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Histopathological Analysis of Invasive Bladder Carcinomas Induced by 3,2′-Dimethyl-4-aminobiphenyl in Hamsters

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Histopathological characteristics of urinary bladder tumors induced in Syrian golden hamsters by 3,2′-dimethyl-4-aminobiphenyl (DMAB) were analyzed. DMAB was subcutaneously injected in corn oil at a concentration of 100 mg/kg once a week for 20 weeks and ethinyl estradiol (EE) was administered in the diet at a dose of 0.75 ppm throughout the experiment. A small group of animals was killed at week 20 and all survivors were killed at week 50. Urinary bladder carcinomas were induced in 14 of 18 hamsters (78%; 0.89/animal) in the DMAB+EE group and 11 of 17 (65%; 0.88/animal) in the DMAB alone group in males, and in 11 of 14 (79%; 0.79/animal) in the DMAB+EE group and 4 of 5 (80%; 0.80/animal) in the DMAB alone group in females examined between weeks 20 and 50. All were non-papillary invasive transitional cell carcinomas partly demonstrating glandular and/or squamous differentiation, and most carcinomas developed in the bladder neck. Degree of invasion was clearly correlated with degree of morphological atypism in the transitional cell carcinomas, but not with squamous or glandular differentiation. No sex difference or modifying effect of EE on DMAB urinary bladder carcinogenesis was evident. No bladder carcinomas were observed in non-DMAB-treated animals.

Key words: Urinary bladder carcinogenesis — Invasive cancer — 3,2′-Dimethyl-4-aminobiphenyl — Ethinyl estradiol — Hamster

3,2′-Dimethyl-4-aminobiphenyl (DMAB) is a wide-spectrum carcinogen in rodents. Its reported major target organs are the small and large intestines, lung, skin, preputial glands, Zymbal glands, prostate and pancreas for the rat[1–8] and the urinary bladder, forestomach, gallbladder and small and large intestines for the hamster.[2,9,10] The difference in tumor spectra between the rat and the hamster has been explained in terms of the different levels of the N-hydroxy-N-glucuronide metabolite of DMAB in the urine and bile.[11]

Using this carcinogen, we have established a prostate carcinogenesis model by combined treatment of male rats with sex hormones, such as estrogens and androgens, and surgical castration.[4–6,12–15] Besides the prostate carcinomas, however, a variety of tumors were induced in a series of experiments in male rats,[6–8] and DNA-adduct formation was demonstrated not only in target organs, but also in non-target organs and tissues.[15,16]

Ethinyl estradiol (EE) is carcinogenic or promotes carcinogenesis in the liver[17–19] and kidney[20,21] of rodents and influences a variety of organs, in particular the hormone-dependent tissues. Shirai *et al.*[22] reported inhibitory effects of EE in the N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)-induced urinary bladder carcinogenesis when EE was given in the diet at a concentration of 0.001% in rats. This observation is in line with a well-known sex difference in susceptibility to carcinogens of this organ of rats.[23] For the purpose of elucidating species differences in the DMAB plus EE carcinogenesis model, we have conducted several experiments using Syrian golden hamsters. In one of these, gallbladder tumors were induced at much higher incidence in animals receiving both DMAB and EE than those receiving DMAB alone.[24] In the same experiment, urinary bladder tumors were also induced at high incidences in both male and female hamsters.

In the present study, histopathological characteristics of urinary bladder carcinomas and modifying effects of EE on bladder carcinogenesis of hamsters were evaluated.

MATERIALS AND METHODS

Male and female 5-week-old Syrian golden hamsters, 70 animals each, were obtained from Japan SLC Inc., Hamamatsu. The animals were housed in plastic cages with wood chip bedding, in an air-conditioned room at 23±2°C with a 12h-12h light-dark cycle, and given food (Oriental MF; Oriental Yeast Co., Tokyo) and water *ad libitum*. DMAB with a purity of more than 98% was obtained from Matsugaki Pharmaceutical Co., Osaka. EE was purchased from Sigma Chemical Co., St. Louis, MO, and given as a supplement in the basal powdered diet at a dose of 0.75 ppm (EE diet).

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The hamsters in group 1 were maintained on the EE diet throughout the experiment and received weekly subcutaneous injections of DMAB dissolved in corn oil at a dose of 100 mg/kg body weight administered into the back for 20 weeks. Other animals were given subcutaneous DMAB injections without EE administration (group 2), the EE diet and 20 subcutaneous corn oil injections (5 ml/kg body weight as group 3), or the corn oil injections alone (group 4) as controls. Ten animals of each sex in groups 1 and 2 were killed 1 week after the last subcutaneous injection of DMAB or oil and all surviving animals were killed at week 50 of the experiment.

At autopsy, after intravesical injection of 10% phosphate-buffered formalin, the urinary bladder, prostates, seminal vesicles and urethra were removed together. Urinary bladders were thereafter cut into 8 longitudinal strips, including adjacent tissues when bladder tumor invasion was present. After careful gross observation, the other main organs and all pathologic lesions were removed, fixed in formalin, and routinely histopathologically examined. Moribund or dead animals were also examined grossly and, where possible, the tissues were processed for histopathological examination as above.

Established criteria for rat and/or mouse lesions were adopted for classification of tumors, and for evaluation of the grade and stage of carcinomas.[23, 25–27] Proliferating lesions were classified into simple hyperplasia, papillary and/or nodular (PN) hyperplasia, dysplasia, papilloma, and carcinoma categories. Carcinomas were transitional cell carcinomas (TCC) with or without glandular and/or squamous differentiation. Grading of carcinomas, based on both cellular and structural atypism, was into mild (grade I), moderate (grade II), and severe (grade III). Stages of carcinomas were intramucosal (*in situ*), submucosal, intramuscular, subserosal and serosal tumors. Both grading and staging were determined from the most advanced portion of each tumor. For parts without serosa, tumors that invaded between the muscular layer of the bladder and the adjacent tissue were regarded as subserosal.

Statistical analysis of differences between pairs was carried out using Fisher's exact probability test. Student's t test or Welch's t test after application of the preliminary F-test for equal variance was used for means. The cumulative chi-square test was used for analysis of carcinogenic potential, and grading and staging of carcinomas.[28]

RESULTS

Weight gain was retarded in the DMAB-treated animals throughout the experiment. Toxic effects of the EE diet in terms of body weight gain were slight in males

Table I. Histopathology of the Urinary Bladder in Hamsters Treated with DMAB

Sex	Weeks[b]	Treatment[c]	No. of animals	No. of animals with[a]					Multiplicity of carcinomas
				Hyperplasia		Dysplasia	Carcinoma	Significance	
				Simple	PN[d]				
Male	20	DMAB+EE	9	2 (22)	0	6 (66)	1 (11)		0.11±0.33
		DMAB	9	4 (44)	4 (44)	1 (11)	0		0
	50	DMAB+EE	18	0	0	4 (22)	14 (78)	*f, g*	0.89±0.58[h, i]
		DMAB	17	0	2 (11)	4 (23)	11 (65)	*e, g*	0.88±0.93[h, j]
		Oil+EE	10	0	0	0	0		0
		Oil	7	0	0	0	0		0
Female	20	DMAB+EE	7	2 (28)	3 (42)	1 (14)	1 (14)		0.14±0.38
		DMAB	7	2 (28)	0	5 (71)	0		0
	50	DMAB+EE	14	0	0	3 (21)	11 (79)	*e, g*	0.79±0.43[h, i]
		DMAB	5	0	1 (20)	0	4 (80)	*g*	0.80±0.45[h, k]
		Oil+EE	8	0	0	0	0		0
		Oil	9	0	0	0	0		0

a) Each animal is tabulated in the column of the most advanced lesion present in the urinary bladders detected by histological examination.
b) Several animals were killed at week 20. Animals which died thereafter or were killed before 50 weeks were included into the 50-week group.
c) DMAB was subcutaneously injected weekly at 100 mg/kg body wt. for weeks. EE was added into the diet at 0.75 ppm throughout the experiment. Oil was the vehicle for DMAB.
d) Papillary or nodular.
e, f) Significant by the cumulative chi-square test from the corresponding 20-week group at $P<0.001$ and $P<0.01$, respectively.
g) Significant by the cumulative chi-square test from the corresponding oil group at $P<0.001$.
h) Significantly different from the 20-week group at $P<0.05$.
i, j, k) Significantly different from the oil group at $P<0.001$, $P<0.01$, $P<0.05$, respectively.

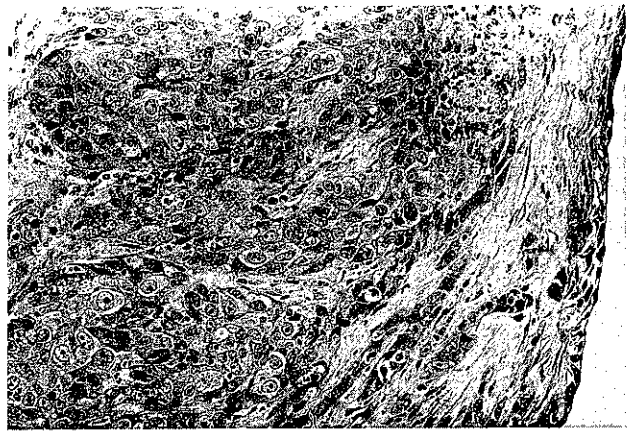

Fig. 1. TCC evaluated as "serosa" in depth of invasion and "grade III" in urinary bladder from a female hamster treated with DMAB and EE. Severe cellular atypism and a number of mitoses are observed. ×200.

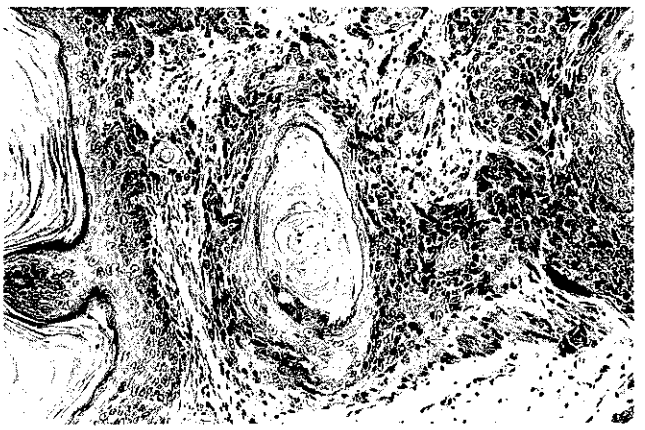

Fig. 2. A part of squamous differentiation in TCC observed in urinary bladder of a male hamster treated with DMAB and EE. The tumor was classified as grade II and stage "muscular layer." ×100.

and not observed in females. No animal died before week 20, but thereafter several animals were found dead or moribund and autopsied before the scheduled termination of the experiment (50 weeks). Most of them had bladder or hematopoietic tumors.

The incidences of hyperplastic, preneoplastic and neoplastic lesions are summarized in Table I. In this experiment, dysplasia was regarded as a more advanced lesion than PN hyperplasia, and each animal is tabulated in the column of the most advanced lesion present in the urinary bladder as determined by histopathological examination for the purpose of statistical analysis with the cumulative chi-square test. Minor lesions such as hyperplasia and dysplasia were already frequently observed at week 20, and more advanced lesions developed by week 50 in the DMAB-treated groups irrespective of EE treatment in both sexes. One male and one female in the DMAB+EE group developed TCC at week 20, but no significant difference in carcinogenic activity between groups 1 and 2 was evident at this time point by the cumulative chi-square test. In animals that were found dead or killed after week 20, carcinomas were observed in 14 of 18 hamsters (78%) in the DMAB+EE group and 11 of 17 (65%) in the DMAB alone group in males, and in 11 of 14 (79%) in the DMAB+EE group and 4 of 5 (80%) in the DMAB alone group in females. Multiplicities of carcinomas were 0.89, 0.88, 0.79 and 0.80 per rat, respectively. All tumors were non-papillary invasive-type TCCs, partly associated with glandular and squamous differentiation, and most carcinomas developed in the bladder neck. Bladder tumors were concluded to be the cause of death in some animals. No urolithiasis was identified in any animal. No bladder

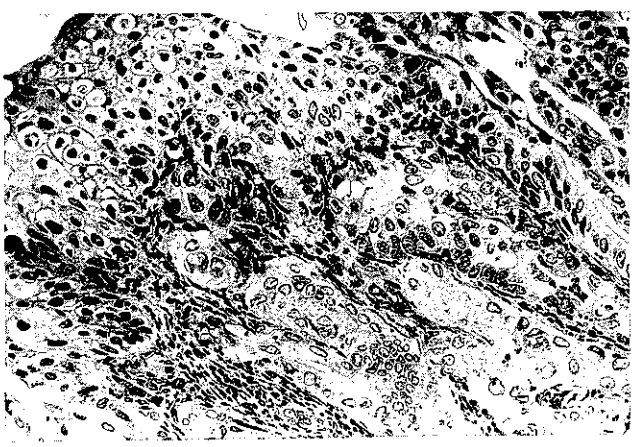

Fig. 3. A part of glandular differentiation in TCC observed in a male hamster treated with DMAB and EE. The tumor was classified as grade II and stage "muscular layer." ×200.

lesions were observed in animals that were not treated with DMAB.

Figs. 1–3 show typical histopathological features of bladder carcinomas. All tumors were non-papillary invasive transitional cell carcinomas, and carcinomas of high staging (invaded deeply) were commonly of high grading (more atypical) (Fig. 1). Several carcinomas showed association with squamous (Fig. 2) and glandular (Fig. 3) elements. Results for grading and staging of each bladder carcinoma and the association of TCC with squamous and glandular differentiation are summarized in Table II. Neither grading nor staging was affected by

Table II. Grading and Staging of Urinary Bladder Carcinomas in Hamsters

Sex	Treatment	No. of animals[a]	Total No. of tumors observed	Grade of dysplasia[b]			Depth of invasion[c]					Association with (%)	
				I	II	III	Mucosa	Sub-mucosa	Muscular layer	Sub-serosa	Serosa	Squamous	Glandular
Male	DMAB+EE	27	17	2	11	4	1	7	5	2	2	4 (25)	8 (47)
	DMAB	26	15	0	10	5	1	4	7	1	2	1 (7)	4 (27)
Female	DMAB+EE	21	12	1	6	5	0	5	3	0	4	2 (17)	4 (33)
	DMAB	12	4	0	3	1	0	3	0	0	1	1 (25)	2 (50)

a) All animals were combined irrespective of the survival time.
b) Grade of dysplasia was evaluated as the highest in the tumor; I, mild; II, moderate; III, severe. See "Materials and Methods."
c) Tumor staging was evaluated from the most advanced portion in each case. For parts without serosa, tumors that invaded between the muscle layer of bladder and the adjacent tissue were included in the "subserosa" category.

Table III. Association of Malignancy Grade with Squamous Components in Transitional Cell Carcinomas in Hamsters

Association with squamous component	Sex	Total No. of tumors observed[a]	Grade of dysplasia[b]			Depth of invasion[c]				
			I	II	III	Mucosa	Submucosa	Muscular layer	Subserosa	Serosa
+	Male	5	0	4	1	0	2	1	2	0
	Female	3	0	2	1	0	1	2	0	0
	Total	8	0	6	2	0	3	3	2	0
−	Male	27	2	17	8	2	9	11	1	4
	Female	13	1	7	5	0	7	1	0	5
	Total	40	3	24	13	2	16	12	1	9

a) Carcinomas observed at all time points were combined for each male and female group.
b) Grade of dysplasia was evaluated as the highest in the tumors; I, mild; II, moderate; III, severe. See "Materials and Methods."
c) Tumor staging was evaluated from the most advanced portion in each case. For parts without serosa, tumors that invaded between the muscle layer of bladder and the adjacent tissue were included in the "subserosa" category.

Table IV. Association of Malignancy Grade with Glandular Components in Transitional Cell Carcinomas in Hamsters

Association with glandular component	Sex	Total No. of tumors observed[a]	Grade of dysplasia[b]			Depth of invasion[c]				
			I	II	III	Mucosa	Submucosa	Muscular layer	Subserosa	Serosa
+	Male	12	1	9	2	0	5	3	1	3
	Female	6	0	4	2	0	3	2	0	1
	Total	18	1	13	4	0	8	5	1	4
−	Male	20	1	12	7	2	6	9	2	1
	Female	10	1	5	4	0	5	1	0	4
	Total	30	2	17	11	2	11	10	2	5

a) Carcinomas observed at all time points were combined for each male and female group.
b) Grade of dysplasia was evaluated as the highest in the tumors; I, mild; II, moderate; III, severe. See "Materials and Methods."
c) Tumor staging was evaluated from the most advanced portion in each case. For parts without serosa, tumors that invaded between the muscle layer of bladder and the adjacent tissue were included in the "subserosa" category.

the EE treatment in either sex as analyzed by the cumulative chi-square test. Several animals demonstrated advanced carcinomas in the urinary bladder, but no metastasis were observed. Squamous and glandular differentiation of TCCs was frequently observed. The EE treatment showed a tendency to increase such squamous and glandular differentiation in males, although the differences were not statistically significant. Such histological variation was not correlated with histologic malignancy based on grading and staging for each sex, as seen in Tables III and IV. No statistical significance was seen for either grading or staging with the cumulative chi-square test.

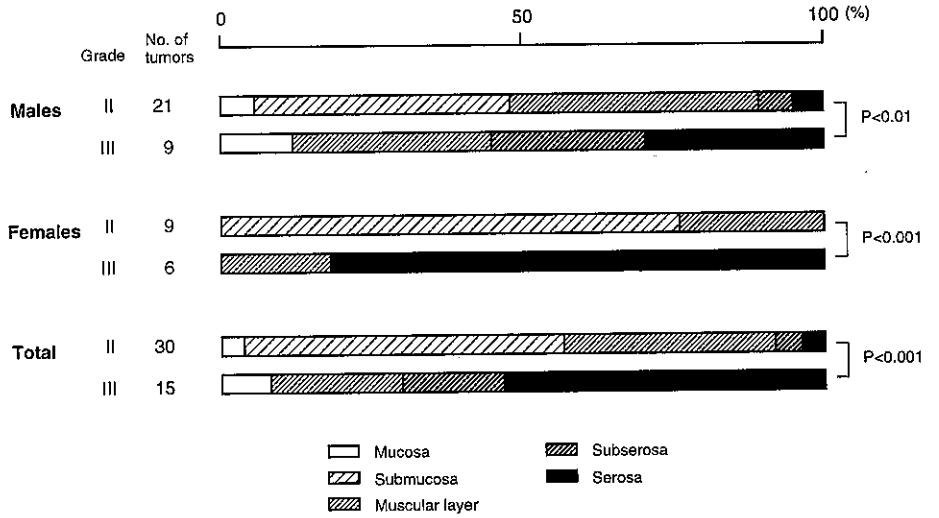

Fig. 4. Proportions of tumors in each category of malignancy. A good positive correlation was found between staging (level of invasion) and grading (degree of atypism) in DMAB-induced bladder carcinomas in hamsters. The cumulative chi-square test was carried out for statistical analysis of the difference between grades II and III for each of males, females, and all animals.

Thus, no sex dependence or modifying effect of EE on DMAB urinary bladder carcinogenesis was observed.

The relationship between degree of atypism (grade) and depth of invasion (stage) was analyzed as shown in Fig. 4. A good positive correlation between depth of invasion and cellular and structural atypia was observed. The correlation was best in females. The cumulative chi-square test was carried out for statistical analysis and *P* values for males, females, and all animals between tumors evaluated to be grades II and III were 0.01, 0.001, and 0.001, respectively.

DISCUSSION

In our previous report, we described the gallbladder carcinogenicity of DMAB and an enhancing effect of EE on this tumorigenesis in hamsters.[24] In the same study, we confirmed DMAB exerted urinary bladder carcinogenicity in both sexes in line with previous reports.[2, 9, 10] Bladder tumors developed within a shorter period than other lesions and were the presumptive main cause of death in some animals.

A number of nitroso compounds, nitrofurans and nitrosoureas have been extensively examined as bladder carcinogens in rats[23, 25, 29, 30] and in mice.[26, 27, 29] Recently, uracil was identified as a bladder carcinogen in both rats and mice, with associated marked urolithiasis.[31] This is a typical model of compound-linked cell proliferation and carcinogenesis, but the effects have not been examined in hamsters. Lijinsky[32] compared the carcinogenic potential of a variety of nitrosamines in rats, mice and hamsters and identified bladder carcinogenicity for 4 compounds in hamsters. Hirose *et al.*[29] reported, however, that the carcinogenic potential of some nitrosamines is far less in hamsters than in either rats or mice. Therefore, DMAB may be the most potent bladder carcinogen in hamsters.

In 1972, So and Wynder[9] first reported DMAB-induced bladder carcinomas in Syrian golden hamsters. Thereafter, Williams and his colleagues[2, 10] showed, in male hamsters, that DMAB-induced bladder carcinomas were mostly of invasive transitional cell type, with most TCCs being moderately well differentiated with a prominent squamous component. Shirai *et al.*[22] reported that EE significantly increased the development of tumors of the liver and kidney in F344 male rats, but inhibited the development in the lungs and urinary bladder. In the present experiment, DMAB induced invasive bladder carcinomas irrespective of additional EE treatment in both sexes. Although the incidence was low, invasive bladder carcinomas were already present in hamsters killed after just 20 subcutaneous injections of DMAB with EE treatment. This observation is comparable with those in previous reports describing induction of invasive carcinomas after about 7 months in a similar experimental protocol to that used in the present study.[2, 10]

The model offers a good contrast to rat bladder carcinomas, which are generally of papillary non-invasive type, irrespective of the carcinogen.[18, 22, 23, 27] Similarly to the mouse models,[26, 27] the DMAB-induced hamster bladder carcinoma is a good animal model for non-papillary invasive type of bladder cancer.

It has been considered that TCCs with squamous cell differentiation and squamous cell carcinomas are biologically more malignant than TCCs without any association with squamous components.[33] In rat lesions, Fukushima *et al.*[25] reported a positive correlation between grading of malignancy and degree of squamous cell differentiation. In the present hamster model, how-

ever, although many tumors were associated with glandular and/or squamous differentiation these histological features were not directly linked with grade and stage of carcinomas. However, the depth of invasion was significantly correlated with the degree of cellular and structural atypism of tumors, as shown in Fig. 4.

EE treatment is known to modify tumorigenesis in a variety of organs in animals[17–19, 22, 34, 35] and liver carcinogenicity is suspected in man.[36, 37] In rats, significant promoting potential of EE for liver and kidney carcinogenesis, and inhibition of lung and urinary bladder tumorigenesis were demonstrated.[22, 38] It is well established that a sex difference is generally present in rat bladder carcinogenesis[23, 28] and our previous data were in line with this observation.[28] In contrast, sex difference has not been established in hamsters. In the present experiment, no sex difference was observed and the effects of coadministration of EE with DMAB were not obvious in terms of morphological findings in the liver, gallbladder or urinary bladder.

In conclusion, the DMAB-hamster bladder cancer model appears appropriate for studies of non-papillary invasive type bladder carcinoma. Present data do not support any correlation between squamous and/or glandular components in TCCs and histopathological parameters of tumor malignancy such as increasing grade and stage.

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