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Effects of α-difluromethylornithine on the development of deeply invasive urinary bladder carcinomas in mice*

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Summary. α-Difluoromethylornithine (DFMO), was examined for its ability to suppress the development of invasive urinary bladder carcinoma in C3H/He male mice. Continuous administration of 0.2% DFMO in water following carcinogen treatment (0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine, BHBN, in drinking water for 8 weeks) was effective in suppressing urinary bladder carcinomas ($P < 0.05$) as compared with the control group. However, when comparison was made based on tumors involving the entire urinary tract, protective effects could not be demonstrated. Coadministration of DFMO (0.2%) and BHBN (0.01%) did not alter tumor induction by the latter. These results were in sharp contrast to the protective effects in rats. Since bladder tumors in rats are of low grade and superficial whereas those in mice are of high grade and deeply invasive, our data indicate that DFMO has little to no effects against the development of aggressive forms of bladder carcinoma.

Key words: Urinary bladder carcinoma – Mouse – Ornithine decarboxylase – α-Difluoromethylornithine – Polyamines

Ornithine decarboxylase (ODC) catalyzes the first committed step of polyamine biosynthesis [5], and the cellular content of polyamines is closely correlated to cell proliferation, differentiation and tumor development [10]. DFMO, an irreversible inhibitor of ODC, inhibited tumor-promoter-induced polyamine accumulation and carcinogenesis in mouse skin [14]. Inhibitory effects of DFMO were also demonstrated in oncogenesis studies involving other organs [4, 13]. We have shown that DFMO is effective in inhibiting (retarding) the development of bladder cancer in the rat when administered topically [1, 12] or orally [3]. This protective effect was attributed to suppression of polyamine biosynthesis in the urothelium stimulated by normal urine and a specific urinary component which stimulates ODC activities in vitro [2]. This notion is supported by the reversion of DFMO protection by exogenous putrescine [6] which is the ODC-catlyzed reaction product [11]. In these studies, however, tumors induced were mostly of low biologic grade and were rarely invasive. The present study was conducted to determine if DFMO was similarly effective in suppressing the development of a more aggressive form of bladder carcinoma. Mice were used because carcinomas induced after BHBN treatment are deeply invasive [7].

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Materials and methods

Animals and chemicals

C3H/He male mice, 5 to 7 weeks old, (Harlan Sprague Dawley Inc., Indianapolis, IN) were housed 5 to 6 per cage in an air-conditioned room at 22°C with 50% humidity under a 12-h light/dark cycle. Diet (Purina 5012, Ralston Purina Co., St. Louis, MO) and drinking water were given *ad libitum*. The carcinogen, BHBN was obtained from Tokyo Kasei Organic Chemicals, Tokyo, Japan and DFMO was a generous gift of the Merrell Dow Research Institute, Cincinnati, OH.

Experimental design

Experiment 1. Animals were divided into 8 groups, each consisting of 35 or 36 mice (Fig. 1). In group A, mice were treated initially with 0.05% BHBN in distilled water for 8 weeks. They were then arbitrarily divided into 3 subgroups, A1, A2 and A3 receiving 0.5, 0.2, and 0% DFMO, respectively, in distilled water for an additional 16 weeks. In group B, DFMO and BHBN were coadministered throughout the experiment: Groups B1, B2 and B3 receiving water containing 0.01% BHBN together with, respectively, 0.5%, 0.2%, and 0% DFMO for 26 weeks. Mice of Groups C and D were given water containing 0.5% and 0% DFMO, respectively, without carcinogen for 26 weeks. Drinking water was prepared fresh twice a week, and consumption per cage was measured at each water change. DFMO solution was adjusted to pH 7.0 with 10 N NaOH. The concentration of sodium in water without DFMO was adjusted

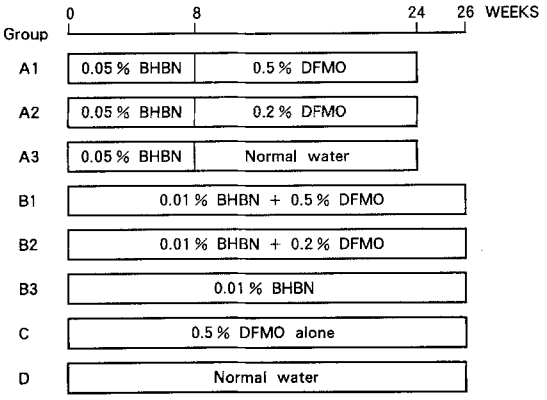

Fig. 1. See text

by adding NaCl solution. Stability of DFMO and/or BHBN in drinking water at room temperature had been confirmed previously [3]. Mice were weighed once every 2 weeks.

Autopsy

At autopsy all major organs except for the brain were inspected. From 80 randomly selected mice (10 in each group), the following organs were examined microscopically; lung, heart, liver, spleen, jejunum, colon, testis, abdominal skin, skeletal muscle, and bone and bone marrow of lumbar vertebrae. Enlarged lymph nodes, when found in paraaortic or iliac regions were examined microscopically. The genitourinary system including the kidneys, bladder, prostate and seminal vesicles were resected *en bloc*. The bladder was inflated with 10% neutral formalin instilled per urethra. Following overnight fixation, the specimen was bisected, gross alterations were recorded, bladder tumors were counted, their size was measured, and finally bladders were cut longitudinally into 8 to 10 pieces and submitted *in toto* for microscopic study. Urothelial lesions were examined according to the criteria described previously [8, 9]. Undifferentiated spindle or pleomorphic carcinoma was defined as grade 4 carcinoma.

Experiment 2. An additional experiment was conducted to determine whether DFMO's failure to suppress tumorigenesis is correlated with its failure to suppress polyamine concentration in urothelial cells. Sixty male mice were assigned to the following 4 groups, each consisting of 15 mice. These were group B1′ (0.01% BHBN and 0.5% DFMO in drinking water), group B2′ (0.01 BHBN), group C′ (0.5% DFMO) and group D′ (untreated). After 12 weeks of treatment, animals were killed. Five bladders were pooled, the urothelia were separated from the bladder walls and polyamine concentrations were measured by the method previously described [1]. Results were expressed as concentration per 10^6 urothelial cells.

Results

Because of the increasing mortality of mice receiving BHBN, the study was terminated at 24 weeks instead of the originally scheduled time at 40 weeks. Retardation in weight gain was noted in animals receiving BHBN as early

Table 1. Microscopic findings of urinary bladder

Group	Treatment	No. of mice effective	No. of mice with (%) tumors	Total no. of tumors	No. of mice with bladder tumors[a]							
					Grade				Stage			
					1	2	3	4	P0	P1	P2	P3[b]
A1	0.05% BHBN →0.5% DFMO	30	11[c] (34)	13	0	6	4	1	2	3	1	5
A2	0.05% BHBN →0.2% DFMO	34	7[d] (21)	7[e]	0	1	6	0	2	1	0	4
A3	0.05% BHBN →0% DFMO	32	16 (50)	24	0	3	13	0	3	2	3	8
B1	0.01% BHBN + 0.5% DFMO	31	31[f] (100)	74	0	0	19	12	1	6	8	16
B2	0.01% BHBN + 0.2% DFMO	34	34[f] (100)	85	0	0	21	13	1	6	4	23
B3	0.01% BHBN + 0% DFMO	32	32[f] (100)	62	0	0	19	13	0	2	7	23
C	No BHBN → 0.5% DFMO	31	0									
D	Untreated	35	0									

[a] The highest grade or stage of tumors was scored; [b] Invasion to surrounding tissue is included; [c] A mouse with lung metastasis is included; [d] Statistically different from Group A3 ($P < 0.05$, x^2 test); [e] Statistically different from Group A3($P < 0.05$, student's t test); [f] A mouse with lymph node metastasis is included

Table 2. Microscopic findings of urinary tract other than bladder

Group	No. of Mice evaluable	Urethra or[a] periurethra		Ureter		Renal pelvis		Overall incidence of urinary tract (%)
		CIS[b]	CA[c]	CIS	CA	CIS	CA	
A1	30	0	2	0	1	3	1	12 (40)
A2[d]	34	1	3	1	0	1	1	10 (29)
A3	32	0	1	2	1	3	2	17 (53)
B1	31	0	4	7	1	0	0	31 (100)
B2	34	2	1	3	4	2	0	34 (100)
B3[d]	32	1	4	3	2	0	1	32 (100)
C	31	0	0	0	0	0	0	0
D	35	0	0	0	0	0	0	0

[a] Considered to bedistinct from bladder carcinoma or invasion; [b] Carcinoma *in situ*; [c] Carcinoma; [d] One mouse in Group A2 and 4 in Group B3 were not evaluated

Table 3. Polyamine concentrations (nmol)/10^6 urothelial cells

	B1′	B2′	C′	D′
BHBN (%)	0.01	0.01	0	0
DFMO (%)	0.5	0.5	0.5	0
12 weeks				
Putrescine	12 ± 6	14 ± 2	11 + 5	13 ± 4
Spermidine	195 ± 43	247 ± 39[1]	73 ± 8	54 ± 21
Spermine	296 ± 64	202 ± 33	246 ± 64	193 ± 22

as 8 weeks ($P < 0.05$) and persisted throughout the experiment. In group B where BHBN and DFMO were coadministered, DFMO at 0.5% in drinking water retarded weight gain more strikingly. DFMO alone (group C) also resulted in decreased weight gain which was noted as early as 12 weeks and persisted throughout the study. Water consumption in group A animals (BHBN followed by DFMO) was higher than in the control groups (group C and D) and the difference became more obvious during the latter half of the experiment. No significant difference was noted among group A or B animals, however. Altogether 6 mice (2 in group A1, 1 in group A2, 2 in group A3 and 1 in group B3) were not available for microscopic evaluation due to autolysis.

Microscopic findings of the urinary tract are shown in Tables 1 and 2. The incidence of bladder carcinoma in group A (DFMO after BHBN treatment) ranged from 21 to 50%. Protective effects were demonstrated in only group A2 (0.2% DFMO); in this group the incidence of tumors and the total number of tumors were lower than those in group A3 ($P < 0.05$, for each comparison). However, when the tumor incidence involving the entire urinary tract was compared, DFMO inhibition could not be demonstrated. In group B where BHBN and DFMO were administered simultaneously, the tumor incidence reached 100% in all subgroups. As expected, all carcinomas were of high grade, mostly grades 3 and 4 showing squamous differentiation in a great majority of the cases. Most tumors were in stage B (muscle layer involved) or C (extension beyond the muscle layer) and frequently invaded the perivesical tissues including the seminal vesicles, prostate and pelvic skeletal muscles. Foci of severe dysplasia and carcinoma *in situ* were common in carcinogen-treated bladders, particularly in group B (BHBN and DFMO coadministered). Metastasis to retroperitoneal lymph nodes was documented in 3 mice of group B and lung metastasis in 1 mouse of group A.

Significant alterations were found in kidneys of animals treated with the carcinogen: hydronephorosis of varying severity with or without acute and chronic pyelonephritis was common. These changes were attributed to obstruction of urine flow due to carcinoma involving the trigone of the bladder or urethra. Changes which were attributable to DFMO administration were not demonstrated in any organs examined microscopically.

The polyamine concentrations in urothelial cells are shown in Table 3. These was no difference in putrescine concentration among the groups. Treatment with BHBN (group B2′) resulted in significant increase in spermidine level as compared to the untreated control group ($P < 0.05$).

Discussion

In one of our recent studies with rats, oral DFMO effectively suppressed the development of urinary bladder carcinoma [3]. In that study DFMO effects were investigated under 2 different carcinogen exposure schedules. In the first, DFMO treatment was delayed until completion of carcinogen treatment. In second, carcinogen at a lower level was co-administered with DFMO. In both schedules, the majority of tumors were preinvasive and were transitional cell carcinomas of grades 1 and 2. DFMO not only suppressed the incidence of tumors but also reduced the average volume of tumor [3].

The present investigation extended our earlier findings by studying a more aggressive form of bladder carcinoma using the identical experimental protocol. Some degree of inhibitory effects was demonstrated in 1 group of animals that had received 0.2% DFMO after BHBN initiation (group A, $P < 0.05$). Yet, in this group, when comparison was made for tumors involving the entire urinary system (renal pelvis and ureters included) no significant inhibitory effects were found. In the second group of study involving the coadministration of carcinogen and DFMO, the tumor incidence reached 100% in all animals irrespective of DFMO treatment. A crucial question is whether the treatment schedule was inadequate in suppressing polyamine synthesis in tumor tissue below critical levels and whether this may have been responsible for the failure of DFMO treatment. This possibility is supported by the failure of DFMO treatment to lower putrescine and spermidine levels at 12 weeks when BHBN treatment induces early neoplastic lesions. Suppression of polyamine syntheis could have been achieved if higher DFMO doses had been used. It is unlikely, however, that mice can tolerate such doses for an extended period of time because DFMO alone (0.5%, group C) resulted in decrased weight gain.

Now that chemopreventive use of DFMO is being seriously considered as a means of suppressing human cancer recurrence, we believe that our studies provide an important message to urologists and oncologists that DFMO treatment not be considered in patients with the prior history of high grade carcinoma, either invasive or preinvasive.

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