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Therapeutic Effect of a Retinoid (Ro 10-9359) on Rats with Bladder Tumours Induced by N-Butyl-N-(4-Hydroxybutyl)-Nitrosamine Upon Administration Alone or in Combination with Mitomycin C

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Summary. The therapeutic effect of an aromatic retinoic acid analogue (Ro 10-9359) and mitomycin C (MMC) on rats with bladder tumours induced by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) was examined. Eight-week-old female Wistar rats were given 0.05% BBN in drinking water for 8 weeks. Therapy was started at week 26 and all rats were killed at week 30. MMC at a dose of 0.3 mg/kg twice a week ip for 3 weeks significantly reduced the incidence and the mean number of tumours. With oral Ro 10-9359 at a dose of 100 mg/kg once weekly for 4 weeks, no significant effect was observed. The combination of MMC and Ro 10-9359 significantly reduced the mean number, but not the incidence, of tumours. The difference between the effect of MMC alone and that of MMC given in combination with Ro 10-9359 was not statistically significant. Thus no favorable effect of the retinoid could be demonstrated either alone or in combination with MMC.

Key words: Retinoid, Mitomycin C, Bladder carcinoma, N-butyl-N-(4-hydroxybutyl)nitrosamine, Combination chemotherapy.

Introduction

The preventive effects of retinoids against chemically or virally induced tumours of various organs including the urinary bladder have been demonstrated in both rats and mice [2, 10]. However, clinical trails of the retinoids, 13-*cis*-retinoic acid and ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (Ro 10-9359), as agents which might inhibit the recurrences of bladder tumours have not been so successful as was initially expected, which might at least partly be due to their severe side effects [9, 16].

Retinoids have also been shown to inhibit the proliferation of various malignant cells in culture, and the development and growth of tumours in vivo [6, 10]. Recently, we have observed that Ro 10-9359, given orally, significantly inhibits the growth of BC50-TC cells, an established tissue culture line originating from the bladder carcinoma produced by N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN), inoculated subcutaneously into the dorsum of ACI/N rats [7]. The combined use of Ro 10-9359 with MMC, however, had a no more favorable effect on the tumour than did either treatment alone, but rather MMC had a suppressive effect on the action of Ro 10-9359. In the present experiment, we assessed whether Ro 10-9359 alone or in combination with MMC has a similar effect on bladder tumours induced in situ by BBN to that on subcutaneously inoculated BC50-TC cells.

Materials and Methods

Chemicals. Ro 10-9359 was obtained from Hoffmann-La Roche & Co., Ltd., Basel, Switzerland and suspended in peanut oil at a concentration of 40 mg/ml. MMC was purchased from Kyowa Hakko Kogyo, Tokyo, Japan, and dissolved in sterile water to 60 μg/ml. Both solutions were freshly prepared before use. BBN was from Nakarai Chemical Co., Kyoto, Japan and given as a 0.05% solution in water from light-proof bottles, which were filled every other day.

Animals. Female Wistar rats (Shizuoka Experimental Animal Farm, Shizuoka, Japan) were 8 weeks old at the time of the first carcinogen administration. Rats were housed 4 per cage and given a commercial pelleted diet F-2 (Funahashi Farm, Chiba, Japan) and water with or without BBN ad libitum.

Design of the study. Animals were divided into 6 groups and treated as shown in Fig. 1. Group 1, the control, was given neither carcinogen nor drugs throughout the experimental period. The rats in groups 2–6 were given 0.05% BBN for 8 weeks. The rats in groups 2 and 3 were sacrificed with no further treatment at week 25 and 30, respectively. Group 4 was given MMC alone. Group 5 was given Ro 10-9359 alone, and group 6 was given Ro 10-9359 within a few minutes after the MMC administration. MMC was given ip at a dose

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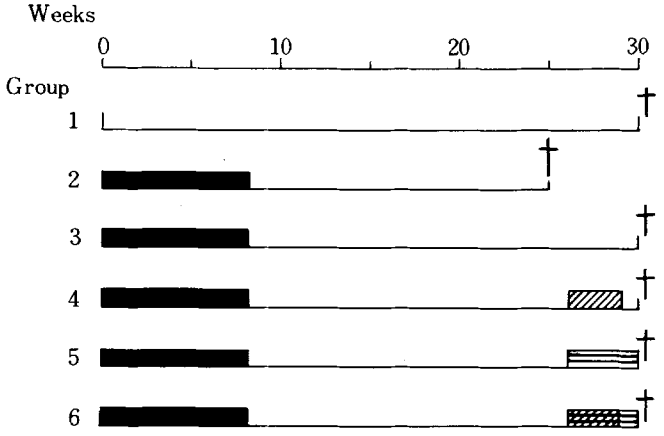
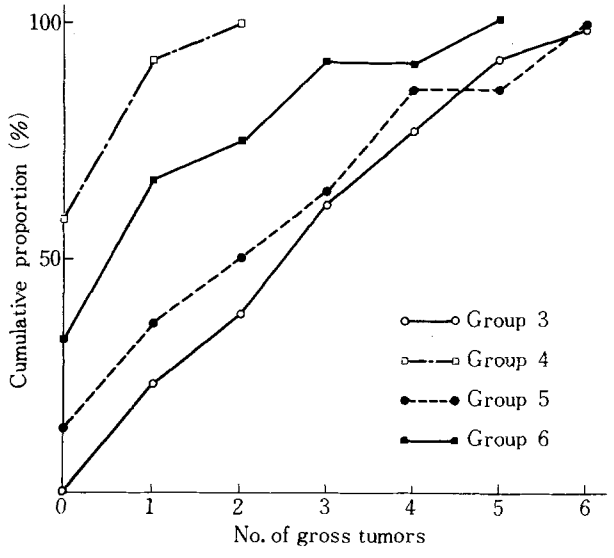


Fig. 1. Experimental protocol. ▬ 0.05% BBN in drinking water, ▨ MMC, 0.3 mg/kg twice/week x 3 weeks, ip, ▤ Ro 10-9359, 100 mg/kg/week x 4 weeks, po, † sacrifice



of 0.3 mg/kg body weight twice weekly from week 26 to 28. Ro 10-9359 was given orally at a dose of 100 mg/kg once a week from week 26 to 29. The rats were weighed every week and observed daily for external signs of retinoid toxicity.

All animals except those in group 2 were killed at week 30. At sacrifice, the urinary bladders were distended with 10% formaldehyde solution and a ligature was placed around the neck of each bladder to maintain proper distention. After fixation and counting of the number of gross tumours using a magnifying glass, each bladder was sliced with 2 cuts into 3 areas according to the method of Squire et al. [14]. Tissues were embedded in paraffin and stained with hematoxylin and eosin. Bladder lesions were classified as described by Ito et al. [8]. χ^2-test or Student's t-test was used to assess the significance of the differences in the incidence and the number of tumours among the groups.

Results

Table 1 shows the incidence and the number of urinary bladder tumours among the various groups. MMC had a marked effect: The incidence of gross bladder tumours was reduced from 100% (Group 3) to 42% (Group 4) ($p < 0.01$), and the mean number of tumours per rat was reduced from 3.2 (Group 3) to 0.5 (Group 4) ($p < 0.05$) (Fig. 2). On the contrary, Ro 10-9359 (Group 5) had no significant effect. The combination of MMC and Ro 10-9359 (Group 6) reduced both the incidence and the number of tumours, but only the latter was statistically significant ($p < 0.05$).

Histopathological examination revealed that the incidences of cancer, papilloma and hyperplasia were all reduced in rats given either MMC, Ro 10-9359 or a combination of the two. However, only the reductions in the incidences of cancer and papilloma observed in rats given MMC alone were of statistical significance ($p < 0.01$). For all the parameters evaluated, the effect of MMC alone (Group

◀ **Fig. 2.** Cumulative distribution of the number of gross tumors. Female Wistar rats were treated as shown in Fig. 1. and killed at week 30. The number of the tumors in each bladder was counted using a magnifying glass

Table 1. Effect of Ro 10-9359 and/or Mitomycin C on BBN-induced bladder tumours in female wistar rats

Group[a]	No. of rats		No. of rats with gross tumour (%)	No. of tumours/rat (mean ± SD)	Histopathological changes (%)		
	Initial	Final			Hyperplasia	Papilloma	Carcinoma
1	10	10	0 (0)	0	0 (0)	0 (0)	0 (0)
2	10	9	8 (89)	2.3 ± 2.5	9 (100)	7 (78)	6 (67)
3	13	13	13 (100)	3.2 ± 1.9	13 (100)	12 (92)	12 (92)
4	13	12	5 (42)[c]	0.5 ± 0.6[c]	8 (67)[b]	4 (33)[d]	4 (33)[d]
5	16	14	12 (86)	2.6 ± 1.9	14 (100)	11 (79)	8 (57)
6	12	12	8 (67)[b]	1.4 ± 1.5[c]	11 (92)	6 (50)[b]	6 (50)[b]

[a] Female Wistar rats were housed 4 per cage and treated as shown in Fig. 1. All rats except those in group 2 were killed at week 30. Rats in group 2 were killed at week 25

[b] $p < 0.1$

[c] $p < 0.05$

[d] $p < 0.01$ significantly different from group 3

Table 2. Effect of Ro 10-9359: average histological criteria scores[a]

Histological criteria	Control (n = 13)	Ro 10-9359 (n = 14)
Flat proliferative lesions[b]	3.36	2.61
Papillary and polyploid proliferative lesions[b]	2.09	1.35
Squamous metaplasia[b]	1.45	0.5
Cellular atypia	1.72	1.5

[a] Criteria for histopathological evaluation and the method of statistical analysis according to those described by Squire et al. [14]. A score per slide ranges between 0 and 5

[b] The focal score plus twice the diffuse score

4) was more favorable than that of MMC combined with Ro 10-9359 (Group 6), but the difference was not statistically significant. The effect of Ro 10-9359 was further assessed by Squire's method of histopathological evaluation [14], which allows semiquantitative comparisons and statistical analysis of the severity and extent of lesions. Although the severity of lesions was suggested to be lessened, the difference was not statistically significant (Table 2).

No lesions were observed in the control animals given no treatment (Group 1). Neither calculi nor parasites were noted in any of the bladders examined. The body weights of rats in the 6 groups were similar, and there was no sign of hypervitaminosis A such as epilations or bone fractures.

Discussion

Vitamin A deficiency in rats has been reported to result in squamous metaplasia of the urinary bladder and a high incidences of cystitis, ureteritis, and pyelonephritis, and to accelerate the carcinogenesis induced by N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) [4]. Prophylactic effects of retinoids against bladder carcinogenesis have also been experimentally proved [13, 15], although there are some conflicting reports [5]. Clinically, a retrospective analysis of data on dietary habits from bladder cancer patients has revealed increases in risk for lower levels of vitamin A intake [11]. Retinoids, however, have failed to show definite effects on recurrences of bladder tumours [9, 16]. To be of significance in prevention of cancer in humans, retinoids may have to possess antitumour or anticarcinogenic activity when administered at a time somewhat removed from the initiation of the neoplastic process, since the earliest stages of tumour development in humans cannot be readily identified. Our present study was aimed at assessing the therapeutic, but not prophylactic, effect of Ro 10-9359 on bladder carcinoma. The results obtained might be considered to reflect mostly the therapeutic effect of the drugs, since gross tumours were found in 89% of rats at week 25 (Group 2) and the drug administration was started from week 26.

Administration of Ro 10-9359 has a significant effect on the growth of tumours in ACI/N rats inoculated subcutaneously with BC50-TC cells [7]. In the present experiment, however, there was no apparent therapeutic effect of Ro 10-9359 on the incidence or the number of BBN-induced autologous bladder tumours by administration in a similar dose and route. Even with semiquantitative histopathological analysis, no significant effect was detected. The difference between the results of the previous study and those of the present study may be due to several factors. In the present experiment, female Wistar rats were used to avoid bladder calculi formation and parasites, and the effect of Ro 10-9359 was expressed in terms of the incidence and number of bladder tumours, whereas in the previous experiment male ACI/N rats were used, and the size and weight, but not the presence or absence, of the subcutaneous tumour were compared. If the retinoid exerts its therapeutic effect via an immunological mechanism [3], the difference in the origins of the tumour, one being established in another rat and then transplanted, and the other induced by orally administered carcinogen, may also be important. Further investigation is necessary, since Ro 10-9359 has been reported to be effective against tumours induced by other chemical carcinogens or viruses [2, 6, 12].

Retinoids are known to potentiate both the in vitro and in vivo antitumour effects of several cytotoxic agents [1]. In accordance with the previous report [7], MMC, at 0.3 mg/kg ip twice a week for 3 weeks, showed significant therapeutic effects in the present experiment. However, the effect of MMC combined with Ro 10-9359, which alone had no effect, was less favorable than that of MMC alone, although the difference was not statistically significant. We have also observed the lack of synergism between these two agents in the previous experiment using BC50-TC cells inoculated subcutaneously. In consideration of the fact that the effect of Ro 10-9359 in combination with MMC on these cells is additive in vitro (Fujita J and Yoshida O, unpublished data), there seems to be a complex in vivo interaction between these two agents. Therefore, in prescribing retinoids clinically, we should assess the effect promptly and adequately taking in account not only the side effects but also the possibility of antagonism with other drugs.

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