The induction of LAK cells in the peritoneal cavity of mice by combined treatment with OK-432 and rIL-2

Akira Nakamura, Katsuhiro Shimoda, Takao Saito and Michio Kobayashi

The First Department of Surgery, Oita Medical University

[Summary]

In ongoing studies on immunotherapy against peritoneal carcinomatosis, LAK cells were introduced into the peritoneal cavity of mice. Antitumor effects of i.p. treatments of OK-432 and rIL-2 given sequentially were studied. When OK-432 was given in a dose of 1 KE, the number of peritoneal exudate cells (PEC) and the ratio of asialo GM 1 (asGM 1)+ cells significantly increased in murine peritoneal cavity and a peak was reached on day 5 after treatment. The mice were then injected i.p. with rIL-2 at a dose of 5×10^4 U. LAK activity of PEC against NK-resistant target cells, EL-4, on day 6 was significantly higher than that in case of PEC with OK-432 alone. In vivo pretreatment of the PEC with anti-asGM 1 antibody prior to rIL-2 eliminated the augmentation of LAK activity. Sequential injection of OK-432 and rIL-2 to mice bearing X 5563 plasmacytoma augmented the LAK activity of PEC and the survival rate of tumor bearing mice was increased. Thus, the in vivo induction of LAK cells by the combined sequential injection of OK-432 and rIL-2 may be given consideration for treating patients with peritoneal carcinomatosis.

Key words: OK-432, rIL-2, LAK, peritoneal dissemination
I. Introduction

Although adoptive immunotherapy (AIT) with lymphokine-activated killer cells (LAK) has been prescribed for cancer patients, the efficacy in clinical trials was unsatisfactory and was much less than seen in experimental models. These limited effects of AIT could be explained by the low cytotoxic activity of LAK cells to tumor cells and because of the poor accumulation of cytotoxic effectors at tumor sites. Furthermore, conventional LAK therapy was clinically hampered because a large number of LAK cells were needed and the subsequent administration of a large amount of rIL-2 often resulted in severe side effects such as pulmonary edema and hypotension. Therefore, alternative procedures need to be designed, which can induce high LAK activity in vivo with a relatively low dose of rIL-2 and can accumulate LAK cells at the tumor sites.

OK-432, a streptococcal biological response modifier preparation exhibits potent antitumor effects against both animal and human cancers, and was given to mice with peritoneal and pleural malignant effusions. The i.v. injection of OK-432 effectively induced LAK precursor cells in vivo in the normal murine spleen and these cells differentiated into LAK cells when exogeneous rIL-2 was administered. Based on these results, we investigated whether the combined intraperitoneal injection of OK-432 and rIL-2 could effectively induce LAK cells in the peritoneal cavity.

II. Materials and methods

1. Mice and tumor

C3H/HeN female mice, 7 to 8 weeks of age, were purchased from Charles River Japan Inc. (Kanagawa, Japan). EL-4 leukemia cells, resistant to NK cells, were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat inactivated fetal calf serum (FCS) (Flow Laboratories, Inc., Virginia). X5563 plasmacytoma, derived from the C3H/HeN strain, was maintained by i.p. passage in syngeneic C3H/HeN mice.

2. OK-432 and rIL-2

OK-432, a penicillin- and heat-treated, lyophilized preparation of S strain with low virulence and of the streptococcus group A3, was provided by Chugai Pharmaceutical Company, Ltd. (Tokyo, Japan). One Klinische Einheit (KE) corresponds to 0.1 mg of dried streptococci. The lyophilized preparation was suspended at a concentration of 1 KE per 1.0 ml saline.

Purified recombinant interleukin 2 (rIL-2), S-6820 (Biogen Geneva, Switzerland), was a generous gift from Shionogi Pharmaceutical Company, Ltd. (Osaka, Japan). The specific activity exceeded than 1×10⁷ units/mg.

OK-432 was injected into mice intraperitoneally (i.p.) at a dose of 1 KE per mouse and rIL-2 was given i.p. at a dose of 5×10⁴ units per mouse, once in
3. Peritoneal exudate cells
Mice were injected i.p. with 5 ml of RPMI 1640 medium supplemented with 5% heat-inactivated FCS and after a 1 min-abdominal massage, peritoneal exudate cells (PEC) were harvested from the peritoneal cavity using a syringe with a 21 gauge needle. PEC was washed three times in the same medium and stained with trypan blue dye for calculation of cell numbers.

4. Cytotoxicity assay
A standard 4-hr 51 Cr release assay was used to measure cytotoxic activity of LAK cells against target cells, the EL-4 leukemia cell line. Target cells were labeled with 100 µCi of sodium 51 CrO4 (New England Nuclear, Boston) for 60 min in 0.5 ml of 10% FCS RPMI. The labeled target cells at 1×10⁴ cells per 0.1 ml and effector cells at 1×10⁶ cells per 0.1 ml were placed in Linbro round-bottomed microtiter plates (Flow Laboratories, Inc., Virginia), and incubated for 4 hrs at 37°C in 5% CO₂. After incubation, 100 µl of supernatants were harvested and the radioactivity was counted with a gammacounter (Aloka Co. Ltd., Tokyo, Japan).

Maximum release was determined by incubation of the target cells with 1% Triton X (Nakarai Chemicals Ltd., Kyoto, Japan), and spontaneous release occurred with incubation of the target cells with medium alone. The percentage of specific lysis was calculated by the following formula: % lysis = (Experimental CPM − Spontaneous CPM)/(Maximum CPM − Spontaneous CPM) × 100.

All experiments were done in triplicate and data were expressed as % cytolysis. Spontaneous release usually ranged from 0 to 10%.

5. Analysis of cell surface antigens
PEC were adjusted to 2×10⁷ viable cells/ml in phosphate-buffered saline (PBS, Sigma Chemical Co., St. Louis). The cells (2×10⁶/0.1 ml) were sequentially incubated with the following fluorescence-labeled monoclonal antibodies, FITC-conjugated anti Thy-1.2, FITC-conjugated anti Lyt-2, and PE-conjugated anti L 3 T 4 (Becton Dickinson, Mountain View, CA) at a final dilution of 1 : 20 in PBS for 30 min at 4°C, in the dark, and were then washed three times in PBS.

Rabbit anti asGM 1 antiserum (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to the cells (2×10⁶/0.1 ml) at a final dilution of 1 : 200. After incubation at 4°C for 30 min, the cells were washed three times in PBS, and resuspended to 2×10⁶/0.1 ml in PBS containing a 1 : 20 diluted of FITC conjugated goat anti rabbit IgG (TAGO, Burlingame, CA). The cells were incubated at 4°C for 30 min in the dark and were washed three times in PBS.

Fluorescence-labeled cell surface antigens of PEC were examined by one color analysis of flow cytometry on a fluorescence-activated cell sorter (FACS) III (Becton Dickinson, Mountain View, CA) at a flow rate of approximately 1,000 cells/sec.

6. Tumoricidal activity of PEC in tumor-bearing mice
Mice were inoculated with 1×10⁴ syngeneic X 5563 plasmacytoma cells and 10 days later, were injected i.p. with OK-432 at a dose of 1 KE per mouse. Five days after the OK-432 treatment, the mice were injected i.p. with rIL-2 at a dose of 5×10⁴ U per mouse and the following day, PEC was harvested and centrifuged on 100% and 80% Ficoll-Conray discontinuous gradients at 500 G for 40 min. There were erythrocytes and debris in the bottom pellet, a tumor cell enriched fraction in 80% of the layer and a mononuclear cell rich fraction in the 100% layer, each with a purity of 75%. Cytotoxicity of the mononuclear cell rich fraction as effector cells was measured in a 4-hr 51Cr release assay.

7. Effects of combined injection on survival rate
Survival rates were compared among tumor-bearing mice treated with OK-432 and rIL-2, those treated with OK-432 alone, and those untreated.
8. Statistical analysis
The mean difference between control and treated groups was evaluated statistically by Student's t test, and a P of less than 0.05 was defined as being statistically significant. Survival rates were determined by the Kaplan-Meier method. Differences in survival rates were calculated using the Cox and Mantel tests.

III. Results

1. Effects of OK-432 alone or OK-432/rIL-2 injections on the number of PEC
After i.p. injection of OK-432 alone or OK-432 and rIL-2, the number of PEC significantly increased in both groups of mice, compared to findings in the non-treated mice, by approximately four fold, and reached the peak on days 4 to 6 after treatment (Fig. 1). There was no statistically significant difference between the two groups.

2. Effects of OK-432 alone or OK-432/rIL-2 injection on cell cytotoxicity of PEC
Cell cytotoxicity of PEC increased more in mice treated with OK-432 and IL-2 than in those treated with OK-432 alone in each time course (Fig. 2). There were significant differences between the two groups on days 4 and 6. The cell cytotoxicity of PEC was increased in mice treated with OK-432 alone, presumably because OK-432 induced anomalous killer cells (OK-AK cells)\(^\text{12}\). The peak of OK-432 induced cytotoxicity was noted on day 4 with \(38.2 \pm 5.6\%\) and decreased thereafter. In contrast, the peak of the cytotoxicity in mice treated with OK-432 and rIL-2 was noted on day 6 and the mean \(\pm SD\) was \(64.8 \pm 6.5\%\), a value significantly higher than in mice treated with OK-432 alone \((35.2 \pm 8.3\%)\).

3. Analysis of cell surface antigens of PEC
On the 6th day after injection of OK-432, a time when the cell cytotoxicity was the most augmented, cell surface antigen on PEC was analyzed (Table 1). AsGM \(1^+\) cells and L3T4\(^+\) cells significantly increased in PEC treated with OK-432 while Thy1.2\(^+\) cells, L3T4\(^+\) cells and asGM1\(^+\) cells significantly increased in PEC of mice treated with OK-432 and rIL-2.

4. Effects of anti asGM 1 antibody pretreatment on cytotoxicity
In vivo pretreatment with anti asGM 1 antibody prior to rIL-2 injection eliminated this augmentation of LAK activity (Fig. 3). These results suggest that asGM 1\(^+\) LAK precursor cells were induced into murine peritoneal cavity by i.p. injection of OK-432 and differentiated into LAK cells following treatment of rIL-2.

5. Effects of combined treatment with OK-432 and rIL-2 in tumor-bearing mice
Effect of combined injections of OK-432 with rIL-2 on LAK activity were examined in tumor bearing mice. Mononuclear cells were separated from PEC...
Fig. 2 Effects of combined injection of OK-432 and rIL-2 on LAK activity
OK-432 was i.p. injected at the dose of 1 KE on day 0. RIL-2 was i.p. injected at the
dose of 5×10^4 U, on days 1, 3, 5, 7, and 10, respectively. Cytotoxicity of PEC against EL
-4 tumor cells was determined with a 4 hr-^51Cr-release assay at E/T ratios of 100/1.
Bars represented the mean ± SD for triplicate cultures. OK-432 and rIL-2 treated
mice; OK-432 treated mice; and control mice. *P<0.05

Table 1 Analysis of cell surface antigens of PEC from mice 6 days
after OK-432 injection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phenotype (%)</th>
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<tr>
<td></td>
<td>Thy 1.2</td>
</tr>
<tr>
<td>control</td>
<td>28.8±3.1</td>
</tr>
<tr>
<td>OK-432</td>
<td>32.4±6.3</td>
</tr>
<tr>
<td>OK-432 and rIL-2</td>
<td>47.8±9.3**</td>
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*p<0.05, **p<0.01, differences between control and other treated groups.

of mice inoculated with X 5563 plasmacytoma cells
using Ficoll-Conray discontinuous gradient, and cell
cytotoxicity of the mononuclear cells to EL-4 cells
was determined. Cytotoxicity tended to be higher in
OK-432 treated mice compared with normal and OK
-432 untreated mice (Fig. 4). The additional injec-
tion of rIL-2, subsequent to OK-432, significantly
augmented the cytotoxicity from 29.3±8.4% to
41.4±7.8%.

Effects of combined injections of OK-432 and rIL
-2 on survival of C 3 H mice bearing X 5563 plas-
macytoma cells were also examined. The survival
time was 17.3±1.5 d in non-treated mice, and
19.3±1.7 d in mice treated with OK-432 alone, and
21.7±5.3 d in mice treated with OK-432 and rIL-2
(Fig. 5). There was a significant difference in sur-
vival time between OK-432 and rIL-2 treated mice
and control mice (p<0.05). However, the differ-
ence between OK-432 treated mice and control mice
was not significant.

IV. Discussion
Cancer immunotherapy using rIL-2 alone has
shown little merit in clinical trials, it is toxic4-7 and
clearance is rapid14-16, though the effectiveness of
rIL-2 in experimental models has been reported17,18.
Fig. 3 Effects of anti asialo GM1 antibody on LAK activity

OK-432 was i.p. injected at the dose of 1 KE on day 0. rIL-2 (5×10^4 U) and anti asialo GM1 antibody were i.p. injected on day 5. The cytotoxicity of PEC against EL-4 tumor cells was determined on day 6. Group I control mice; group II OK-432 treated mice; group III OK-432 and rIL-2 treated mice; and group IV OK-432, rIL-2 and anti asialo GM1 antibody treated mice. *p<0.05

Adoptive immunotherapy of LAK cells proposed by Rosenberg and associates1–3) has problems of cost, labor, and time. In attempts to induce high effective LAK activity in vivo by using biological response modifiers (BRM), we studied the in vivo local induction of LAK cells by combined treatments with OK-432 and rIL-2. The i.v. injection of OK-432 induced a large number of asGM 1+ LAK precursor cells in the normal murine spleen and these cells differentiated into LAK cells following the well-timed in vivo treatment with rIL-213. This would suggest that LAK precursor cells which were directly stimulated by OK-432 accumulate in the regions.

Fig. 4 Effects of combined injection of OK-432 and rIL-2 on LAK activity in tumor bearing mice

X 5563 plasmacytoma cells were inoculated on day 0, OK-432 was injected i.p. on day 10, and rIL-2 was on day 15. On day 16, PEC was harvested and cytotoxicity was determined. Group I control mice; group II tumor bearing mice; group III tumor bearing mice treated with OK-432 alone; and group IV tumor bearing mice treated with OK-432 and rIL-2. *p<0.05

Fig. 5 Effects of combined injection of OK-432 and rIL-2 on survival of C 3 H mice bearing X 5563 plasmacytoma cells

OK-432 was injected i.p. on day 10 and rIL-2 was on day 15. ○ control mice, ● OK-432 treated mice and ■ OK-432 and rIL-2 treated mice. There was a significant difference between OK-432 and rIL-2 treated mice and control mice (p<0.05).
In the present work, i.p. pretreatment with OK-432 increased asGM 1+ cells in the murine peritoneal cavity, and these cells differentiated into LAK cells following the subsequent intraperitoneal treatment with exogenous rIL-2. Treatment with OK-432 and rIL-2 in tumor-bearing mice also more effectively induced LAK cells and prolonged the survival time, as compared to treatment with OK-432 alone.

Patients are rarely rescued from peritoneal carcinomatosis. Other investigators reported that i.p. injection of OK-432 reduced ascites and destroyed tumor cells in ascites in patients with peritoneal carcinomatosis. OK-432 activates natural killer cells and macrophages and also induces cytokines such as, TNF, IL-1, IL-2, and IFN-γ. Accurate effectors augmented by OK-432 remain to be identified because cell populations induced by OK-432 appear dependent on the time after treatment. It has been reported that 90% BALB/C mice bearing syngeneic BAMC-1 tumor were cured by i.p. injection of OK-432 given in a dose of 1 KE 5 times every other day. In these mice, several cell populations seemed to depend on the time course, neutrophils 3 hours after injection, cytotoxic macrophage 30 hours, L3 T4+ T cells 7 days and pantropic killer cells 12 days.

In our experiment, the bolus injection of OK-432 at a dose of 1 KE given into the peritoneal cavity of mice greatly increased PEC, especially asGM 1+ cells compared to other cell populations, and a peak was reached at 4 days. As the majority of LAK precursor cells express asGM 1 surface antigen, we examined whether such increased asGM 1+ cells in PEC differentiated into LAK cells by injection of rIL-2. A high LAK activity was evident after the successive administration of rIL-2 and such augmented LAK activity was greatly diminished by pretreatment with anti asGM 1 antibody. These results suggested that a part of asGM 1+ cells in mice treated with OK-432 contained LAK precursor cells.

Treatment of OK-432 induces anomalous killer cells (OK-432-activated killer cells, OK-AK cells) similar to LAK cells with respect to target specificity and cell surface marker. In our experiment, OK-AK cells were detected in the mouse peritoneal cavity 3 to 4 days after OK-432 injection and the cytotoxicity was gradually reduced as the number of PEC increased (Fig.2). Since our OK-432 induced asGM 1+ LAK precursor cells were detected 6 days after OK-432 treatment, these cells were considered to be different from OK-AK cells. The relationship between OK-AK cells and OK-432 induced asGM 1+ LAK precursor cells was not elucidated.

Combined immunotherapy and various of immunomodulators were sometimes prescribed for cancer patients. In such trials, it is important to decide the most suitable combination, the order and route of administration, and the optimal dose. Crump III et al. showed that the timing of rIL-1 addition was important in synergistic augmentation of LAK activity induced by rIL-2 in human peripheral blood monocytes. Addition of rIL-1 at or before treatment of rIL-2 was necessary to induce synergistic augmentation. In our system of OK-432 and rIL-2, the number of PEC, asGM 1+ cells and cell cytotoxicity were most increased and augmented after sequential injections of OK-432 and rIL-2. Conversely, sequential injections of OK-432 and rIL-2, reverse order of the two BRM failed to induce synergistic augmentation of LAK activity (data not shown).

Sequential well-timed injection of OK-432 and rIL-2 effectively induced LAK cells in peritoneal cavity of normal mice. A similar combined injection of OK-432 and rIL-2 for tumor bearing mice also improved the survival rate. Thus, this combined immunotherapy of OK-432 and rIL-2 seems worthy of consideration for treatment of patients with peritoneal carcinomatosis in humans.
References


16) Papa, M.Z., Yang, J.C., Vetto, J.T. et al.: Combined effects of chemotherapy and inter-


