Effect of clarythromycin on the distant metastases of human lung cancer cells in SCID mice

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Abstract: Recently, the use of macrolides is suggested to be therapeutically effective in prolonging the survival of patients with inoperable non-small cell lung cancer. The purpose of this study was to examine therapeutic effects of a macrolide, clarythromycin (CAM) on the metastastic developments of two different human non-small cell lung cancers (squamous cell lung carcinoma RERF-LC-AI, and adenocarcinoma PC-14) in severe combined immunodeficient (SCID) mice depleted or undepleted of natural killer (NK) cells, respectively. CAM, injected subcutaneously at doses of 5 and 10 mg/kg body weight/day from day 7 to 41 after i.v. inoculation of human lung cancer cells, was not effective in inhibiting their distant organ metastases in SCID mice. CAM at concentrations of less than 10 μg/ml did not have a direct influence on the proliferation of these tumor cells in vitro. Although CAM alone was not effective in augmenting NK activity, it augmented the IL-2-induced killer (LAK) activity against Daudi cells *in vitro*. These results suggest that CAM alone may not be enough to control the spread of non-small cell lung cancer in the patient with T cell dysfunction. J. Med. Invest. 44 : 205-210, 1998

Key Words : *Macrolide, human lung cancer, metastasis, SCID mice.*

INTRODUCTION

Among various human cancers, lung cancer is the most prevalent in Japan and elsewhere (1) and has a poor prognosis (2). The major obstacle in the therapy of lung cancer is the development of distant organ metastases, which cause most of the cancer-related deaths. Since lung cancer, even on the time of the early diagnosis, is frequently associated with distant micrometastases, conventional therapeutic modalities such as chemotherapy, radiation therapy and surgical resection, are little effective in terms of prolongation of the survival. For this, lot of research work are being focused on the development of new therapeutic modalities against lung cancer, particularly against its metastatic spread.

In the recent years, there has been a considerable interest in the role of macrolides as biological response modifiers (3-5) and the possibility of using macrolides such as erythromycin and clarythromycin (CAM) in the treatment of cancer as an adjuvant to anticancer chemotherapy and/or radiation therapy is being explored. There is a report showing the effects of erythromycin on reducing the tumor load and prolonging the survival of mice inoculated with different types of tumor cells (6). Moreover, long term CAM treatment has also been reported to have prolonged the survival of patients with inoperable non-small cell lung cancer (7). However, clinical effect of CAM on the survival periods of lung cancer patients remains debatable because of the limited numbers of patients evaluated. There is no study whether these macrolides have any therapeutic effect on the distant organ metastases of lung tumors, which accounts for the maximum cancer-related deaths.

Recently, we have developed novel metastasis models of non-small cell lung cancers in severe combined immunodeficient (SCID) mice which lack T cells and B cells (8, 9). Human squamous cell lung carcinoma RERF-LC-AI cells were found to develop distant metastases into various organs such as liver, kidneys and lymph nodes 6 to 8 weeks after their i.v. inoculation into the NK-depleted SCID mice (8). On the other hand, human lung adenocarcinoma PC-14 cells formed multiple metastases predominantly in lungs and regional lymph nodes, when inoculated i.v. into the SCID mice (9). We also found that these models allowed us to study effect of macrophage activating cytokine (i.e. macrophage colony stimulating factor) on distant organ metastases of human lung cancer (10). Macrolides such as erythromycin and CAM are shown to stimulate and/or potentiate functions of monocyte/macrophages in terms of cytokine productions (4-6). In this study, we have examined the effect of CAM on the metastasis of two different non-small cell human

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lung cancer in our SCID mouse models.

MATERIALS AND METHODS

Cell lines and cell culture

The human squamous cell lung carcinoma RERF-LC-AI cells were kindly provided by Dr. M. Akiyama (Radiation Effects Research Foundation, Hiroshima, Japan). The human lung adenocarcinoma PC-14 cells were kindly provided by Dr. N. Saijo (National Cancer Institute, Tokyo, Japan). Human Burkitt lymphoma (Daudi) cell line was obtained from American Type Culture Collection (Rockville, MD). Cell cultures were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and gentamycin (Schering-Plough, Osaka, Japan), designated as complete RPMI-1640 (CRPMI), at 37°C in a humidified atmosphere of 5% CO_2 in air. Growth inhibition assays were performed when the cultured target cells were in the exponential phase of growth.

Reagents

Clarythromycin (CAM) was kindly supplied by Taisho pharmaceutical Co., (Tokyo, Japan). Gum arabic was from Katayama Chemicals (Osaka, Japan). Recombinant human IL-2 (specific activity, 1.14×10^7 U/mg protein) was kindly provided by Takeda Pharmaceutical Co. (Osaka, Japan). Anti-mouse IL-2 receptor β chain monoclonal antibody, TM-β1 (IgG2b), was obtained as described previously (11). None of these materials contained endotoxins, as judged by *Limulus* amebocyte assay (Seikagaku Kogyo Co., Tokyo, Japan : minimum detection level 0.1 ng/ml).

Models of experimental metastases of human lung cancer cells

For experimental production of metastases of squamous cell lung carcinoma (RERF-LC-AI) cells, SCID mice were injected i.p. with TM-β1 antibody (1 mg/0.3 ml PBS/mouse) 2 days before tumor inoculation to deplete them of NK cells, as described (8, 9). However, for experiments with lung adenocarcinoma (PC-14) cells, SCID mice were used without the injection of TM-β1 antibody, because these cells formed metastatic colonies in the lungs of NK cell-undepleted mice in our preliminary experiments. The viable tumor cells suspended in 0.3 ml of PBS were injected into a lateral tail vein. Mice were treated s.c. with PBS, 5% gum arabic or CAM daily from day 7-41. They were sacrificed on day 42 and number of metastatic colonies were counted. Nodules more than 1 mm in diameter in the lungs were counted with the aid of a dissecting microscope.

In vitro growth inhibition assay

In vitro cell proliferation was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye-reduction method (12). In brief, tumor cells were seeded in 96-well tissue culture plates at a density of 5×10^3 cells/well. After incubating the cells overnight, drug treatments were initiated. The cells were exposed to the drugs for 72 hours, and then 50 μl of stock MTT solution (5 mg/ml) was added to all wells. After two hour incubation, the culture media were removed and 100 μl of dimethyl sulfoxide (DMSO) was added to dissolve the dark blue crystals. Absorbance was measured with a microplate reader (Corona Electric, Ibaragi, Japan) at test and reference wavelengths of 550 and 630nm, respectively.

Lymphokine-activated killer (LAK) cell cytotoxicity assay

LAK mediated cytotoxicity was assayed by measuring ^{51}Cr release in a 4 h test as described previously (13). Briefly, peripheral blood mononuclear cells (MNC) were separated from the blood of healthy donors in lymphocyte separation medium. MNC (1×10^5/well) were incubated in CRPMI with the IL-2 (100 U/ml) in presence or absence of indicated concentration of CAM for 4 days. The cells were washed and used as effectors, incubated with Daudi cells labeled with ^{51}Cr at effector target ratio (E : T) of 10 : 1. After incubation for 4 h, the radioactivities of the supernatants were determined. Results are expressed as percent cytotoxicity, calculated using the following formula : (E-S/M-S) X 100 (where, E=experimental cpm, S=spontaneous cpm and M=maximum cpm). the spontaneous release in different experiments ranged from 5 to 15% of the maximum release.

Statistical analysis

The statistical significance of differences between groups was analyzed by Student's two-tailed *t* test.

RESULTS

Effect of CAM on the metastases of RERF-LC-AI cells in NK-depleted SCID mice.

We examined the antimetastatic effect of s.c. administration of CAM on the metastasis of human squamous cell lung carcinoma RERF-LC-AI in NK cell-depleted SCID mice as described in the materials and methods. As shown in Table 1, RERF-LC-AI cells formed numerous metastases in the liver and kidneys, with a few metastases in the lymph nodes. No significant difference was observed in the formation of metastases among the groups of mice treated with PBS, gum arabic and CAM, indicating the ineffectiveness of CAM to inhibit distant metastases in this model. Macroscopically, no difference between untreated and CAM-treated groups was seen in the sizes of metastatic nodules (Fig.1).

Effect of CAM on the metastasis of PC-14 cells in SCID mice with intact NK activity

Next, we examined therapeutic efficacy of CAM to inhibit metastasis of PC-14 cells in SCID mice undepleted of NK cells as described in the materials and methods. CAM was injected s.c. daily from day 7-41.On day 42, the formation of metastatic colonies in the lungs and lymph nodes were examined. As shown in Fig.2, PC-14 cells formed metastatic colonies in the lungs and lymph nodes in SCID mice treated with CAM at 10 mg/kg, and the number of colonies of CAM-treated mice were not signifi-

Table 1. Metastasis of RERF-LC-AI cells in NK-cell deleted SCID mice treated with CAM

Treatment	No. of mice	No. of metastatic colonies (Mean±SD)		
		Liver	Kidneys	Lymph nodes
Experiment 1				
PBS	5	98.6±21.6	41.2± 5.7	9.8± 3.4
Gum arabic	5	106.8±18.5	41.8± 6.5	8.0± 3.8
CAM (5 mg/kg)	5	94.2±30.7	36.0±11.6	8.0± 2.6
CAM (10 mg/kg)	5	115.2±21.2	40.4± 8.4	8.4± 4.3
Experiment 2				
PBS	4	108.8±19.6	32.1±10.7	15.6± 4.0
Gum arabic	4	115.6±19.6	31.8± 9.8	19.0± 5.0
CAM (5 mg/kg)	4	110.5±32.3	33.8± 8.7	15.3± 7.9
CAM (10 mg/kg)	4	95.5±38.7	30.8±11.5	16.3±11.5

SCID mice were pretreated with TM-β1 (1 mg) for NK cell depletion, then RERF-LC-Al cells (1 x 10^6) were injected i.v. into the mice. These mice were treated with PBS, 5% Gum arabic or CAM daily from day 7-41. Mice were sacrificed on day 42 and number of metastatic colonies were counted.

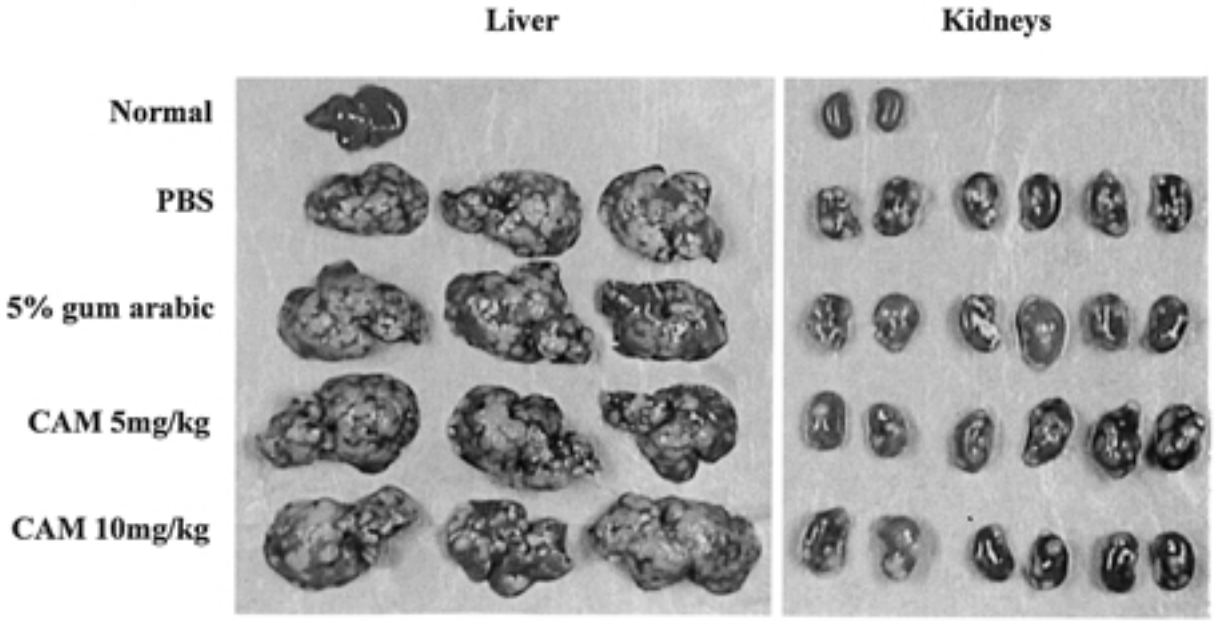

Fig.1. Metastasis of human lung cancer cells in NK cell-depleted SCID mice treated or untreated with CAM. RERF-LC-AI cells were inoculated into a tail vein of SCID mice pre-treated with TM-β1 antibody. Mice were injected s.c. with PBS, 5% gum arabic or CAM (5 mg/kg or 10 mg/kg) from day 7-41, killed on day 42 and metastatic nodules were counted. Each treatment group consisted of five mice, representative three are shown in the figure.

cantly different from the control groups, i.e., mice treated with PBS or gum arabic alone. Moreover, there was no remarkable difference in the formation of pleural effusion in the CAM-treated mice compared with the mice treated with gum arabic or PBS alone (data not shown).

Effect of CAM on the proliferation of human lung cancer cells in vitro

To examine if the failure of CAM to inhibit metastases of human lung cancer cells in SCID mice was due to it's ineffectiveness in controlling their proliferation, we treated two human lung cancer cells with various concentrations of CAM and assayed their proliferation in vitro. CAM at concentrations less than 10 mg/ml were not effective in inhibiting the cell proliferation of RERF-LC-AI (Fig.3 A)

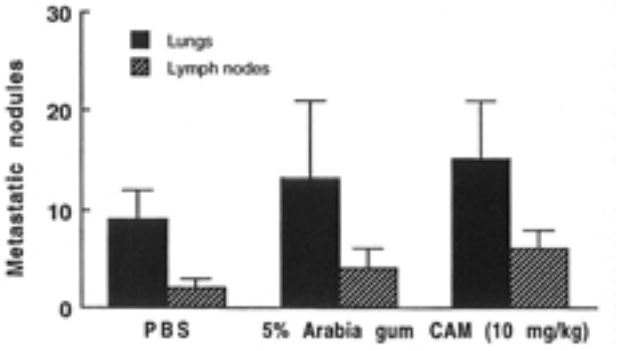

Fig.2. Metastasis of PC-14 cells in SCID mice treated with CAM. PC-14 cells (1 x 10^6) were injected i.v. into SCID mice without TM-β1 treatment. These mice, in groups of five, were treated with PBS, 5% gum arabic or CAM daily from day 7-41. Mice were sacrificed on day 42 and the number of metastatic colonies were counted.

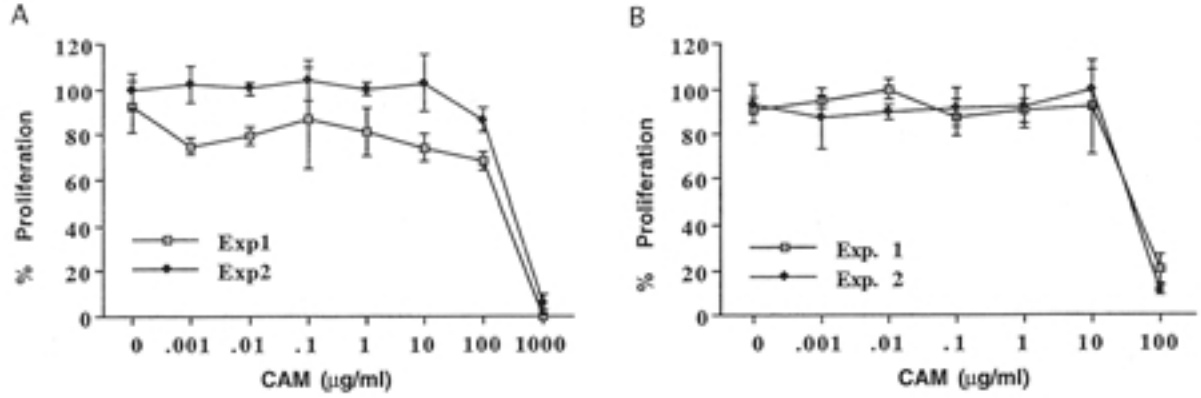

Fig.3. Proliferation of lung cancer cells in presence of CAM in vitro. Human lung cancer cells RERF-LC-AI (A) and PC-14 (B) were incubated in medium with different concentrations of CAM. After 72 h, the proliferation was evaluated by MTT assay as described in the materials and methods. Values are mean±SD of triplicates.

and PC-14 (Fig.3 B), while CAM at concentrations of higher than 100 μg/ml was very toxic to the cells.

Augmentation by CAM of the IL-2-induced LAK activity of MNC

In order to check if CAM could modulate the anti-tumor function of the immune system, we studied the effect of CAM on the LAK activity of MNC induced with IL-2. As shown in Fig.4, MNC incubated with IL-2 in presence of 1 and 10 μg/ml CAM showed significantly enhanced LAK activity compared with MNC incubated with IL-2 alone.

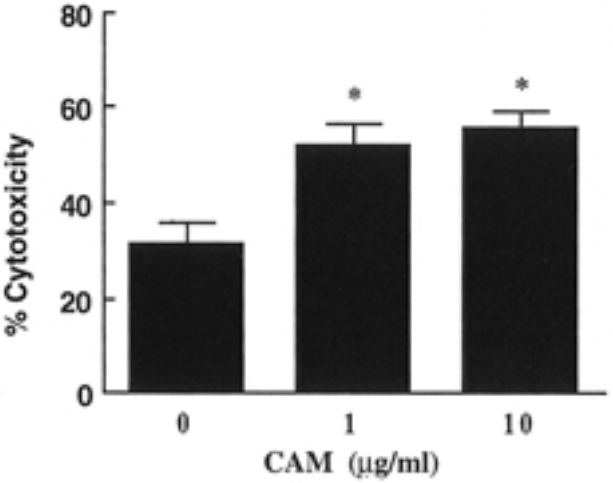

Fig.4. Effect of CAM on the IL-2-induced LAK activity of MNC. MNC were incubated in medium with IL-2 in presence of different concentrations of CAM. After 4 days, cells were harvested and their LAK activity was assayed against ^{51}Cr-labeled Daudi cells as described in the materials and methods. Values are mean±SD of one representative of two experiments done in triplicates.
*** p value<0.05 vs. LAK induced with IL-2 alone.**

DISCUSSION

The purpose of this study was to examine therapeutic effect of CAM on the metastasis of lung cancer cells in SCID mice models. We observed that the treatment with CAM did not have any effect on the metastasis of human squamous cell lung carcinoma RERF-LC-AI (Table 1 and Fig.1) and lung adenocarcinoma PC-14 in SCID mice models (Fig.2).

Recently much attention has been paid to antitumor effect of macrolides such as erythromycin and CAM, because of several suggestive reports. For example, erythromycin in the dose ranges of 1-10 mg/kg body weight were reported to be effective in reducing the tumor volume and prolonging the survival of mice bearing Ehrlich ascites carcinoma and P388 leukemia (6). Particularly, a recent finding (7) that oral administration of CAM resulted in significant prolongation of survival of patients with inoperable non-small cell lung cancer stimulated us to examine whether CAM treatment could affect metastases of human non-small cell lung cancer in SCID mouse models, because we have established novel metastases models of non-small cell lung cancer in SCID mice (8, 9). First, in NK cell-depleted SCID mice, we examined whether daily treatment with CAM at doses of 5 mg/kg/day and 10 mg/kg/day, could eradicate the development of established micrometastases of squamous cell lung carcinoma (RERF-LC-AI). Although RERF-LC-AI cells were highly metastatic to liver, kidneys and lymph nodes, antimetastatic effects of CAM were not seen on the distant metastases at all (Table 1). Next, we also examined whether CAM treatment could affect formations of tumor nodules in the lungs with malignant pleural effusion and lymph node metastases in SCID mice with intact NK cell activity. When the SCID mice were sacrificed 42 days after i.v. inoculation of lung adenocarcinoma (PC-14), multiple metastatic nodules could be seen in the lungs and lymph nodes with malignant pleural effusions as reported previously (9). CAM (10 mg/kg/day) when administered on day 7-41 after i.v. inoculation of PC-14 cells did not have any antitumor effect on tumor metastases in lungs and lymph nodes (Fig.2). We also observed that daily treatments with CAM did not affect neither the numbers nor the sizes of metastatic nodules formed in both metastasis models of squamous cell lung cancer (RERF-LC-AI) in SCID mice (Fig.1). Thus, our present findings are not in accordance with previous report by others. The reasons for this discrepancy between our and other observations (7) may be due to several differences such as tumor growing sites, origins of tumor, host immunocompetence and treatment

schedule. Since CAM was not toxic to lung cancer cells at physiological doses (Fig.3), it seems that antitumor effect of CAM, as observed by other investigators, may be mediated through the anti-tumor activities of host.

Regarding effect of CAM on host defense mechanisms, there are accumulating evidence suggesting it's function as biological response modifier. For example, CAM were reported to have a stimulatory effect on the production of IL-1β, TNF-α and IL-4 by monocytes (4-6). The SCID mice do have intact monocyte/macrophage populations, and even if NK cells were depleted, human lung cancer cells which had been genetically engineered to produce M-CSF could not develop distant metastases into liver and lymph nodes in the SCID mice (10). Since daily treatments with CAM were not effective in the present study, it appears that only monocytes/macrophages are not enough to mediate anti-metastatic effect *in vivo*.

Other effector cells in the antitumor system like cytotoxic T cells, NK cells, or lymphokine activated killer (LAK) cells could be involved in mediating the anti-tumor effect of CAM in vivo. Particularly, NK cells are known as primary effector cells responsible for inhibition of cancer dissemination (14, 15). To examine this possibility, we cultured human blood MNC with IL-2 in the presence or absence of CAM and measured their NK and LAK activities. CAM alone did not augment NK activity, but it caused significant increase in the LAK activity induced by IL-2 (Fig.4). The mechanism of stimulation of LAK activity by CAM is not clear at present. Nevertheless, it is possible that the monocytes, upon activation by CAM, lead to the stimulation of LAK activity, because cytokine-activated monocytes are known to stimulate the IL-2 induced LAK activity (13). Thus, no effect of CAM on NK activity in vitro may account for the therapeutic failure of CAM in the pulmonary and lymph node metastases of adenocarcinoma PC-14 cells in SCID mice bearing intact NK cells.

In summary, the present study showed that CAM alone did not have therapeutic effect on the growth and organ metastasis of human non-small cell lung cancer cells in SCID mouse models. Nevertheless, the present finding does not rule out a possibility that CAM may mediate antitumor effect in the immunocompetent host through stimulation of immune T cells. Further studies are required to explore this possibility, particularly in combination with other biological response modifier (s).

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