Original

Lack of Inhibition of BBN-induced Bladder Carcinogenesis in C57BL/6 Mice by Intravesical Instillation of KRN 7000

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Abstract: The immunostimulatory α -galactosylceramide, KRN 7000 or (2S, 3S, 4R)-1-0-(α -D-galactopyranosyl)-2-(N-hexacosynoylamino)-1,3,4-octadecatrienol, might be anticipated to have antitumor activity *in vivo* apart from any direct toxicity to cancer cells. We investigated inhibition of mouse bladder carcinogenesis by intravesically instilled KRN 7000. C57BL/6 mice were divided into 4 groups; all first receiving the carcinogen 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine in drinking water for 8 weeks. Next, groups 1 and 2, respectively were administered 10 and 0.1 μ g/kg of KRN 7000 intravesically once weekly for 17 weeks. Group 3 received only 0.3 ml of saline (vehicle control). Group 4 did not undergo bladder catheterization. By histologic examination at 26 weeks, the incidence of bladder carcinoma of all types tended to be higher in group 1 than in group 3, but without significance. The incidence of bladder carcinoma in group 4, (no catheterization), was similar to that in group 1. Only one precancerous lesion (papillary or nodular dysplasia) was seen in each of groups 3 and 4. Thus vesical instillation of KRN 7000 did not inhibit bladder carcinogenesis in mice, exposed to the carcinogen studied. (J Toxicol Pathol 2003; 16: 19–23)

Key words: KRN 7000, C57BL/6 mouse, intravesical instillation therapy, bladder carcinoma

Introduction

Patients with urinary bladder cancer have a high risk of recurrence after transurethral resection¹. Further, a tumor in recurrence is often biologically more aggressive than initially¹. Although the mechanism underlying this high risk of recurrence have not been well explained, two hypotheses have been proposed. One is that cancer cells might be transplanted into urothelium at another site through the urinary stream. Urothelial trauma during endoscopy may provide an opportune site for such tumor cells to implant and grow. Laboratory studies in animals and circumstantial evidence in patients have provided evidence supporting this mechanism for some recurrences^{1,2}. Soloway *et al.*³ reported high susceptibility of cauterized urinary bladder to tumor cell implantation in a mouse model. In contrast, Nakamura *et al.*⁴ suggested the presence of a system in intact

urothelium, especially involving umbrella cells, that works to prevent implantation. The other hypothesis is one of urothelial multifocal carcinogenesis. Some reports describing histologic mapping of bladders resected for invasive cancer have documented additional findings of multifocal carcinoma or carcinoma *in situ* (CIS) in approximately 80% of specimens^{1,5}. Other reports based on biopsy specimens showed an incidence of atypia, CIS, or cancer from normal-appearing mucosa at distant sites from an evident tumor, ranged between 20% and 80%¹. Various anticancer agents have been instilled into the urinary bladder to treat such high-risk epithelia. Intravesical instillation therapy with bacillus Calmette-Guerin (BCG) has been used for more than 20 years to treat multifocal CIS and also as an adjuvant therapy after transurethral resection of Ta (non-invasive papillary carcinoma) and T1(subepithelial connective tissue invasion tumor) papillary bladder cancer, this is now considered the most successful immunotherapy for bladder tumors. Sometimes, however, the therapeutic protocol cannot be completed because of serious side effects,

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Abbreviations: BBN: N-butyl-N-(4-hydroxybutyl)nitrosamine, DC: dendritic cell, NK: natural killer, NKT: natural killer T, BCG: bacillus Calmette-Guerin, CIS: carcinoma *in situ*.

such as, pyrexia, generalized fatigue, and bladder pain. Recently developed agents with less potential for side effects are hoped to be effective for intravesical immunotherapy.

The immunostimulatory α -galactosylceramide, KRN 7000 or (2S,3S,4R)-1-O-(α -D-galactopyranosyl)-2-(N-hexacosnoylamino)-1,3,4-octadecatrienol ($C_{50}H_{99}NO_9$; MW. 858.33; Fig. 1) might be anticipated to have antitumor activity *in vivo* apart from any direct toxicity to cancer cells⁶, and there are many proofs in the literature about its antitumor effects in mice. We investigated inhibition of mouse bladder carcinogenesis by intravesically instilled KRN 7000.

Materials and Methods

Experimental animals

Female C57BL/6 mice, 5 weeks old and weighing about 25 g, were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were housed in plastic cages with a bedding of paper chips, five mice per cage, in an environmentally controlled room maintained at a temperature of $22 \pm 2^\circ\text{C}$, a relative humidity of $55 \pm 10\%$, and a 12 h /12 h light-dark cycle. Standard diet (Oriental MF; Oriental Yeast, Co. Tokyo, Japan) and water were available *ad libitum* throughout the experiment.

Chemicals

N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) was obtained from Tokyo Kasei (Tokyo, Japan). KRN 7000 was kindly provided by KIRIN Brewery (Tokyo, Japan).

Experimental design

At 6 weeks of age, the mice were divided into four groups as specified below (Fig. 2) and all mice were given 0.05% BBN in the drinking water for 8 weeks to initiate bladder carcinogenesis. One week later after the initiation period, groups 1 and 2 (15 mice each) respectively were

administered 10 or 0.1 $\mu\text{g}/\text{kg}$ of KRN 7000 once weekly for 17 weeks in 0.1 ml of saline, given by intravesical instillation under anesthesia with intraperitoneal pentobarbital (Nembutal, ABBOTT Laboratories, Illinois, USA). As a catheter for intravesical instillation, we used the sheath of an intravenous injection catheter, inserting it through the urethra into the urinary bladder, after expulsion of the urine in the bladder, KRN 7000 was instilled using a 1 ml syringe pump. About 2 h after injection, the agent was allowed to drain from the bladder through the catheter. Group 3 (15 mice) received 0.1 ml of saline intravesically as a control. Group 4 (10 mice) underwent no catheterization or anesthesia.

During the experiment, the health of mice was checked daily. The mice were weighed every 2 weeks during the initiation period and then before each intravesical instillation. Water consumption and food intake were recorded every 4 weeks over a period of 2 days. The experiment was terminated at 26 weeks after it began, 1

KRN 7000 (α -galactosylceramide; $C_{50}H_{99}NO_9$, MW = 858.34)

Fig. 1. Chemical structure of KRN 7000.

Fig. 2. Experimental protocol for our study, 6-week-old female C57BL/6 mice. Black, 0.05% BBN in drinking water for first 8 weeks. Dark gray, 10 $\mu\text{g}/\text{kg}$ KRN 7000 by intravesical instillation once weekly for 17 times. Light gray, 0.1 $\mu\text{g}/\text{kg}$ KRN 7000 by intravesical instillation once weekly for 17 times. Crosshatched saline by intravesical instillation once weekly for 17 times.

week after the last instillation. Surviving mice were killed under light ether anesthesia. The urinary bladder was inflated with 10% buffered formalin as a solution fixative and then removed. The bladder was then weighed. The entire bladder was cut longitudinally into multiple strips according to the number and size of macroscopic tumors. These tissues were embedded in paraffin and examined histopathologically. Sections were cut and stained with hematoxylin and eosin (H and E) for microscopic examination. Liver, kidney, and spleen were also removed, weighed, examined macroscopically, and stained with H and E for histopathologic assessment. Mice in a critical condition were sacrificed and examined similarly at an earlier period.

Statistical analysis

Fisher's exact probability test was used to analyze the incidence of bladder tumors, and Student's *t* test was used for testing differences in body weights and relative organ weights between the groups. A value of *p* less than 0.05 was considered to indicate significance.

Results

By the middle of the experiment, eight mice died of overdoses of pentobarbital anesthesia as a complication of instillation treatment.

Body weight, food consumption, water intake, and organ weight

Body weights were similar in all groups during the initiation period, but after commencement of instillation treatment, body weight gains were less in groups 1 to 3 than

in group 4. Body weights were similar between groups 1 to 3 throughout the instillation period (Table 1).

No remarkable differences in the water intake or food consumption were noted between groups 1 to 3 during the experiment. Mice in group 4 consumed more food and water than others during the instillation period (Table 1).

Mean weights of liver and kidney (Table 1), as well as spleen (data not shown), were similar between all groups. Mean urinary bladder weights were similar in groups 1 to 4 (Table 1).

Macroscopic findings

Bladder tumors were evident macroscopically in 5 of 11 mice in group 1 (45.5%); 2 of 13 in group 2 (15.4%); 2 of 13 in group 3 (15.4%); and 3 of 13 in group 4 (25.1%). Almost all tumors were solitary, and they were examined histopathologically. No significant macroscopic findings were noted in liver or kidney.

Histopathological findings

Histopathologic changes of urinary bladder epithelium were classified as showing either preneoplastic change specifically, papillary or nodular (PN) dysplasia, (7–9) or carcinoma (transitional cell or squamous cell). No other bladder neoplasm occurred in these experiments. Data are summarized in Table 2. Total incidence of bladder carcinoma tended to be higher in group 1 than group 3, but the differences were not significant. The total incidence of bladder carcinoma in group 4 was similar to that in group 1. PN dysplasia was seen only twice, one in each of groups 3 and 4. No specific histopathologic changes were seen in liver or kidney between KRN 7000-treated and controls groups.

Table 1. Final Body Weight, Food Consumption, Water Intake, and Liver and Kidney Weight in Mice

Group	Treatment	No. of mice examined	Body weight (g) at week 26	Urinary bladder weight (g)	Liver weight (g)	Kidney weight (g)	Food consumption (week 26) (g/mouse/day) (mean)	Water intake (week 26) (ml/mouse/day) (mean)
0.05% BBN intravesical instillation								
1	+	10 μ g/kg b.w KRN 7000	11	22.7 $\pm$ 1.5 ^a	1.1 $\pm$ 0.1 ^a	1.1 $\pm$ 0.1 ^a	0.32 $\pm$ 0.03 ^a	6.0
2	+	0.1 μ g/kg b.w KRN 7000	13	22.4 $\pm$ 1.2*	1.1 $\pm$ 0.1	1.1 $\pm$ 0.1	0.32 $\pm$ 0.05	5.6
3	+	0.9% saline	13	23.2 $\pm$ 1.4*	1.1 $\pm$ 0.2	1.1 $\pm$ 0.2	0.31 $\pm$ 0.04	5.5
4	+	no treatment	10	27.6 $\pm$ 3.6	1.1 $\pm$ 0.1	1.1 $\pm$ 0.1	0.30 $\pm$ 0.03	7.1

BBN; N-butyl-N-(4-hydroxybutyl)nitrosamine, a; Values are mean $\pm$ SD, *; Significantly different from group 4, *P* < 0.05.

Table 2. Histologic Findings in Mouse Urinary Bladder

Group	Treatment			No. of mice examined	Carcinoma			PN dysplasia (%)
	0.05% BBN	intravesical	instillation		TCC (%)	SCC (%)	Total (%)	
1	+	10μg/kg b.w	KRN 7000	11	3 (27.3)	2 (18.2)	5 (45.5)	0 (0)
2	+	0.1μg/kg b.w	KRN 7000	13	2 (15.4)	0 (0)	2 (15.4)	0 (0)
3	+	0.9% saline		13	1 (7.7)	0 (0)	1 (7.7)	1 (7.7)
4	+	no treatment		10	2 (16.7)	2 (16.7)	3 (25.1)	1 (8.3)

BBN; N-butyl-N-(4-hydroxybutyl)nitrosamine, TCC; transitional cell carcinoma, SCC; squamous cell carcinoma, PN; papillary or nodular.

Discussion

KRN 7000 is isolated from extracts of the sponge, *Agelas mauritianus*¹⁰. Kobayashi *et al.* reported that this agent markedly augmented natural killer (NK) activity in spleen cells *in vivo*, and also showed significant antitumor activity in mice with lung metastasis following transplantation of B16 melanoma cells¹¹. Nakagawa *et al.*¹² reported that KRN 7000 had a strong antitumor activity in mice with Colon 26 hepatic metastases, accompanied by marked activation of NK cells in the liver, and intravenous injection of KRN 7000 resulted in a high percentage of cures in their mice, which thus acquired tumor-specific immunity¹³. Moreover, the antitumor effect was stronger than those of known chemotherapeutic agents such as mitomycin C, 5-fluorouracil, or adriamycin; the effect was equal to that of interleukin-12. Accordingly, KRN 7000 is hoped to be useful for the treatment of various liver metastases. Reports have suggested a sequential mechanism of antitumor activity. First, the molecule of KRN 7000 is taken up by a dendritic cell (DC) and then presented on the DC membrane together with a CD1 molecule. The CD1/KRN 7000 complex is recognized by natural killer T (NKT) cells with V α 14/V β 8¹², attacking tumor cells¹⁴ and releasing various cytokines such as interleukin-4 and interferon- γ ¹². In turn, NK cells and macrophages are activated and injure tumor cells non-specifically¹⁵. Furthermore, CD8⁺ cytotoxic T lymphocytes with a tumor-specific effect are induced. This combination of antitumor mechanisms for KRN 7000 is considered unique, differing in important ways from those of other antitumor agents. In recent studies, activation of DC by KRN 7000 *in vitro*, a significant antitumor effect was seen when DCs were injected into cancer-bearing mice^{6,16}. Such treatment of various cancers using KRN 7000 has been considered promising.

As specialized leukocytes, DCs in different human organs show various degrees of maturity and sometimes have an organ-specific name¹⁷, such as Langerhans cells in skin. DCs also circulate in the blood circulation, accounting for 0.14% of peripheral blood leukocytes. Although the mechanism of DC activation has not been fully elucidated, DCs apparently infiltrate cancerous tissue, take up tumor antigens, and migrate to the periphery of the cancer where they present the antigen to T lymphocytes. In tumors, DC has been often observed in association with T cells. According to another report, cancer cells expressing CD1d molecules become targets of NKT cells activated by KRN 7000¹⁸. Thus detection of CD1d expression by cancer cells might be an indication of a potential anticancer effect¹⁹. Little has been reported about localization of DC in the urinary bladder or its cancers, but Hart *et al.*²⁰ found MHC class II⁺ DC beneath the epithelial layer, as has been seen in other epithelial surfaces²¹. Inoue *et al.*²² reported that S100 protein-positive DC, suspected to be a prognostic factor in various cancers, were also present in transitional cell carcinomas of the urinary bladder.

Antitumor effects of intravesical BCG therapy are well

known, and involve activation of the immunologic response²³. Yet BCG often has serious side effects and new immunotherapeutic agents have been sought for bladder tumors. We expected that intravesical instillation of KRN 7000 would be effective against bladder cancer, as well as being essentially free of serious side effects. In mice, piloerection soon after intravenous administration and liver toxicity from KRN 7000 have been reported¹², but we did not find any abnormalities in our samples of liver, spleen, or kidney, and observed no piloerection upon treatment.

In the present experiments, we planned to use an intravesical concentration of KRN 7000 that corresponded to the most effective dose in *in vitro* studies (100 ng/ml). We therefore used a similar dose in high-dose mice and a 1:100 dilution of this in low-dose mice. The ideal duration of drug retention in the bladder and the volume to be instilled effective are uncertain. Agents for intravesical instillation therapy in humans (pirarubicin hydrochloride or cytarabine) are usually retained for only 30 min, and are instilled in a solution volume of 20 ml. We based duration and relative amounts on these protocols, in order to avoid missing the therapeutic effect of KRN 7000. The frequency of instillation corresponded to chemotherapy instillation protocols for the human bladder, once weekly. Intraperitoneal administration every 4 days has been reported to show a clear anticancer effect in mice, while overly frequent administration of the agent was avoided so as not to decrease cytokine induction (unpublished data).

In the present experiment, long cumulative intervals of respiratory insufficiency related to anesthesia may have caused lower body weights in catheterized mice. No significant differences in incidence of bladder carcinoma or PN dysplasia noted between groups, so KRN 7000 neither inhibited nor facilitated bladder carcinogenesis. Bladder tumor incidence in KRN 7000-instilled mice (groups 1 and 2) was higher than in saline controls (group 3), but this tendency was not significant. This minor increase could reflect mucosal injuries from catheterization rather than tumor-stimulating effects of KRN 7000; some studies of intravesical instillation therapy have suggested that catheterization itself might promote bladder carcinogenesis²⁰. So the total bladder carcinoma incidence in normal saline instillation group (group 3) must be higher than that of no instillation group (group 4). However, in the present experiment, that of group 3 was lower than that of group 4. Those of bladder instillation groups (groups 1 to 3) seemed to become higher dose dependently, but they were not significant. KRN 7000 seemed to stimulate bladder carcinogenesis, but the total bladder carcinoma incidence KRN 7000-low dose group (group 2) was rather lower than that of group 4. Variation in bladder carcinoma incidence between our groups most likely was random, and this should be originated from the failure of appropriate induction of bladder carcinogenesis by BBN treatment and/or the absence of antitumor effect of KRN 7000 to bladder tumor. We can only say that intravesical instillation treatment with KRN 7000 did not inhibit bladder carcinogenesis in these

mice. KRN 7000 may not have an appropriate character for intravesical instillation therapy, for example, permeability to bladder epithelium. Further investigation is necessary, however, to avoid premature generalization of this specific negative result.

In conclusion, we could not observe an inhibitory effect from intravesical instillation of KRN 7000 in C57BL/6 mice exposed to a bladder carcinogenesis.

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