Several investigators have demonstrated that bladder carcinomas in rats are typically multiple, papillary and non-invasive.[3,10–12] On the other hand, non-papillary and invasive types of cancer, associated with metastatic spread, generally develop in the mouse bladder.[1,11,13–18] The reported histological types of most human bladder carcinomas are transitional cell carcinoma (TCC), squamous cell carcinoma (SCC) and undifferentiated carcinoma (UC),[19,20] the malignancy of TCC depending on their grading, usually being less than in the SCC and UC cases where non-papillary and invasive patterns are more common.[21,22] Many studies on the development of bladder carcinoma in rats which have been performed using various carcinogens have demonstrated that N-ethyl-N-(4-hydroxybutyl)nitrosamine (EHBN), with an N-ethyl base substituted for the N-butyl base in N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), is the most reliable inducing agent.[23] In order to cast light on the process of EHBN-induced bladder carcinogenesis in mice, we examined the dose-dependence and the sequential changes leading to invasive bladder carcinomas.

MATERIALS AND METHODS

Animals A total of 155 male inbred B6C3F$_1$ mice were purchased from Charles River Japan, Inc., Atsugi, and maintained on basal diet (pellet Oriental MF, Oriental Yeast Co. Ltd., Tokyo). The mice, 6 weeks of age at the beginning of the experiment, were housed 4 or 5 to a plastic cage with hard-wood chips for bedding under constant conditions (room temperature, 21±3°C; humidity, 55±10%; light/dark cycle, 12 h : 12 h). The animals were observed daily for abnormalities, and body weights and food and water consumption were measured weekly up to week 4, and every 4 weeks from weeks 8 to 36.

Chemicals EHBN synthesized at Tokyo Biochemical Research Institute, Tokyo, was used in the present study. Stock solution containing 0.25% EHBN dissolved in distilled water was stored at 4°C in the dark, and then diluted ten-fold with tap water before administration *ad libitum* from black bottles which were refilled 3 times a week with newly prepared solution.

Experimental design (Fig. 1) The animals were randomly divided into 6 groups comprising 42 mice (group I), 34 mice (group II), 26 mice (group III), 18 mice (group IV) and 25 mice (group V) given water containing 0.025% EHBN for 4, 12, 20, 28 and 36 weeks, respectively. Eight animals from each group were killed at the cessation of treatment and every 8 weeks thereafter until week 36. Deaths and moribund cases resulted in reduction to 7 mice in group II after 20 weeks, and 5 mice in group IV, 7 mice in group III and 6 mice in group

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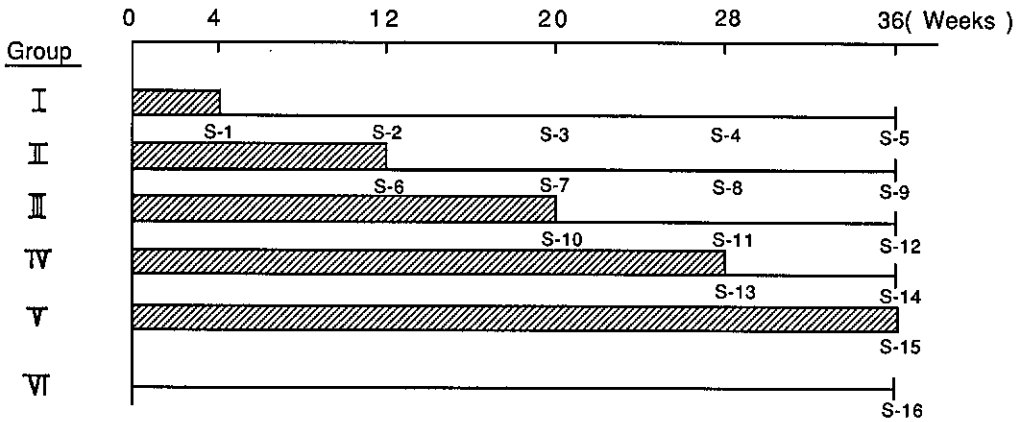

Fig. 1. Experimental design. ▨, 0.025% EHBN in drinking water; ——, tap water; S, animals killed.

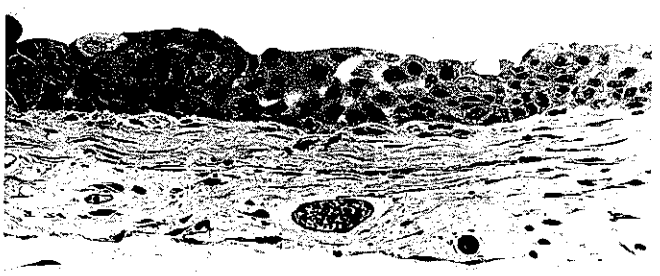

Fig. 2. Dysplasia. H-E, ×350.

Fig. 4. Carcinoma *in situ*. H-E, ×350.

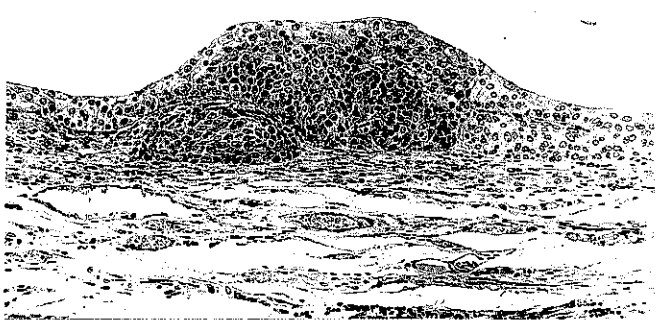

Fig. 3. PN dysplasia. H-E, ×175.

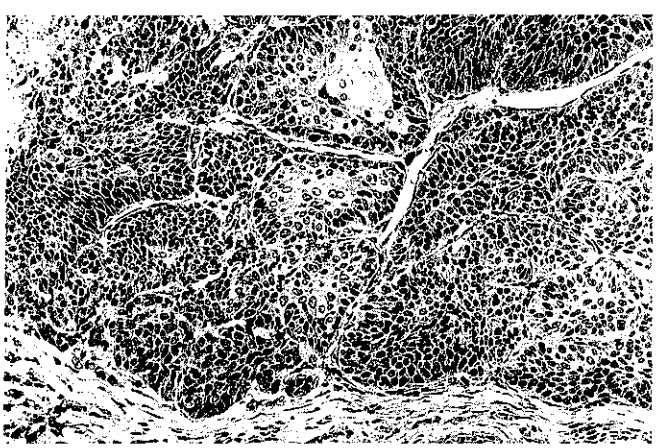

Fig. 5. Transitional cell carcinoma. H-E, ×175.

Fig. 6. Squamous cell carcinoma. H-E, ×175.

II after 28 weeks. These moribund and dead cases were reassigned appropriately, according to the survival period (Table I). After 36 weeks, all of the remaining animals (10 mice from group I, 5 mice from group II, one mouse from group III, 3 mice from group IV and 2 mice from group V) and the 10 mice (group VI) given tap water alone as controls were killed.

Histopathological examination Animals were killed under ether anesthesia and autopsied. The urinary bladder and stomach from each animal were inflated with 10% phosphate-buffered formalin solution. After routine embedding in paraffin, sections were cut and stained with hematoxylin and eosin (H-E).

Lesions observed in the urinary bladder could be classified histologically as follows: dysplasia — epithelium of more than four layers with moderate or severe anaplasia demonstrating diffuse proliferation (Fig. 2); papillary or nodular dysplasia (PN dysplasia) — moderate or severe anaplastic epithelial lesion of localized cellular proliferation resulting in nodular or papillary forms involving infolding of capillaries and connective tissues (Fig. 3); papillomas, without severe anaplasia, characterized by more massive upward growth into the lumen supported by small vessels and fibrous connective tissues; carcinoma *in situ* (CIS) — severe epithelial cellular atypia with mitosis and loss of polarity not including nodular or papillary proliferation (Fig. 4); carcinomas classified into TCC (Fig. 5), SCC (Fig. 6) and adenocarcinoma (AC). The tumors were graded into 3 steps regarding atypia and the extent of invasion was assessed as involving the epithelial layer, submucosa, muscle layer, subserosa or surrounding tissues and/or metastasis to distant regions.

Transmission electron microscopy (TEM) examination Tissues were fixed in buffered glutaraldehyde and OsO_4, and embedded in Epon resin. Sections were cut on an LKB Ultratome with a glass knife. They were mounted on Formvar-copper specimen grids and double stained with aqueous uranyl acetate followed by lead citrate. Sections were examined with a Hitachi 200CX electron microscope.

RESULTS

In the mice given 0.025% EHBN for 20 weeks or longer (groups III, IV and V), mean body weights declined gradually from week 20 up to 32 or 36 weeks (Fig. 7). The first deaths occurred in week 18 for group V, week 24 for group IV, weeks 22 for group III and week 19 for group II. Deaths or moribund cases were encountered up to week 36 in each group given 0.025% EHBN for 12 weeks or longer. The resultant decrease in percentage terminal survivals was dose-dependent (Table I). EHBN intake (mg/kg/day) did not differ between groups at any time and therefore total EHBN intake (g/kg body wt.) was proportional to the period of administration.

Gross pathological findings included grayish-white, flat or irregularly-shaped masses, in some cases filling the urinary bladder lumen. Grayish-white nodules were also observed attached to abdominal tissues in some animals.

Histopathological findings are summarized in Table II. The first cancer, a TCC, was found in an animal treated with 0.025% EHBN for 12 weeks followed by no treatment for 8 weeks. The total yield of 81 cancers consisted of 55 SCCs (68%), 25 TCCs (31%) and 1 AC (1%). The cancers induced were almost all non-papillary and

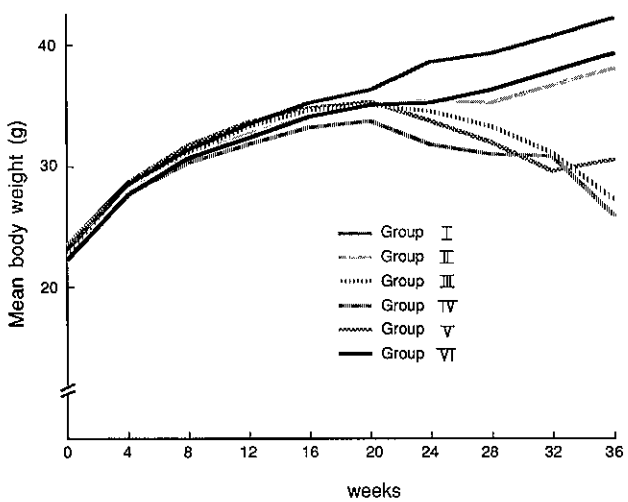

Fig. 7. Growth curves of mice maintained on tap water containing 0.025% EHBN.

Table I. Experimental Details, Mean Body Weight and EHBN Intake Data

Group	Treatment (weeks)		Terminal survival rate (%)	No. of mice examined (moribund and dead cases)	Terminal body weight (g)	Total EHBN intake (g/kg body wt.)
	EHBN	without EHBN				
I S-1[a]	4	0		8 (0)	28.5±2.2	1.69
S-2	4	8		8 (0)	33.5±2.8	
S-3	4	16		8 (0)	36.4±3.3	
S-4	4	24		8 (0)	39.3±3.4	
S-5	4	32	42/42 (100)	10 (0)	42.2±4.4	
II S-6	12	0		8 (0)	32.7±2.4	4.58
S-7	12	8		8 (1)	35.1±2.6	
S-8	12	16		9 (4)	35.3±3.5	
S-9	12	24	25/33 (75.8)	8 (3)	38.2±1.8	
III S-10	20	0		10 (2)	35.1±3.6	6.79
S-11	20	8		12 (4)	33.3±3.7	
S-12	20	16	17/27 (63.0)	5 (4)	27.3	
IV S-13	28	0		18 (16)	31.0±2.2	10.18
S-14	28	8	5/26 (19.2)	8 (5)	26.0±4.4	
V S-15	36	0	2/15 (13.3)	15 (13)	30.6±0.5	13.00
VI S-16	0	36	10/10 (100)	10 (0)	39.4±5.0	0

a) Animals were killed at the time points indicated in Fig. 1.

Table II. Incidences of Urinary Bladder Lesions in Mice Treated with 0.025% EHBN for Different Periods

Group	No. of mice examined	Dysplasia	PN dysplasia[a]	Papilloma	Carcinoma			CIS[d]
					TCC[b]	SCC[c]	Adenocarcinoma	
I S-1[e]	8	7 (88)[f]	0	0	0	0	0	0
S-2	8	2 (25)	0	0	0	0	0	0
S-3	8	1 (13)	0	0	0	0	0	0
S-4	8	2 (25)	0	0	0	0	0	0
S-5	10	0	0	0	0	0	0	0
II S-6	8	8 (100)	1 (13)	0	0	0	0	0
S-7	8	5 (63)	6 (75)	1 (13)	1 (12)	0	0	0
S-8	9	9 (100)	3 (33)	1 (11)	1 (11)	3 (33)	0	0
S-9	8	8 (100)	5 (63)	0	2 (25)	4 (50)	0	1 (13)
III S-10	10	10 (100)	3 (30)	0	1 (10)	1 (10)	0	1 (10)
S-11	12	10 (83)	9 (75)	1 (10)	2 (16)	9 (75)	0	4 (33)
S-12	5	5 (100)	3 (60)	0	2 (40)	4 (80)	0	1 (20)
IV S-13	18	18 (100)	10 (56)	0	5 (27)	13 (72)	0	6 (33)
S-14	8	8 (100)	5 (63)	0	4 (50)	7 (88)	1 (13)	3 (38)
V S-15	15	15 (100)	12 (80)	0	7 (47)	14 (93)	0	11 (73)
VI S-16	10	0	0	0	0	0	0	0

a) Papillary or nodular hyperplasia.
b) Transitional cell carcinoma.
c) Squamous cell carcinoma.
d) Carcinoma *in situ*.
e) Animals were killed at the time points indicated in Fig. 1.
f) Numbers in parentheses represent percentage incidences.

Table III. Anaplastic Grade and the Extent of Invasion of Transitional Cell Carcinomas (TCC)[a]

Anaplastic grade	No. of mice bearing TCC	Extent of invasion				
		Mucosa	Submucosa	Muscle layer	Subserosa	Perivesical tissue and/or other sites
1	6	1 (17)	5 (83)	0	0	0
2	10	0	4 (40)	5 (50)	1 (10)	0
3	9	0	2 (22)	1 (11)	5 (56)	1 (11)

a) Not including carcinoma *in situ*.

Table IV. Anaplastic Grade and the Extent of Invasion of Squamous Cell Carcinomas (SCC)

Anaplastic grade	No. of mice bearing SCC	Extent of invasion				
		Mucosa	Submucosa	Muscle layer	Subserosa	Perivesical tissue and/or other sites
1	0	0	0	0	0	0
2	10	0	0	7 (70)	2 (20)	1 (10)
3	45	0	0	0	24 (53)	21 (47)

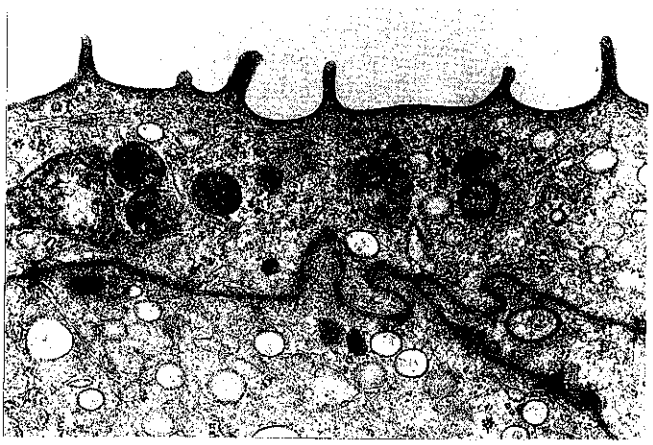

Fig. 8. Electron microscopic appearance of dysplastic epithelium in a mouse given 0.025% EHBN for 12 weeks. ×12,000. Short microvilli and a decrease in the number of fusiform vesicles are evident.

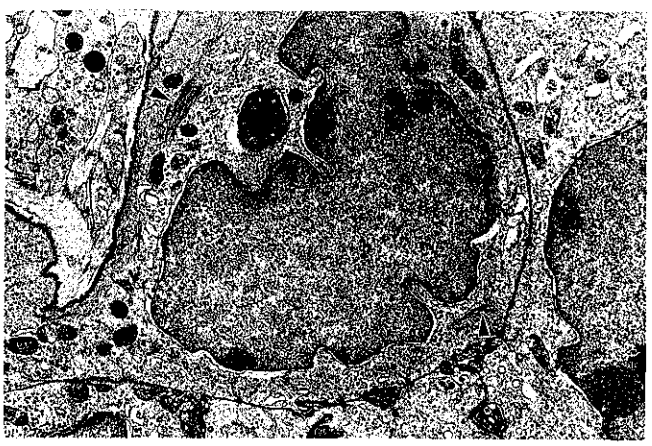

Fig. 9. Electron microscopic appearance of dysplastic epithelium in a mouse given 0.025% EHBN for 12 weeks. ×4,500. Tonofibrils (arrows) are present in the basal epithelial cells.

invasive. A significant time-dependent increase in incidence of cancer was apparent. CIS was noted in animals treated with EHBN for more than 12 weeks with an increase in incidence in parallel with increasing period of administration continuing to the end of the experiment. CIS were often found in association with dysplasias and invasive cancers.

Dysplastic epithelium was already present in animals treated with EHBN for only 4 weeks, this lesion demonstrating partial reversibility in group I. Almost all animals treated with EHBN for more than 12 weeks possessed dysplastic epithelium. PN dysplasias were observed in animals treated with EHBN for more than 12 weeks with increasing incidence dependent on the period of administration. Papillomas only occurred at a relatively early stage of the neoplastic process with very low incidence.

The relationships between histologic grades of TCC and SCC and the extent of invasion are shown in Tables III and IV, respectively. Anaplastic grade showed a close

positive link to the extent of invasion in both TCC and SCC cases, the latter being the more malignant. Invasion of the surrounding tissues and/or metastases were observed in 23 invasive cancers which consisted of 22 SCCs and 1 TCC (23 cancers invaded the prostate, metastases were found in the lung in 3 cases, and spreading occurred by peritoneal seeding in 2 cases).

TEM examination of mice given 0.025% EHBN for 12 weeks revealed luminal surface cells in areas of dysplasia to possess short microvilli. Cytoplasmic vesicles decreased and changed from fusiform to round or elliptical shape (Fig. 8). Tonofibrils, characteristic of squamous cells, were present in the basal epithelial cells (Fig. 9).

DISCUSSION

In the present study, EHBN was found to induce invasive urinary bladder cancers associated with a high incidence of metastasis in male $B6C3F_1$ mice. Substitution of an N-ethyl for an N-butyl base in BBN has been indicated to confer stronger carcinogenicity, which is both dose- and time-dependent, in rats and mice.[5, 23] In the present study, rapid increases in carcinoma development after cessation of carcinogen treatment in groups administered 0.025% EHBN for 12, 20 and 28 weeks were observed. The invasive cancers induced by EHBN in mice were essentially similar in character to lesions reported in BBN bladder carcinogenesis,[16, 18, 24] in spite of the different strain of mouse used, although the level of malignancy was higher since no metastatic spread was observed with BBN. The incidence of SCC was about 2 times higher than that of TCC, in accordance with previous reports.[14, 16, 25] The malignancy of SCC in terms of anaplastic grade and extent of invasion significantly exceeded those of TCC.

In the human bladder carcinoma case, it has been concluded that non-papillary lesions might be the most frequent precursors of the typical malignancies, which frequently show metastasis and have a very poor prognosis.[21, 22, 26] Invasive cancers are very uncommon in rats, in which the histogenesis of TCC involves PN hyperplasias and papillomas.[10, 11] In contrast, the malignancy grades of mouse bladder tumors associated with metastasis are higher than those of rats.

The morphological characteristics and behavior of early lesions involved in experimental bladder carcinoma induction in mice appear radically different from those concerned with the genesis of invasive cancer in rats. Firstly, in mice, the PN type mainly develops from dysplasias and is postulated to result in non-invasive carcinomas which eventually form invasive cancers. Ohtani *et al.*[18] reported similar morphological characteristics along with dose-dependence of tumor induction in male C3H/He mice. The present data confirm this histogenesis involving early dysplastic lesions. The second pathway, which is a minor one developing to invasive carcinoma, is CIS through dysplasia. We could not find evidence that papillomas turn to non-invasive carcinoma, although this point must remain open since papilloma development was very limited. In rat bladder carcinogenesis, papillomas mainly turn to non-invasive carcinoma, leading to invasive carcinomas.[20] Thus, the histogenesis of invasive bladder carcinomas seems to be quite different between mice and rats.

The present investigation further revealed ultrastructural lesions including tonofibrils, which are elements characteristic of squamous cells. The high incidence of SCC was consistent with such evidence of squamous cell transformation possibly occurring in early stages of carcinogenesis. These findings suggest that SCC also develop from dysplastic cells going through PN dysplasias and/or CIS instead of developing by metaplasia at the TCC stage. Although this latter possibility involving progression from TCC to SCC cannot be precluded, it seems unlikely from our data.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from the Ministry of Health and Welfare and by a Grant from the Society for Promotion of Pathology of Nagoya.

(Received December 19, 1990/Accepted March 16, 1991)

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