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## Abstract

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**KEYWORDS:** indomethacin, interleukin-2, mouse, hepatoma

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# Combined Therapy with Interleukin 2 and Indomethacin in Mice Inoculated with MH134 Hepatoma

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The antitumor effects of indomethacin and interleukin 2 (IL-2) were studied in C3H/HeJ mice inoculated with MH134 hepatoma cells. Combined treatment with indomethacin and IL-2 augmented natural killer (NK) cells in mice with MH134-induced peritoneal carcinomatosis, and the survival of the treated mice was significantly longer than the non-treated mice. In animals with subcutaneous MH134 tumors, the combined therapy with indomethacin and IL-2 significantly suppressed tumor growth and induced complete regression of the tumor in three out of five mice. These results suggest that indomethacin and IL-2 therapy could be effective on human gastrointestinal cancer cells as well.

**Key words:** indomethacin, interleukin-2, mouse, hepatoma

Various causes have been reported to lead to the suppression of immunological activity in cancer-bearing hosts. Prostaglandins (PG), which are primarily secreted by host macrophages in the lymphoid organs, as well as at the tumor site (1), are reportedly one of the main causes of natural killer (NK) cell suppression in cancer-bearing hosts (2). Prostaglandin E (PGE) has also been known to prevent T cell activation by two mechanisms: the down-regulation of interleukin 2 (IL-2) receptors and inhibition of IL-2 production. The suppressive action of $PGE_2$ on NK and lymphokine-activated killer (LAK) cell activation may also be explained by similar mechanisms (3-5). Lala and Parher reported that long-term indomethacin therapy combined with multiple doses of IL-2 activates killer cells at the tumor site, resulting in a lasting cure of experimental metastasis (6). In this study, we examined the antitumor effects of long-term indomethacin therapy combined with repeated doses of IL-2 on MH134 tumors inoculated subcutaneously or intraperitoneally in C3H/HeJ mice.

## Materials and Methods

***Animals.*** Specific pathogen-free C3H/HeJ mice (6-7 weeks of age) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The mice were housed in group of 10 or fewer per cage, and were fed with a solid diet (Oriental Yeast Co., Tokyo, Japan).

***Tumor.*** The murine hepatoma cell line MH134 (7) was used which has been successfully transplantable in C3H/He mice. The tumors are specific for the strain in which they arose and will not grow in mice of different H-2 histocompatibility. It has been demonstrated that these tumors are weakly immunogenic. A single-cell suspension was inoculated into the mice, at $1 \times 10^6$ or $3 \times 10^6$ cells per body, either into the peritoneal cavity or under the dorsal skin, respectively. The tumor diameters were measured at the longest (a) and shortest (b) arms every two or three days using a caliper; and the size was calculated using the formula: $(a + b)/2$.

***Spleen cells.*** Spleens were removed from the inoculated mice, cut finely in the medium, passed through a 150 mesh, and placed in 0.835 $NH_4Cl$-Tris buffer to lyse the erythrocytes, thus isolating the lymphocytes. The lymphocytes were then washed 3 times and resuspended in a comlete medium (CM): RPMI 1640 medium, Nissui Pharmaceutical Co., Tokyo, Japan, supplemented with 25 mM *N*-2-hydroxyethyl piperazine-*N*-2 ethane sulfonic acid (Sigma Chemical Co., St. Louis, MO, USA), 2 mM L-glutamine (Wako pure Chemical Industries, Ltd., Osaka, Japan), 50 $\mu$M 2-mercaptoethanol (Sigma Chemi-

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cal Co.), streptomycin (100 $\mu$g/ml), penicillin G (100 U), and 10 % heat-inactivated fetal calf serum (FCS, Grand Island Biological Co., Grand Island, NY, USA).

***Cytotoxic assay for LAK and NK activity.*** Cytotoxic activity was examined by $^{51}$Cr-release assay. YAC-1 cells from a Moloney virus-induced lymphoma in A/St mice (8) were labeled with radioactive chromium for use as target cells. Various numbers of effector cells were incubated with $1 \times 10^4$ $^{51}$Cr-labeled target cells in 0.2 ml of CM in 96-well round-bottomed microtiter plates. The plates were centrifuged at $300 \times g$ for 5 min and incubated at 37°C in air saturated with 5 % $CO_2$ for 12 h. Radioactivity in 0.1 ml of the supernatant was then determined using a gamma counter. Cytotoxicity was calculated by the following formula:

$$\% \text{ specific lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100$$

***Drugs and experimental protocols for therapy.*** Indomethacin (Sigma Chemical Co.) was dissolved in 95 % ethyl alcohol before use; further dilutions to the final concentration were made in the culture medium. Mice were inoculated with MH134 tumor cells on day 0, and randomly assigned to four groups (each $n = 5$). Group 1 received indomethacin 14 $\mu$g/ml in drinking water from day 1 throughout the experimental period; group 2 received recombinant human IL-2 (Shionogi Pharmaceutical Co., Osaka, Japan) 10,000 units i.p. twice a day, for two 5-day rounds (on days 10 through 14 and 17 through 21 for the treatment of peritoneal tumors, or on days 5 through 9 and 19 through 23 for the treatment of subcutaneous tumors); group 3 received indomethacin plus IL-2; and control animals received drinking water only.

***Statistical analysis.*** Survival times and tumor size were analyzed in the control, indomethacin, IL-2, and indomethacin plus IL-2 groups. Cumulative suvival rates were calculated by the Kaplan-Meier method and their statistical significance by the log-rank and generalized Wilcoxon tests. Statistical significance of median survival was evaluated by the Wilcoxon 2-sample test. Comparison of tumor size between each group was analyzed by Tukey's test.

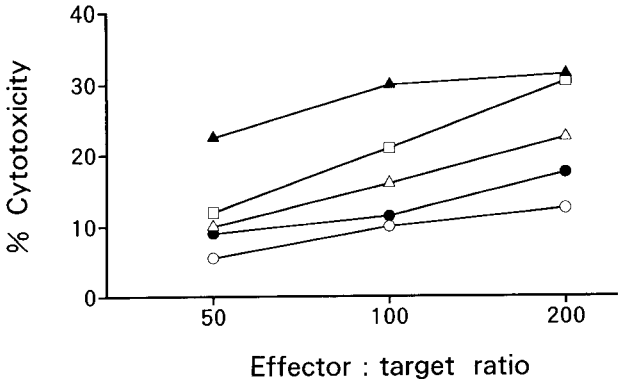


**Fig. 1** Cytotoxicity of spleen cells against YAC-1 targets in tumor-inoculated mice subjected to various treatments. ●, tumor-free mice; ○, tumor-bearing mice, no treatment; △, indomethacin; □, Interleukin 2 (IL-2) (days 5-9); ▲, IL-2 plus indomethacin.

## Results

***In vivo augmentation of NK activity by interleukin 2 and indomethacin.*** MH134 hepatoma cell line cells were injected intraperitoneally at an inoculum dose of $10^6$ cells/mouse. Five days after the inoculum, the animals were subjected to the following treatment. One group of C3H mice was allowed free access to the indomethacin solution from day 1, while another group of mice was treated with the intraperitoneal injection of $10^4$ U of IL-2 on each of days 5 through 9, and the third group underwent the combined administration of indomethacin and IL-2, according to the doses previously described. Spleen cells of each group were obtained on day 10, and the NK activity was examined. The administration of either indomethacin or IL-2 alone augmented the NK activity of spleen cells to levels exceeding that in the tumor-free donor or tumor-bearing mice with no treatment, and the combined administration of the two drugs further enhanced NK activity (Fig. 1).

***Effects of indomethacin and IL-2 on peritoneal carcinomatosis and subcutaneous tumor induced by MH134 cancer cells.*** We examined the effects of indomethacin and IL-2 on the survival of mice with peritoneal carcinomatosis induced by MH134 tumor cells. MH134 cells were injected intraperitoneally at $1 \times 10^6$ cells/mouse, and the therapy was

performed according to the protocol described in Materials and Methods. The survival rate was significantly improved in the indomethacin plus IL-2 group ($P < 0.05$). The median survival time of the control, indomethacin, and IL-2 group was 16.0, 19.6, and 20.6 days, respectively. By the combined treatment with these two drugs, the median survival was markedly prolonged to more than 54.8 days, showing a significant difference from the control group ($P < 0.05$). Two mice survived for more than 100 days (Fig.2).

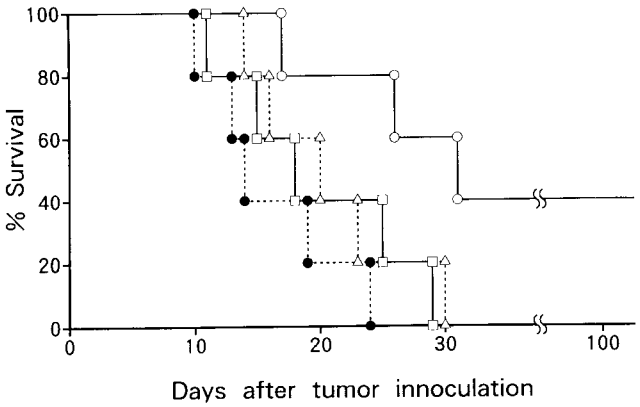


**Fig. 2** Survival curves of four groups of tumor-injected mice placed under various regimens. $1 \times 10^6$ MH134 cells were inoculated intraperitoneally on day 0. Interleukin 2 (IL-2) was administered on days 10 through 14 and 17 through 21. ●, Control; □, Indomethacin; △, IL-2; and ○, IL-2 + Indomethacin.

Next, the combined effects of IL-2 and indomethacin were examined using mice with subcutaneous MH134 tumors. Treatment with IL-2 or indomethacin alone could not induce marked suppression of tumor growth. In the indomethacin group, temporary suppression of tumor growth was found on day 14 ($P < 0.05$). In the IL-2 group, temporary tumor regression was observed on day 16 in 2 out of 5 mice. By the combined therapy with IL-2 and indomethacin, significant suppression of the tumor growth was found from day 9 to day 30, in comparison with the control group ($P < 0.01$) (Fig. 3). On the 16th day, the mean tumor size was decreased, and complete tumor regression was observed in 3 out of 5 mice. One of them sustained the tumor free state for 14 days. The other two showed tumor recurrence on the 20th day, and the tumor developed thereafter in spite of readministration of IL-2 from day 20 (Fig. 4).

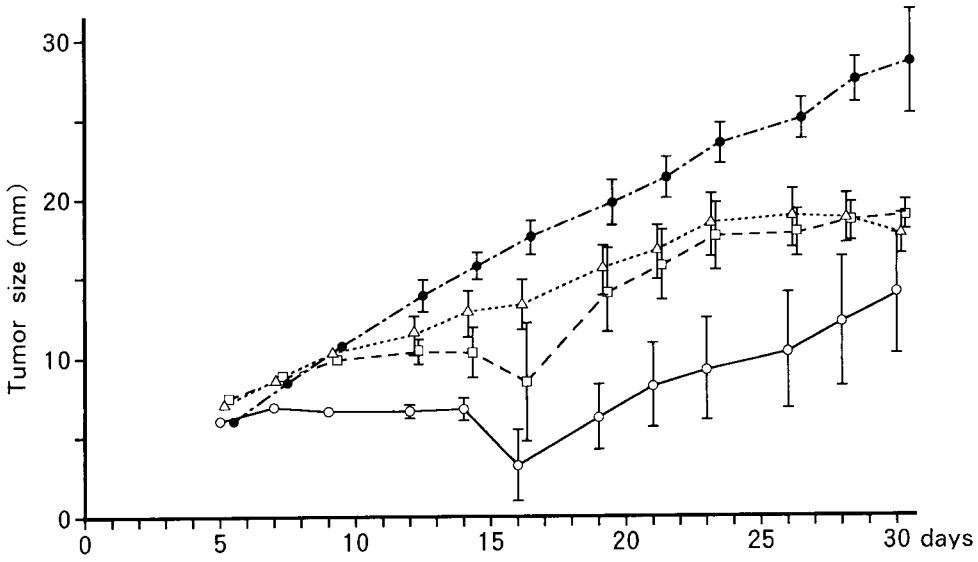


**Fig. 3** Tumor growth in animals treated with various regimens; $3 \times 10^6$ MH134 cells were inoculated under the dorsal skin on day 0. ●, tumor inoculated mice receiving vehicle alone (control); □, indomethacin; △, IL-2 (days 5–9 and days 19–23); ○, indometnacin plus IL-2. Significant difference ($P < 0.01$) in tumor size was detected from day 9 to day 30 between the IL-2 plus indomethacin group and the control group.

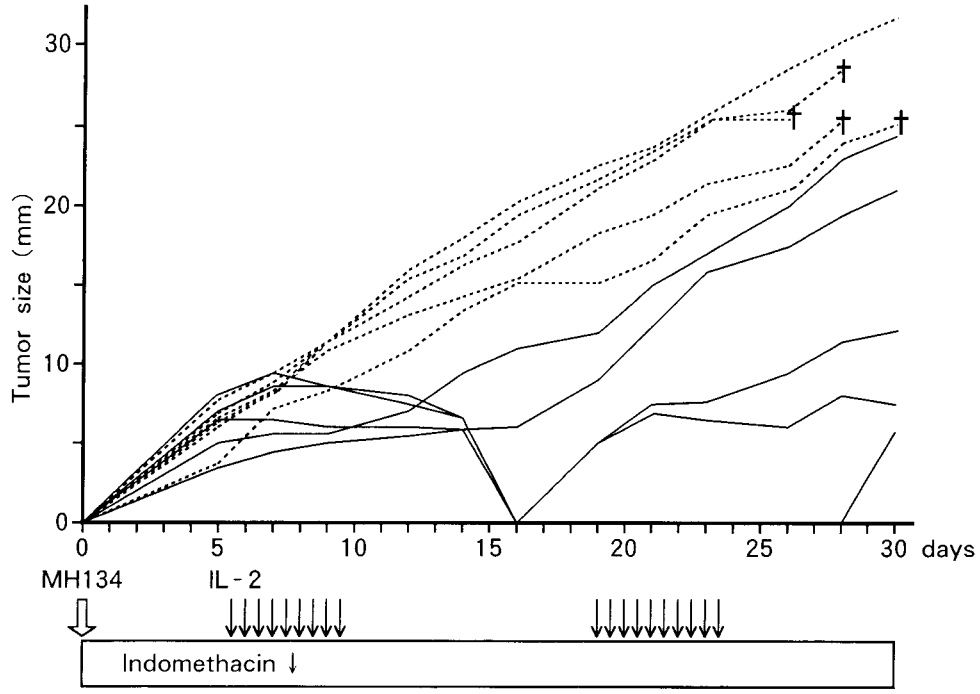


**Fig. 4** Tumor growth of individual mice in the control group and the indomethacin plus IL-2 group. Dotted line, control; solid line, indomthacin plus IL-2.

## Discussion

PGE, which is produced by macrophages in cancer-bearing hosts, or by cancer cells themselves, has been shown to suppress the activation of NK cells, as well as the generation of LAK cells, *in vitro* (9). Further, a high correlation was seen between high levels of PGE in the tumor tissue and high tumorigenicity and metastatic potential (10). PGE also affects such tumor-stroma interrelationships as angiogenic activity (11).

Inhibitors of prostaglandin synthesis have been shown to suppress the growth of transplantable tumors in both mice and rats (12, 13). Indomethacin has been found to be effective in inhibiting the growth of both primary tumors and its metastases. Giardiello *et al.* (14), in a recent controlled study, found that Sulindac, cis-5-fluoro-2-methyl-1- [p-(methyl-sulphinyl)-benzylidenyl] -indene-3-acetic acid (Merck Sharp and Dohme Ltd.), another nonsteroidal antiinflammatory drug, which inhibits the synthesis of prostaglandin, reduced the number and size of colorectal adenomas in familial adenomatous polyposis.

Maca (15) found that *in vivo* treatment with indomethacin significantly inhibited the growth of Lewis lung carcinoma (LLC) cells, while *in vitro* treatment of LLC cells with indomethacin did not suppress their growth. These findings indicate that the *in vivo* antitumor effect of indomethacin is not caused by a direct inhibitory action on these tumor cells.

In this study, long-term indomethacin therapy augmented the NK activity of spleen cells in tumor-bearing mice. Its combined use with multiple doses of IL-2 further enhanced natural killer activity. Using the B16F10 melanoma model of experimental lung metastasis in C57BL/6 mice, Lala and Parhar reported that repeated IL-2 treatment of mice in combination with oral administration of indomethacin has been shown to generate high LAK killer activity in both splenic and pulmonary lymphocytes, and a lasting cure for metastatic disease was achieved (6). In our present study using the MH134 hepatoma cell line in C3H mice, this combined therapy was also very effective. Two out of five mice with peritoneal carcinomatosis survived for a long period with this therapy, exceeding 100 days, whereas the control mice showed median survival of only 17 days. In the mice bearing subcutaneous tumors, the combined therapy sig-

nificantly suppressed tumor growth from day 7 to day 34, and three out of 5 mice showed temporary tumor regression.

This promising therapy should be carefully applied in clinical trials. The dose of indomethacin for mice, which was 14 $\mu$g/ml in drinking water, is equivalent to a clinical dose of 2-4 mg/kg/day. However, it has been suggested that IL-2 and indomethacin interact to cause more severe renal insufficiency through the inhibition of renal prostaglandin synthesis (16). Further studies are needed before the clinical application of this combined therapy of IL-2 and indomethacin can be tried.

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