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Effect of OK-432 on the Lymphnode Metastasis of MCA-sarcoma Cell Lines: A New Therapeutic Approach

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ABSTRACT

The two unique highly metastatic MCA-sarcoma cell lines have been established by the present authors. The inoculation of 1153Ln, one of the cell lines, either into footpad or subcutaneously on the back of syngeneic mice resulted in the development of metastasis exclusively in almost all lymphnodes of the body. We evaluated the therapeutic effect of a streptococcal preparation, OK-432, on the lymphnode metastasis. Two KE per mouse of OK-432 were injected intratumorally (*it*) at 4, 7 or 10 days after the footpad inoculation of 1153Ln. OK-432 injected *it* at 7 days after tumor inoculation showed an inhibition of the lymphnode metastasis. Histological findings indicated that the proliferation of lymphoid cells in the drainage node was most prominent in mice treated with OK-432 at 7 days after the tumor inoculation. A combined treatment of *it* and intraperitoneal (*ip*) injections of OK-432 significantly reduced lymphnode metastasis as compared with that of *ip* injection alone. This can be attributed to the fact that the activated lymphocytes induced by *it*-injected OK-432 exhibited a potent antimetastatic activity together with general administration (*ip*) of OK-432 given after surgical removal of the tumor. Low dose of total-body irradiation (TBI), known to augment the antitumor potential of tumor-bearing animals together with general application of OK-432, showed synergistic action in inhibiting tumor growth. Overall results suggest that the better antitumor effect of OK-432 can be anticipated by combination of the agent itself and with other means.

Key words: *MCA-sarcoma, Lymphnode metastasis, OK-432, Low dose TBI*

Despite major advances in surgical manipulations and adjuvant therapies, metastasis still remains a major clinical problem. Paradoxically, the more effective local treatment is in prolonging life, the greater the risk of metastasis formations. Indeed, the final goal in mastery of cancer is to control metastasis.

We have established two unique, highly metastatic cell lines, 1153Ln and 1153Pn, derived from 3-methylcholanthrene (MCA)-induced fibrosarcoma in a female (C57BL/Ka × C3H/He) F_1 mouse[8]. The 1153Ln cells metastasized to almost all lymphnodes through the lymph vessel when the tumor cells were inoculated s.c. of back. The another cell line, 1153Pn, metastasized to many visceral organs via the blood stream. Each cell line showed distinct and selective propensities for the mode of metastasis.

A streptococcal preparation, OK-432, is known to have anti-tumor and immunoadjuvant effects in both experimental animals and man[5,6,12,13,17-19]. These effects are thought to be partly due to the activation of the host immune system. OK-432 is now in clinical use in Japan. Preliminary studies showed that the survival of mice inoculated with 1153Pn, a highly hematogenously metastatic cell line, was prolonged by the treatment with OK-432. In the present experiment, OK-432 was given generally and locally, in 1153Ln-bearing mice to evaluate the therapeutic effect on the lymphnode metastasis, aiming at exploring the new approach to the treatment of lymphnode metastasis with OK-432.

MATERIALS AND METHODS

Mice Male and female (C57BL/Ka × C3H/He)F_1 (BCF_1) mice were produced in our laboratory and used at two to four months old. C57BL/Ka mice, originally supplied by late Professor H.S. Kaplan (Department of Radiology, Stanford University, School of Medicine, Stanford, California, USA) have been maintained by brother × sister mating in our laboratory since 1964. C3H/He mice were obtained from Charles River Japan Inc., Kanagawa, Japan. The mice were kept, 5-7 per cage, in an air-conditioned room at 24±1°C. They were given commercial pellets and tap water *ad libitum*.

Tumor cells 1153Ln[8], metastasizing to almost

every lymphnode, was used in this experiment. Tumor was maintained by a serial subcutaneous transplantation in syngeneic mice. A tumor cell suspension was obtained by collagenase digestion of tumor fragments. Tumor fragments were agitated for 90 minutes on a magnetic stirrer in Hanks solution (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 0.1% collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 37°C. The tumor cell suspension was passed through stainless-mesh to remove cell clumps, and washed three times by centrifugation at 1,000 rpm for 10 minutes, and the cell pellet was resuspended in Eagle's minimun essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). Cell concentration for injection was determined by the use of hemocytometer. 100,000 cells of 1153Ln were injected into the right footpad of each syngeneic mouse to evaluate the metastatic behavior of 1153Ln.

Preparation of OK-432 OK-432 (Chugai Pharm. Co. Ltd., Tokyo, Japan) is produced from cultures of a nonvirulent strain of type 3, group A Streptococcus pyogenes. These cells are grown in Bernheimer basal medium, and penicillin G is added for 30 minutes followed by increased temperatures for 45 minutes. OK-432 is quantitated by KE units (1 KE unit equals 0.1 mg lyophilized cells which also includes 2.7 mg of medium salts and 2,700 units of penicillin G). Before use, this preparation was suspended in sterilized saline. Our preliminary study showed that survival time of mice inoculated iv with hematogenously metastasizing MCA-sarcoma cell line, 1153Pn, was prolonged by the treatment of iv or *ip* injection of OK-432. The number of lung metastatic nodules was smaller in mice given OK-432 *ip*. We employed the *ip* injection for the general application of OK-432.

Amputation Mice were lightly anesthesized with pentobarbital (Abbot Laboratories, North Chicago, Illinoi, USA) and tumor-bearing legs were amputated at the hip joint. Popliteal lymphnodes were removed at 13 days after the inoculation of 1153Ln for histological examination.

Histological observations The lymphnodes of the sacrificed mice were removed and were fixed in 10% neutral formalin. Paraffin sections were stained with hematoxylin and eosin and examined histologically. Histological grade of lymphnode metastasis was determined as described previously[18]. Briefly, Grade 1: tumor cells proliferate only in marginal sinus; Grade 2: tumor cells proliferate as far as intermediary sinus and invade into parenchyma; Grade 3: tumor cells occupy almost all the lymphnode.

Statistical analysis Group comparisons were made by using Student's t-test χ^2 analysis or ridit analysis for ordered categorical data[3]. Data are presented as mean value ± SD.

Experimental setup

Exp. I. 1153Ln (1×10^5 cells/mouse) were inoculated into the right footpad of each syngeneic mice. OK-432 (2 KE/mouse) was injected intratumorally at 4, 7 or 10 days after the inoculation of 1153Ln. All mice were sacrificed at 14 days after the tumor inoculation to see the effect of OK-432 in inhibiting lymphnode metastasis.

Exp. II. 1153Ln (1×10^5 cells/mouse) were inoculated into the right footpad of each of the sygeneic mice. OK-432 (2 KE/mouse) was injected intratumorally at 6 days after the tumor inoculation, and the right legs were amputated at the hip joint at 13 days after the tumor inoculation. OK-432 (2 KE/mouse) was injected intraperitoneally two times a week, thereafter (at 15 and 18 days). All mice were sacrificed at 21 days after the tumor inoculation to examine the effect of combined *it* and *ip* administrations of OK-432 on the lymphnode metastasis of 1153Ln.

Exp. III. 1153Ln (1×10^5 cells/mouse) were inoculated into the footpad of syngeneic mice. OK-432 (2 KE/mouse) was injected intraperitoneally at 1, 5, 8 and 12 days after the tumor inoculation. Further, the mice were given a low dose (10 rads) of total-body x-irradiation (TBI) at 5, 8 and 12 days after tumor inoculation. All mice were sacrificed at 14 days after tumor inoculation.

RESULTS

Effect of *it* injection of OK-432 (Exp. I)

The incidence of metastasis to the popliteal lymphnode (drainage node) in each treatment group was 11 of 12 (92%), 8 of 10 (80%), 7 of 11 (64%)

Table 1. Effect of intratumorally injected OK-432 on the lymphnode metastasis of syngeneic mice inoculated with 1153Ln

Treatment		Incidence of metastasis to:			
With	at the day after inoculation	Popliteal[a] LN	Inguinal LN	Axillar LN	Lumbar LN
none	———	11/12[b]	3/12	0/12	1/12
OK-432	4 day	8/10	7/10[c]	0/10	0/10
OK-432	7 day	7/11	4/11	0/11	0/11
OK-432	10 day	9/11	4/11	1/11	0/11

[a] Incidence of metastasis to popliteal lymphnode.
[b] Number of mice with metastasis/number of mice examined.
[c] $P<0.05$

Table 2. Histological grade of the lymphnode metastasis in 1153Ln-bearing mice treated with OK-432 intratumorally

Treatment	with at the day after inoculation	none —	OK-432 4 days	OK-432 7 days	OK-432 10 days
Number of mice examined		12	10	11	11
Popliteal LN	no metastasis	0[a]	2	4	2
	Grade 1	1	1	2	1
	Grade 2	10] 11	6] 7	5] 5[b]	8] 8
	Grade 3	1]	1]	0]	0]
Inguinal LN	no metastasis	9	3	7	7
	Grade 1	2	3	2	4
	Grade 2	1] 1	3] 4	2] 2	0] 0
	Grade 3	0]	1]	0]	0]

[a] Number of mice with metastasis in each grade
[b] $P<0.025$

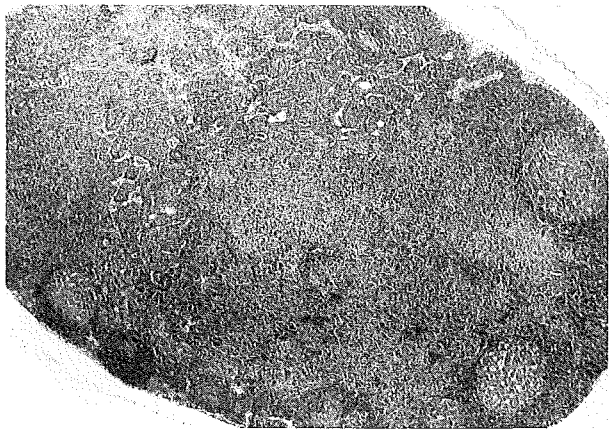

Fig. 1A

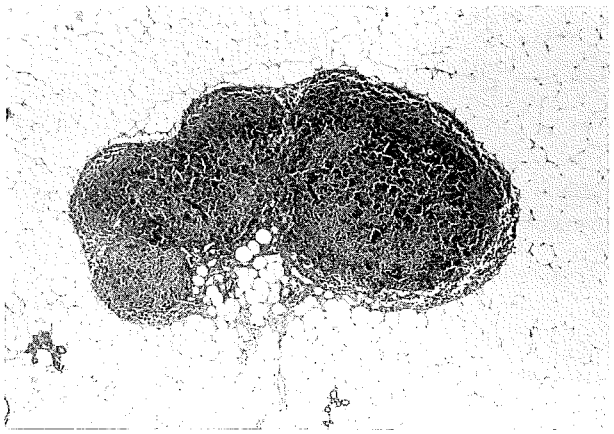

Fig. 1B

Fig. 1. The effect of intratumor injection of OK-432 to the lymphnode of mice bearing 1153Ln tumor in the right footpad.

A. The histological appearance of the right inguinal node of a mouse given OK-432 intratumorally at 7 days after 1153Ln tumor inoculation. The node presents both hyperplastic and hyperreactive pictures; the formation of active germinal centers and thickening of the paracortical area and the medullary cord. There is no evidence of the 1153Ln tumor metastasis. (H & E staining, ×40).

B. The histological appearance of the right inguinal node of a mouse bearing the 1153Ln tumor, but without OK-432 treatment. In contrast to A, the node presents non-reactive and quiet picture. There is no metastasis of 1153Ln tumor grafted in the right footpad 7 days previously. (H & E staining, ×40).

Table 3. Effect of intratumorally and intraperitoneally injected OK-432 on the metastasis of syngeneic mice inoculated with 1153Ln

Treatment		Incidence of metastasis to:				
with	site	Lung	Inguinal LN	Axillar LN	Lumbar LN	Renal LN
none	—	2/12[a]	12/12	12/12	6/12	6/12
OK-432	ip	0/15	14/15	14/15	6/15	3/15
OK-432	it+ip	1/13	12/13	7/13[b]	3/13	3/13

[a] Number of mice with metastasis/number of mice examined.
[b] $P<0.01$

Table 4. Histological grade of lymphnode metastasis in 1153Ln bearing mice treated with OK-432 intratumorally and intraperitoneally

Treatment	with site	none —	OK-432 ip	OK-432 it+ip
Number of mice examined		12	15	13
		$\chi^2=4.18^c$		
Inguinal LN	no metastasis	0[a]	1	1
	Grade 1	0	0	1
	Grade 2	0	0	2
	Grade 3	12] 12	14] 14	9] 11
		$\chi^2=15.6^e$		
Axillar LN	no metastasis	0	1	6[b]
	Grade 1	0	0	0
	Grade 2	2	3	5
	Grade 3	10] 12	11] 14	2[e]] 7[b]
		$\chi^2=3.38$		
Lumbar LN	no metastasis	6	9	10
	Grade 1	0	0	0
	Grade 2	0	1	2
	Grade 3	6] 6	5] 6	1[d]] 3

[a] Number of mice with metastasis in each grade
[b] $P< 0.01$ [c] $P<0.05$ [d] $P<0.025$ [e] $P<0.005$

Table 5. Effect of OK-432 and low dose of total body irradiation (TBI) on the lymphnode metastasis of syngeneic mice inoculated with 1153Ln

Treatment	Weight of primary tumor (g)	Incidence of metastasis to:			
		Popliteal LN	Inguinal LN	Axillar LN	Lumbar LN
none	2.41 ± 0.58[a]	10/11[b]	10/11	1/11	4/11
OK-432	1.91 ± 0.42[c]	12/12	10/12	1/12	3/12
OK-432+TBI	1.63 ± 0.38[d]	11/11	9/11	1/11	1/11

[a] Mean ± SD
[b] Number of mice with metastasis/number of mice examined
[c] $P<0.025$
[d] $P<0.005$

Table 6. Histological grade of lymphnode metastasis in 1153Ln bearing-mice treated with OK-432 and total body irradiation (TBI)

Treatment with		none	OK-432	OK-432 + TBI
Number of mice examined		11	12	11
Popliteal LN	no metastasis	1[a]	0	0
	Grade 1	0	0	2
	Grade 2	7	11	8
	Grade 3	3] 10	1] 12	1] 9
Inguinal LN	no metastasis	1	2	2
	Grade 1	1	4	5
	Grade 2	9	6	4[b]
	Grade 3	0] 9	0] 6	0] 4[b]

[a] Number of mice with metastasis in each grade
[b] $P<0.05$

and 9 of 11 (83%), respectively (Table 1). There was no statistical difference in the rate of metastasis among the groups using χ^2 analysis. But the incidence of metastasis to the inguinal lymphnode was higher in mice given OK-432 at 4 days after the inoculation of the tumor cells than in control mice. We further examined the histological grade of lymphnode metastasis in each group as the indicator

of the effect of *it* injection of OK-432. There was no statistical difference in the grade of draining lymphnode metastasis among the groups using ridit analysis for ordered categorical data. The incidences of grade 2 and 3 metastasis in the popliteal lymphnodes in non-treated, given OK-432 *it* at 4, 7 or 10 days after tumor inoculation were 11 of 12 (92%), 7 of 10 (70%), 5 of 11 (45%) and 8 of 11 (73%), respectively (Table 2). Grade 2 and 3 metastasis in the popliteal lymphnode was significantly lowered by *it* injection of OK-432 at 7 days after the tumor inoculation. Results indicated that *it* injection was most effective in mice given OK-432 at 7 days after the inoculation. Intratumoral injection of OK-432 showed no influence on the growth of the grafted tumor itself (data not shown).

The histological appearance of lymphnode in mice injected with OK-432 *it* showed the formation of active germinal centers, the proliferation of lymphocytes in the paracortical area (T-cell zone), and the proliferation of plasma cells in the thickened medullary cord. The pictures were particularly prominent in the node of mice given OK-432 at 7 days after the inoculation of 1153Ln (Fig. 1A and 1B).

Effect of a combined *it* and *ip* injection of OK-432 (Exp. II)

In the previous section, we showed that *it* injection of OK-432 at 7 days after the inoculation of 1153Ln had a potent effect to suppress the metastatic behavior of 1153Ln. Therefore, we further examined whether *it* combined with general application (i.p) of OK-432 will show the synergistic effect on the lymphnode metastasis. The incidence of metastasis to each lymphnode was shown in Table 3. A combined treatment of *it* and *ip* injection of OK-432 resulted in inhibition of the metastatic spread of 1153Ln, notably, metastasis to the inguinal lymphnode ($P < 0.05$) and the axillar lymphnode ($P < 0.005$) were significantly suppressed by the combined treatment using ridit analysis for ordered categorical data. The number of mice with grade 3 metastasis in axillar and lumbar lymphnode were markedly decreased ($P < 0.005$ and $P < 0.025$) in mice given the combined treatment (Table 4). Results indicated that *it*, followed by *ip* injections of OK-432 was appreciably effective in inhibiting lymphnode metastasis of 1153Ln.

Effect of OK-432 and low dose total-body irradiation (TBI) (Exp.III)

Low dose of TBI has been shown to have an antitumor activity in tumor-bearing mice. We examined the effect of TBI on the lymphnode metastasis in 1153Ln-inoculated mice treated with *ip* injection of OK-432. Table 5 shows that general application (*ip*) of OK-432 could reduce the tumor weight, but did not influence the incidence of metastasis even in mice given both OK-432 and TBI. However, a combined treatment of OK-432 and TBI resulted in a significant suppression of the grade 2 and 3 metastasis in the inguinal lymphnode from 9 of 11 (82%) to 4 of 11 (36%), as compared to untreated mice (Table 6). These findings indicated that *ip* injection of OK-432 alone (Exp. II) showed no apparent effect in inhibiting the metastatic behavior of 1153Ln, but an addition of TBI acted synergistically with OK-432.

DISCUSSION

We have previously shown that *ip* injection of OK-432 resulted in a significant reduction of the number of metastatic nodules in the lung and a prolongation of the survival time of mice inoculated i.v. with 1153Pn through the tail vein. In the present study, we have demonstrated that a combined *it* and *ip* injection of OK-432 inhibited the lymphnode metastasis of 1153Ln, and the synergistic effect of low dose of total-body irradiation (TBI) and *ip* injection of OK-432.

New application methods of OK-432 for cancer therapy, such as oral administration[11] and local injection[1,7] has attracted much attention. As the local injection of OK-432, *ip* injection for malignant ascites[1] and *it* injection[4,7] have been employed in clinical use. More than one hundred and thirty patients with malignant ascites caused by cancer of the digestive tract were treated with *ip* injection of OK-432. Effusions disappeared in two third of the patients[1]. This therapy significantly prolonged the survival time and improved immune response of immunosuppressed patients with advanced cancer. The *it* injection of OK-432 is used for the treatment of head and neck tumors, breast cancer, gastric cancer, hepatoma, gynecological cancer, bladder cancer and malignant lymphoma. The mechanism of the antitumor effect of *it* injected OK-432 has not been fully clarified. Several reports show that tumor-infiltration lymphocytes, probably OKT8+ or Leu7+ cells, are associated with antitumor activity of *it* injection of OK-432[9].

We investigated the effect of *it* injection of OK-432 on the metastatic behavior of 1153Ln. OK-432 was injected at 4, 7 or 10 days after tumor inoculation. Results indicated that *it* injection at 7 days after inoculation had a potent effect to inhibit the lymphnode metastasis. Histological examination showed that the proliferation of lymphnode cells in T- and B-cell zones was most prominent in mice treated with OK-432 *it* 7 days after tumor inoculation, which may support the above-mentioned outcome. The reason for increased lymphnode metastasis in mice given *it* injection at 4 days after the inoculation was obscure. The footpad is a marrow limited space, and the pressure in the footpad will be increased by the injection of tumor cell suspension. We speculated that the increased pressure, caused by *it* injection of OK-432 pushed out the migrating 1153Ln cells into lymphatics soon after the inoculation of cell suspensions. Thus, lymphnode metastasis was augmented in mice given OK-432 at 4 days after cell inoculation.

In order to study the synergistic effect of *it* and *ip* injections of OK-432, OK-432 was injected *it* at 7 days before the amputation and injected *ip* two times a week after the amputation. A combined *it* and *ip* injection significantly lowered the incidence and the histological grade of lymphnode metastasis. Results suggested that the proliferation of activated lymphocytes, induced by *it* injection of OK-432 seemed to have a potent anti-tumor activity, and promote the therapeutic effect of generally applied OK-432, given after the surgical removal of the tumor. The operative stress has been shown to impair cell mediated immunity and enhance metastasis in many experimental models[2,16].

The preoperative general administration (*ip* injection) or OK-432 was effective in the prevention of the adverse effects of operative stress[16]. General administration of OK-432 showed some effects in inhibiting the lymphatic metastasis (Tables 5 and 6). However, we observed that *it* injected OK-432 was more effective than *ip* injection in both protecting metastasis and stimulating the proliferation of lymphocytes: more effective than preoperative *ip* injection of OK-432. We speculate that *it* injection of OK-432, several to 10 days before the surgery, followed by the general application of the same agent might be effective in lowering the lymphnode metastasis.

Low dose of TBI is known to enhance the immunological response of tumor-bearing mice, but not of intact mice[14]. Further study has shown that low dose of TBI decreases the induction of suppressor cells in tumor-bearing mice[10,15]. The effect of TBI on the metastatic behavior of 1153Ln given OK-432 was tested, and the results indicated that the combined treatment exhibited the synergism in suppressing both the growth of primary tumor and lymphnode metastasis. A synergistic effect of TBI and local radiotherapy has been suggested for patients with advanced cancer. The addition of OK-432 therapy to TBI and radiotherapy and/or chemotherapy may prove to be one of the effective strategies in managing patients with advanced cancer.

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