

## Effect of 1-arginine HCl and OK-432 on the inhibition of Lung Metastasis in Mice

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**ABSTRACT:** To investigate the effect of OK-432 and 1-arginine HCl on the artificial metastases in lung, mice were injected mouse colon carcinoma, colon 26 cells ( $1 \times 10^6$ ).

Pulmonary nodules produced by the i. v. injection of  $1 \times 10^6$  colon tumor 26 cells into a caudal vein were evaluated after 3 weeks.

OK-432, 1KE/mouse, was administered by the i. v. injection three times on day 0, 2, 4 after the tumor injection.

L-arginine HCl, 50mg/mouse, was given by the i. p. injection 6-7 times on day 0, 1, 2, 3, 4, 5, 6.

IV administration of OK-432 led to a significant suppression of lung metastases formation in comparison to saline-injected animals. Furthermore, OK-432 and i. p. administration of 1-arginine HCl led to a considerable, synergistically retardation of tumor metastasis.

The suppressive effect of OK-432 and 1-arginine HCl on the artificial lung metastases may be due to its activation of the immunologic system.

And this work suggest that OK-432 and 1-arginine HCl enhanced the phagocytic activity of macrophages in vivo.

### INTRODUCTION

OK-432, a streptococcal preparation, has been developed by Okamoto in Japan.

This agent has been used in cancer patients and beneficial clinical outcome has been noted, OK-432 is a potent immune modulating agent in both animal models and cancer patients and possesses therapeutic efficacy.

Intavenous hyperalimentation was first reported by Dudrick and is now most easily used in every hospitals for nutritional support.

Harper<sup>3)</sup> first suggested the therapeutic value of amino acid imbalance in treatment of malignant disease.

Recently, it has been reported that the addi-

tion of excess 1-arginine HCl resulted in protection against the growth of a transplantable tumor.

In this study, we examined the effect of OK-432 and 1-arginine HCl on the artificial lung metastasis in Balb/c mice.

And combined treatment by OK-432 and 1-arginine HCl was significantly effective in artificial lung metastases.

Then, we examined the therapeutic mechanism of OK-432 and 1-arginine HCl.

It is suggested that the protection of early artificial metastases in mice by OK-432 and 1-arginine HCl may be due to the phagocytotic macrophages elicited by them indirectly.

Harper<sup>1)</sup> first reported the antitumor effect of arginine on dietary imbalance. In fact, it has

been known that arginine supplementation can alleviate the negative effect of recovery from trauma. On the other hand, arginine deficiency impairs wound healing process in rats with trauma.

A large amounts of administration of arginine focus our attention on enhancement of anti-tumor effect. In addition, other reports clarify that antitumor effects are facilitated in conjunction with arginine and biological response modifiers (BRM). The purpose of this study is to certify as to whether a combination with arginine and OK-432, streptococcal lyophilized preparation as a kind of BRM, potentially acts as antitumor effect.

In this study, antitumor effects of arginine and OK-432 were tested in mice lung metastases.

## MATERIALS AND METHOD

### (1) Experimental animals :

Colon tumor 26 was transplanted in Balb/c mice, female, aged 8-10 weeks.

BL-6 melanoma was transplanted in C57BL/6 mice, female, aged 8-10 weeks. The animals were obtained from Shizuoka Animal Facility Center, Shizuoka, Japan and acclimatized for 1 week to our laboratory conditions before experiments.

All animals were kept in a room at a constant temperature of  $22 \pm 1^\circ\text{C}$ .

### (2) Experimental tumors :

Colon Tumor 26<sup>2)</sup> is induced with DMH (Dimethyl hydrazine) and it is an undifferentiated Grade 4 carcinoma. The lung were the major site of metastases. Colon Tumor 26 was grown in monolayer culture in RPMI-1640 medium supplemented with 10% heated-inactivated fetal calf serum in a humidified 5% CO<sub>2</sub> atmosphere at 37°C

B16-BL6 mouse melanoma cells were maintained in vitro at 37°C in a humidified 5% CO<sub>2</sub> plus 95% air atmosphere using RPMI-1640 medium supplemented with 10% heated-inactivated fetal calf serum.

The cells were maintained in vitro as a monolayer culture using plastic tissue culture flasks and subcultured weekly using 0.25% trypsin-0.02% EDTA.

### (3) Accurate Indication of Experimental Pulmo-

nary Metastasis<sup>3)</sup>

Pulmonary nodules produced by the iv injection of  $1 \times 10^6$  colon tumor 26 cells into a caudal vein were evaluated, and mice were sacrificed after 3 weeks.

The sacrificed animal was pinned to a dissecting board in the supine position, and trachea and chest cavity were exposed through a mid line chest incision. The trachea was isolated and a #22 blunt needle was inserted into the transected end of the trachea. 2ml of India ink (15ml India ink, 85ml of distilled water, two drops of ammonia water) was injected with gentle spring pressure.

Then, the lung were dissected, en bloc, from the thoracic cage and placed in a beaker of tap water for at least 5 minutes. The organ was then placed in Feketes solution (100ml of 70% alcohol, 10ml of formaldehyde, and 5ml of glacial acetic acid) for 24 hours. Then, metastatic tumor foci of separating the lobes of the lung were counted easily.

### (4) OK-432 and 1-arginine HCl :

OK-432 was administrated at 1KE per one mouse, on day 0, 2, 4 after i. v. injection of colon 26. OK-432 was diluted with salt solution to 1KE/0.1ml.

OK-432 was supplied by Chugai Co., Japan in vials. This pharmaceutical preparation is standardized by a variety of tests and a unit of "KE" (= Klinische Einheit) is used to express the strength of the preparation.

Daily injections of 1-arginine HCl into mice bearing colon 26 tumors was done for 6 days starting after inoculation of colon 26 tumors. Mice received intraperitoneal (ip) injection of a dose of 50mg 1-arginine HCl for 6 days running.

(5) Anti asialo GM1<sup>4, 5)</sup>, Carrageenan<sup>6)</sup>, Arginase:  
(i) Purified asialo GM1 from bovine brain tissue was repeatedly immunized with mouse methylated bovine serum albumin and with complete Freund's adjuvant.

This product react with mouse natural killer cells, a part of mouse monocytes and immature mouse fetal thymocytes.

20μl anti asialo GM1 was iv injected three times on day of 0, 4, 8.

(ii) Carrageenan was dissolved in saline by being heated to 100°C for 10 minutes in a water bath, mice were given 1mg iv one shot in 0.1ml

saline, immediately after inoculation of colon 26.  
 (iii) Arginase 50units/mouse was injected ip for 5 times, starting immediately after inoculation of colon 26.

One unit will convert  $1.0\mu\text{mole}$  of l-arginine to ornithine and urea per minute at pH 9.5 at  $37^\circ\text{C}$ .

(6) Statistical analyses :

Statistical analyses of changes in metastasis formation were performed by Student's t-test.

## RESULTS

(1) Effect of l-arginine HCL and/or OK-432 on induced lung metastasis (colon 26 cell line) (Fig. 1 and Table 1).

When colon 26 cells were transferred in Balb/c mice, three weeks later, lung metastases developed with an average of  $194\pm 39$  in number.

The addition of OK-432 iv showed an average of  $71\pm 36$  ( $p<0.01$ ) and  $31\pm 17$  ( $p<0.01$ ) in

conjunction with Arg.

When Arg alone was used, these were  $138\pm 35$  without significant difference from the control. The use in combination with Arg and OK-432 enabled lung metastasis to significantly reduce ( $p<0.05$ ). And also this study was undertaken to define immunologic mechanisms of inhibition of the tumor growth.

(2) Effect of combined therapy is dependent on the time period of administration (Table 2)

When Arg and OK-432 were given prior to implantation of tumor cells to mice, the number of lung metastasis showed an average of  $126\pm 70$ . On day 3 after implantation of tumor cells to mice, it was  $105\pm 58$  and  $31\pm 17$  on day 0 respectively.

The results indicated that combination therapy with Arg and OK-432 was of great benefit to reduce lung metastasis regardless of prior to and/or after implantation of tumor cells. In addition, the maximum effect of combined

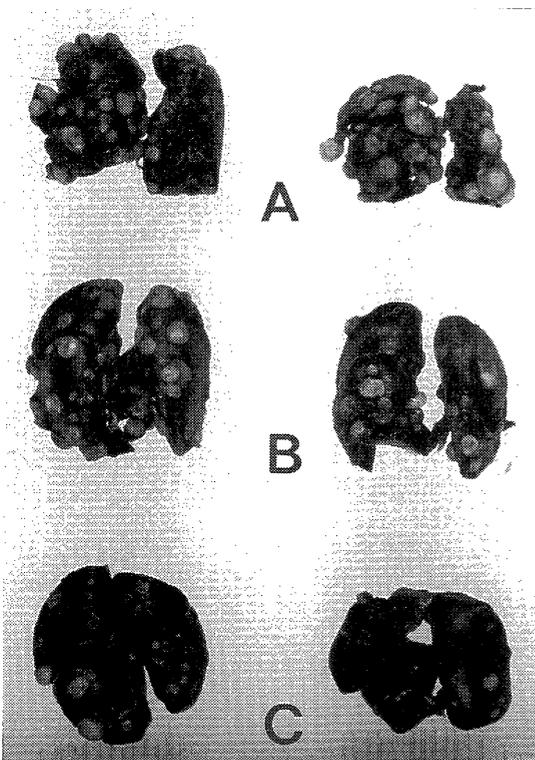


Fig. 1. Metastatic nodules in lung of Balb/c mice.  
 A ; control  
 B ; OK-432  
 C ; Arg+OK-432

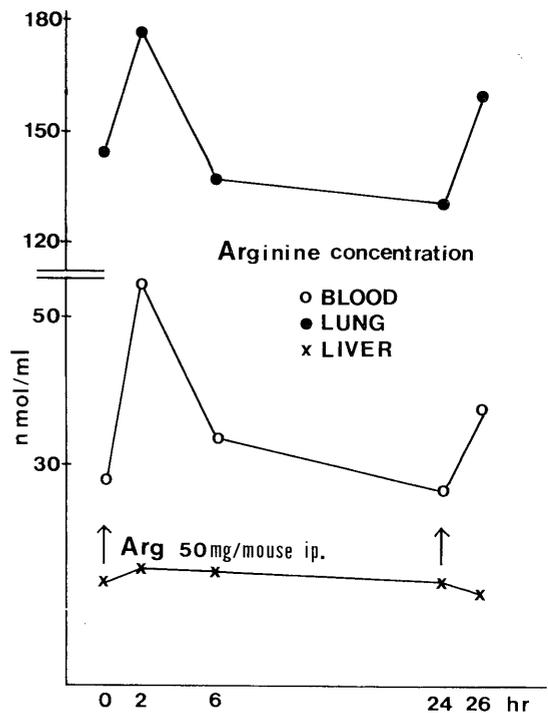


Fig. 2.

therapy on inhibition of tumor cell growth was defined at the same time as implantation of tumor cells.

(3) The concentration of Arg in the plasma, the lung and the liver (Fig. 2)

When a dosis of 50mg of l-Arg HCL was given intraperitoneally, there was no difference in the concentrations of the liver. On the other hand, the concentrations of Arg increased double from 30 nmol/ml to 60 nmol in the blood and from 140 nMol/ml to 180 nMol/ml in the liver.

(4) Effect of arginine on lung metastasis

When arginase, an enzyme of degradation of arginine, in addition to OK-432 was injected intraperitoneally to make clear of the role of arginine on the arginine deficient condition.

Table 3 showed that the number of lung metastasis in Balb/c mice was not significant difference among the control, arginase and arginase+OK-432 groups.

(5) Effect of a decrease in NK activity by

**Table 1.** Effect of OK-432 and/or l-Arginine HCl on artificial metastases of colon 26 cells in Balb/c mice

Treat.	No. of mice	Lung meta. Mean±SD	t-test
Cont.	15	194±39	—
OK-432	10	71±36	P<0.01
OK-432+Arg.	10	31±17	P<0.01
Arg.	10	138±35	N. S.

**Table 2.** Effect of OK-432 and l-Arginine HCl on artificial metastases of colon 26 cells in Balb/c mice (Depend on injection period)

Treated period	No. of mice	Lung meta. Mean±SD	t-test
No treatment	15	194±39	—
Before	5	126±70	P<0.05
After	6	105±58	P<0.01
Synchronous	10	31±17	P<0.01

**Table 3.** Effect of Arginase on artificial metastases of colon 26 cells in Balb/c mice

Treat.	No. of mice	Lung meta. Mean±SD	t-test
Control	15	194±39	—
Arginase	5	232±34	N. S.
Arginase+OK-432	5	225±25	N. S.

anti-asialo GM/Ab on lung metastasis in mice (Table 4)

The number of lung metastasis showed 234±38 when administering OK-432, indicating not significant difference in comparison with the control.

In contrast, while the combination therapy with Arg and OK-432 was instilled, the number of lung metastasis was reduced to 82±77 with a significant difference.

(6) Effect of carrageenan on lung metastasis (Table 5)

An average of 159±73 in pulmonary metastasis was numbered when administering OK-432 in addition to carrageenan in contrast to 194±77 in the control. On the other hand, it was 174±94 in combination with Arg and OK-432.

There was no definitive difference in the number of lung metastasis between Arg and Arg+OK-432 in addition to carrageenan.

(7) Effect of OK-432 and Arg on lung metastasis

**Table 4.** Effect of Anti-asialoGMI Ab on artificial metastases of colon 26 cells in Balb/c mice

Treat.	No. of mice	Lung meta. Mean±SD	t-test
Control	5	256±14	—
OK-432	5	234±38	N. S.
OK-432+Arg.	6	82±77	<0.01

**Table 5.** Effect of Carrageenan on artificial metastases of colon 26 cells in Balb/c mice

Treat.	No. of mice	Lung meta. Mean±SD	t-test
Cont.	5	194±77	—
OK-432	5	159±73	N. S.
OK-432+Arg.	6	174±94	N. S.

**Table 6.** Effect of OK-432 and l-Arginine HCl on artificial metastases of B16-BL6 cells in C57BL/6 mice

Treat.	No. of mice	Lung meta. Mean±SD	t-test
Cont.	5	42±16	—
OK-432	5	19±10	P<0.05
OK-432+Arg.	5	2±4	P<0.01

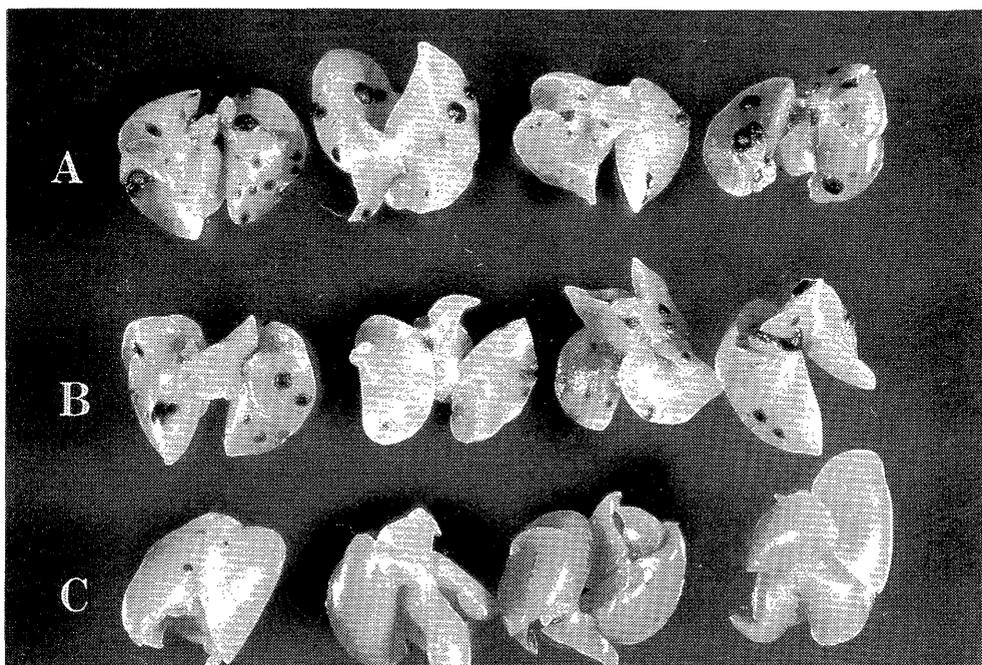


Fig. 3. Metastatic nodules in lung of C57BL/6 mice.  
A; control B; OK-432 C; Arg+OK-432

of B16-B16 cells in C57 BL/6 mice (**Table. 6 and Fig. 3**)

**Table 6** showed significant inhibition of lung metastasis of B16-BL6 cells in C57BL/6 mice when OK-432 or Ar+OK-432 were used. The numbers of lung metastases were diminished from  $42 \pm 16$  in the control to  $19 \pm 10$  in OK-432 group and  $2 \pm 4$  in Arg+OK-432 group with statistically significant difference ( $p < 0.05$  and  $p < 0.01$  respectively).

Diminished metastatic nodules in the lung were shown in **Fig. 3** in cases of the control, OK-432 and OK-432+Arg groups.

## DISCUSSION

Harper<sup>1)</sup> first reported the antitumor effects of nutritional imbalance of amino acids.

There are two ways to clinically make use of antitumor effect of amino acid imbalance, induced supplement and/or deficiency of amino acids.

However, the treatments of amino acids imbalance were mainly used for a deficiency of essential amino acids such as methionin,

tryptophan and phenylalanine. Nutritional imbalance of amino acids provides a great effect on proliferation of tumor cells and promotion of metastasis and recurrence. In particular, a deficiency of essential amino acids influences on the metabolism of cancer cells as well as normal cells.

It is necessary to search for the endpoint of the minimum ill effect on metabolism in normal cells and the maximum ill effect on metabolism in cancer cells. Excess administration of Arg is most suitable for this purpose. Recently, evidence has been accumulated by research work to clarify the antitumor mechanism on excess instillation of Arg. Cho-chung<sup>7)</sup> assumed that antitumor effects of Arg might be in association with cyclic AMP system. Takeda<sup>8)</sup> also reported the fact of the inhibitory effect on cancer cell proliferation and recurrence of breast cancer in rats by the addition of Arg supplement. Barbul<sup>9)</sup> clarified that the enlargement of the thymic gland and the increase in thymic cells were facilitated by the addition of excess administration of Arg. He had postulated that the antitumor effects of Arg should be in associa-

tion with enhancement of immune surveillance system by a stimulation of thymic gland and improvement of nutritional state by the addition of arginine to the hyperalimentation. His clinical trial of giving Arg of 30g for seven days was conducted in volunteers. As a result, it was reported that lymphocytes in the peripheral blood was activated without remarkable side effect except for slight degree of nausea and diarrhea. The results of this study also indicated that excess addition of Arg supplement as the imbalance therapy of amino acids was a better way to inhibit tumor growth and metastasis.

Chany<sup>10)</sup> reported that the anti-tumor effect of Arg in conjunction with C parvum or IFN resulted in prolongation of the survival time of Swiss mice inoculated intraperitoneally with 180 TG Crocker tumor cells although that of Arg alone was not sufficient for elongation of the survival in mice.

In this study, the antitumor effect of Arg in combination with OK-432<sup>11)</sup>, one of BRM, was studied as to whether inhibition of tumor growth and reduction of pulmonary metastasis were experimentally evidenced or not.

Many studies in vitro and in vivo clarified that OK-432 enhanced the immune response to the tumor-bearing hosts. Saito<sup>12)</sup> suggested that the antitumor effect of OK-432 be a direct action of INF  $\gamma$  induced by OK-432 and/or an indirect action of activated macrophages and NK cells or differentiated T cells. Much work reported that OK-432 also induces non-specific increased resistance to tumor growth by activated macrophages. Toge<sup>13)</sup> explained that activation of macrophages by OK-432 is non-T cell dependent. The mechanism for antitumor action of OK-432 had been ascertained that induction of increasing sizes, changes in cell shapes and increases resistance to the tumor cells were main.

When administering Arg in conjunction with OK-432 in BALB/c mice with colon 26 cells, there was no significant difference in the number of lung metastasis between the control and Arg alone. And the number of lung metastasis was significantly reduced by administration of Arg in conjunction with OK-432. In this study, pulmonary metastasis was diminished by admin-

istering Arg in conjunction with OK-432.

It seemed to be based on activation of the immune system. The timing of administration of Arg with OK-432 was also studied in relation to that of transplantation of tumor cells. The timing of administering Arg was divided into the three groups, prior to, at the same time and following transplantation of tumor cells, respectively.

Tanaka<sup>14)</sup> reported that the antitumor effect of OK-432 was prominent when OK-432 was injected before implantation of AH 7974 tumor cells in rats.

In this study, the antitumor effect of OK-432 was manifest when OK-432 was given at the same time as the time of implantation.

The diversity of the antitumor effect of Arg seemed to be in association with differences in tumor cells used.

When 50mg/mouse of Arg was given ip, the concentration of Arg was measured in the blood and the lung.

The concentration of Arg in the blood reached a peak level 2 hours later and that in the lung also raised but it returned to the normal 6 hours after intraperitoneally giving Arg. Maintenance of high concentration of Arg in the blood and the lung was limited during a short time. A slight increase in Arg concentration in the liver was seen. It was a reflection of a presence of a large amount of arginase in the liver. It was confirmed that the addition of Arg supplement was of great effect to eliminate lung metastasis. On the other hand, when arginase was given ip, the number of lung metastasis was the same as the control and the effect of OK-432 was also disappeared.

One must take into consideration that in vivo presence of Arg may play a role in inhibition of tumor growth. It is well known that initial steps of progression in metastasis comprises of embedding and extravascular infiltration of tumor cells and inhibition of this process may relate to activated NK cells, macrophages and Killer T cells which are generated in tumor-bearing hosts. In this series, the association of NK cells and macrophages with the antitumor effect of Arg was studied. The administration of Arg in conjunction with OK-432 resulted in a decrease in lung metastasis on the condition of

reduced activation of NK cells which was produced by anti-asialo GM1Ab.

Combination administration with Arg and OK-432 was beneficial in reducing lung metastasis with statistically significant difference. Experimental pulmonary metastasis was assessed when carrageenan was used for reduced activation of macrophages in tumor-bearing hosts.

Combined administration of Arg and OK-432 was of no use to reduce lung metastasis. The result showed that at the initial stage of metastasis, macrophages play an important role in inhibition of cancer extension including organ metastasis and so it was suggested that the role of Arg and OK-432 be based on enhanced activity of macrophages.

Colon 26 and Balb/c as an experimental tumor was selected in this series for an evaluation of the antitumor effect of Arg and OK-432. In addition, C57BL/6 was also used for tumor-bearing hosts.

OK-432 alone and in combination with Arg was significantly reduced lung metastasis and it had become synergistic in conjunction with Arg regardless of tumor-bearing hosts and/or the kinds of the tumors.

Weisburger<sup>15)</sup> reported that arginine offered great effect on inhibition of tumor growth for liver tumors which was induced by acetamide without any changes in body weight as reported by Yamamoto, Nakanishi, Nakagawa<sup>16)</sup>. Shimke<sup>17)</sup> *et al* clarified that excess of arginine promotes metabolism on the urea cycle. The mechanism of inhibition of tumor growth is that carbonayl-phosphate moves from pyrimidin synthesis to the urea cycle from the result of activated urea cycle. As a result, synthesis of nucleic acid via the *de novo* pathway is inhibited by excess excretion of urea.

Davis<sup>18)</sup> explained that instillation of arginine leads to high levels of prolactin, growth hormone and insulin in blood. Therefore, enhancement of antitumor effect of Arg and OK-432 is associated with a stimulation of hormone secretion which helps facilitate cellular immunity of the host. Currie<sup>19)</sup> stated that the addition of arginine supplementation resulted in damage to and destruction of tumor cells by activated macrophages which secrete arginase.

In cultured cells, the addition of arginine

enables colon 26 cells to increase and proliferate. It is assumed that arginine is not only directly influenced on damage to tumor cells but is needed for nutrient of tumor growth. Activated macrophages are easily in contact with tumor cells and cause a destruction of tumor cells with help of arginase. In consequence, tumor cells have become unable to absorb arginine which is needed for metabolism.

In conclusion, synergistic effects of Arg and OK-432 are explained that OK-432 activate macrophages. The addition of Arg much more enhanced activation of macrophages and easy access to tumor cells and therefore cause damage to tumor cells. Clinical use of amino acid imbalance therapy such as the addition of Arg in conjunction with OK-432 is of great benefit to inhibit lung metastasis.

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