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Antitumor Effect of Interleukin-1β in the Double Grafted Tumor System

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The antimetastatic effect of recombinant human interleukin-1β (rIL-1β) in a new experimental mouse model was studied. Intratumoral administration of IL-1β strongly inhibited the growth of Meth-A solid tumors in male BALB/c mice and led to a complete regression of tumors and resistance to reinoculated tumor. Subsequently, the anti-metastatic effect of IL-1β was examined in the double grafted tumor system, in which mice first received simultaneous intradermal inoculations of Meth-A in both right (10^6 cells) and left (2×10^5 cells) flanks and were then injected with 0.2 μg of IL-1β in the right tumor on days 3, 4 and 5. IL-1β significantly inhibited the growth of the left, non-treated tumor. When mice received only an inoculation of Meth-A (2×10^5 cells) in the left flank and were injected subcutaneously with IL-1β into the right flank on day 3 (single tumor system), there was no inhibition of the growth of the left, non-treated tumor. These findings suggest that intratumoral IL-1β immunotherapy in one region has an effect on tumor growth in another region. Immunized spleen cells were taken from mice which had been cured by the intratumoral administration of IL-1β. Adoptive transfer of the immunized spleen cells caused the complete regression of Meth-A tumors. These results suggest that intratumoral administration of IL-1β might induce cytotoxic cells in the left non-treated tumor of the double grafted tumor system and bring about the regression of metastatic tumors. On the other hand, recombinant tumor necrosis factor was effective only on the treated, right tumor, having no effect on the distant, left tumor in the double grafted tumor system. Recombinant interleukin-2 was effective on neither the right tumor nor the left tumor in this system. These results show that there are major differences of antitumor mechanism among cytokines.

Key words: Interleukin-1 — Antimetastatic effect — Cytokine

Interleukin-1, designated as IL-1,[2] was first defined as a lymphocyte activating factor (LAF) produced by monocytes-macrophages[1] and is a cytokine produced by activated macrophages and several other cell types.[2] It mediates a wide range of biological activities involved in immune and inflammatory responses.[3] IL-1 is defined by its ability to induce the differentiation of thymocytes and peripheral T lymphocytes.[4] Recently, Kikumoto *et al.* succeeded in the purification of recombinant human IL-1β produced in high yields in an *Escherichia coli* expression system.[5] Recombinant IL-1β has been shown to inhibit the growth of A375 human melanoma cells *in vitro*[6] and recombinant IL-1α has been shown to have antitumor activity *in vivo*.[7] However, the antitumor effect of IL-1β *in vivo*, and the mechanisms by which it brings about regression, remain unclear.

Metastasis is one of the most serious problems in cancer, and its prevention is obviously of great importance for improving the prognosis of cancer patients. Many investigators have reported that intratumoral therapy with chemotherapeutics[8] and BRMs (biological response modifiers)[9–11] is effective in causing the regression of local tumors. It has been reported that mice cured by intratumoral therapy of BCG or *Corynebacterium parvum* are resistant to a challenge with the same tumor.[9, 10] It has been shown that intratumoral injection of some agents eradicated the primary tumor and prevented the growth of regional lymph node metastases.[10, 12] These results suggest that the effect of intratumoral treatment is probably caused by a combination of the cytotoxic action of the agents and the development of a more potent antitumor immunity. Therefore, it is very important to clarify the mechanism of the antimetastatic effect of BRMs. In the present report, the antimetastatic effect of cytokines was examined in the double grafted tumor system[13–15] in which mice received simultaneous intradermal inoculations of Meth-A cells in the right (1×10^6 cells) and the left (2×10^5 cells) flanks, and were injected with cytokines (IL-1, IL-2, TNF, IFN) in the right tumor (primary region) 3 days later. The growth of the left, non-treated tumor (metastatic region) was observed for 21 days.

MATERIALS AND METHODS

Drugs Recombinant human interleukin-1β ^{71}Ser mutant[16] (rIL-1β) was supplied by Otsuka Pharmaceutical Co. Ltd., Tokushima (LAF activity: 2×10^7 units/mg protein). Recombinant interleukin-2 (rIL-2) was

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[2] The abbreviations used are: IL-1, interleukin-1; IL-2, interleukin-2; TNF, tumor necrosis factor; IFN, interferon; BRM, biological response modifier; LAF, lymphocyte activating factor

supplied by Shionogi Co. Ltd., Osaka (specific activity: 1.34×10^7 units/mg protein). Recombinant tumor necrosis factor (rTNF) was supplied by Asahi Chemical Industry Co. Ltd., Tokyo (specific activity: 2.3×10^6 units/mg protein). Cyclophosphamide was purchased from Sigma Chemical Co., St. Louis, MO.

Mice and tumor Six-week-old male BALB/c mice were obtained from the Shizuoka Breeding Farm for Laboratory Animals, Hamamatsu. Meth-A fibrosarcoma was administered to syngeneic BALB/c mice in solid form by intradermal inoculation.

Evaluation of antitumor activity Antitumor activity was assessed in terms of tumor weight 21 days after the inoculation of Meth-A cells, and tumor diameter was serially measured with calipers to estimate tumor size, as calculated by the following formula: square root of [long diameter × short diameter] (mm). The difference in tumor growth (tumor size or tumor weight) between the control and experimental groups was tested statistically by using Student's *t* test.

Adoptive transfer test to characterize effector cells *In vivo* assay of effector cells was conducted in recipient mice inoculated intradermally with 1×10^6 Meth-A sarcoma cells. Cyclophosphamide (CY; 2 mg/0.2 ml/mouse) was given intravenously one hour prior to administration of effector cells on day 3. Various effector cells in 0.1 ml of PBS (pH 7.2) were injected intratumorally into the recipient mice.

Treatment of spleen cells with G-10 column G-10 (Pharmacia Fine Chemicals, Uppsala) was soaked, dried and packed into a 10 ml syringe. Spleen cells (2×10^8 in 4 ml of RPMI 1640 medium with 10% fetal calf serum) were loaded on the G-10 column.

Treatment of spleen cells with antibody and complement Monoclonal antibody to Thy-1.2 was purchased from Sera-lab, Compiegne. Rabbit low cytotoxic complement was purchased from Cederlane Laboratories Ltd., Ontario. Spleen cells (10^7/ml) were incubated at 4°C for 60 min with antibody at a dilution of 1:500 in a volume of 0.5 ml. Cells were then washed and incubated at 37°C for 45 min with rabbit complement at a final dilution of 1:10.

RESULTS

Antitumor effect of intratumoral administration of IL-1β and resistance to reinoculated tumors The antitumor effect of rIL-1β (3 μg/mouse) on Meth-A sarcoma was investigated using different administration routes and was generally dose- and route-dependent, being highest by the intratumoral route (3 cured mice/8 tested mice), followed by the subcutaneous route (1 cured mouse) and the intravenous route (no cured mice). BALB/c mice were inoculated intradermally with 10^6 syngeneic Meth-A fibrosarcoma cells on day 0 and given three intratumoral injections of 0.1–0.5 μg of IL-1 starting on day 3, when the tumor was already palpable. As summarized in Table I (left column), 2 to 6 out of 6 mice receiving IL-1 intratumorally became tumor-free on day 21. Moreover, tumor weight in IL-1 treated mice was significantly lower than in control mice. Cured mice were reinoculated intradermally with 5×10^5 Meth-A cells on day 21 and were observed for the following 21 days. All cured mice that had received IL-1 rejected the reinoculated tumor cells (Table I, right column). These results indicated that the intratumoral administration of IL-1 seems to lead to systemic immunity to Meth-A tumors in host BALB/c mice.

Antimetastatic effect of IL-1β in the double grafted tumor system The above-mentioned findings raised the

Table I. Anti-Meth-A Fibrosarcoma Effect of IL-1 and Resistance to the Reinoculated Tumor

Group	First inoculation (1×10^6)		Treatment	Second inoculation (5×10^5)	
	Tumor-free /tested	Tumor weight (g±SD)		Tumor-free /tested	Tumor weight (g±SD)
Control	0/6	5.4±1.57	Control	0/6	2.1±1.33
IL-1 0.5 μg ×3 d3, 4, 5	6/6**	0	None	6/6**	0
IL-1 0.2 μg ×3 d3, 4, 5	3/6	0.6±0.58**	None	3/3*	0
IL-1 0.1 μg ×3 d3, 4, 5	2/6	0.9±0.35**	None	2/2	0

Mice were inoculated intradermally with 1×10^6 Meth-A sarcoma cells on day 0 and with 3 intratumoral injections of IL-1 (0.1, 0.2 or 0.5 μg). Cured mice were reinoculated intradermally with 5×10^5 Meth-A sarcoma cells on day 21, and a 3-week observation period followed. Significant difference from the control group: * $P<0.05$, ** $P<0.01$.

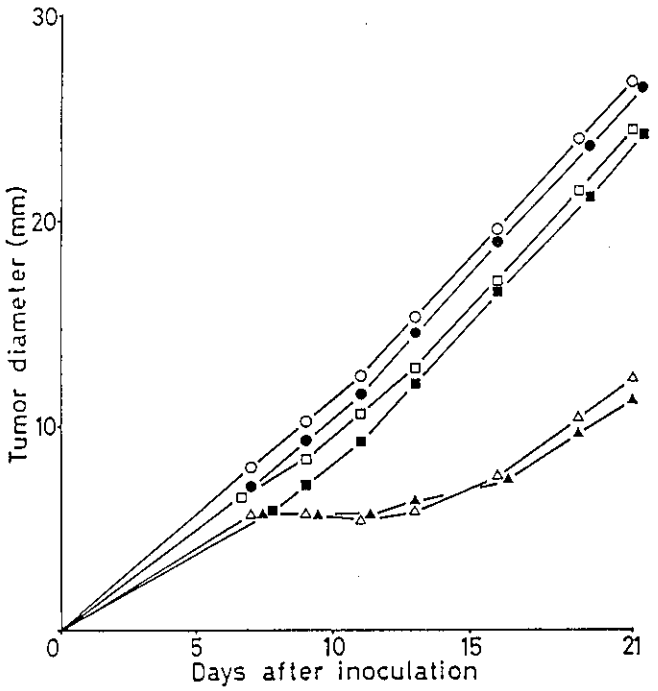

Fig. 1. Effect of IL-1β on the growth of Meth-A tumor in the double grafted tumor system and single tumor system. Experimental conditions were identical with those presented in Table II. ○, control right tumor of the double grafted tumor system; ●, control left tumor of the double grafted tumor system; △, IL-1 treated right tumor of the double grafted tumor system; ▲, IL-1 treated left tumor of the double grafted tumor system; □, control left tumor of the single tumor system; ■, IL-1 treated left tumor of the single tumor system.

possibility that intratumoral immunotherapy for a primary tumor might lead to systemic immunity to metastatic tumors. Accordingly, we devised the double grafted tumor system in which mice received simultaneous intradermal inoculations of Meth-A tumor cells in both the right (1×10^6 cells, primary region) and the left (2×10^5 cells, metastatic region) flanks, IL-1 was injected into the right tumor on day 3, and the left (non-treated) tumor was observed for 18 days so that the antimetastatic effect of IL-1 might be evaluated. As shown in Fig. 1, the simultaneous inoculations of Meth-A cells into the right and left flanks in control mice did not generate concomitant immunity,[17] that is, there was no difference between the growth of the 10^6 tumor cells and that of the 2×10^5 tumor cells. As shown in Table II, IL-1 significantly inhibited the growth of the left, non-treated tumor. That is 1 out of 4 mice was tumor-free, and the average tumor weight in the other 3 mice was significantly lower than that of the control mice.

Effect of IL-1β in the single tumor system The above-mentioned results do not exclude the possibility that IL-1 in the right tumor may directly affect the left tumor via the blood stream, etc. Accordingly, we devised as a control the single tumor system in which mice received intradermal inoculations of 2×10^5 Meth-A tumor cells only in the left flank, and then were injected subcutaneously with IL-1 into the right flank on day 3 and observed for 18 days. As summarized in Table II, no tumor-free mice were found in the single tumor system with IL-1 injection. Moreover, as shown in Fig. 1, IL-1 significantly inhibited the growth of the left non-treated tumor in the double grafted tumor system but had no

Table II. Anti-Meth-A Fibrosarcoma Effect of IL-1 in the "Double Grafted Tumor System" and "Single-tumor System"

Group	Right tumor (1×10^6 cells)		Left tumor (2×10^5 cells)	
	Tumor-free /tested	Tumor weight (g±SD)	Tumor-free /tested	Tumor weight (g±SD)
Double-tumor				
Control	0/4	4.8±0.72	0/4	4.4±0.53
IL-1 0.2 μg ×3	2/4	0.6±0.27[a)]**	1/4	0.6±0.79[a)]**
Single-tumor				
Control	—	—	0/5	4.0±0.97[a)NS]
IL-1 0.2 μg ×3 d3, 4, 5	—	—	0/5	3.6±0.97[b)NS]

Mice received intradermal inoculations of Meth-A sarcoma cells in both the right (1×10^6 cells) and left (2×10^5 cells) (double grafted tumor system) or left (2×10^5 cells) flanks (single-tumor system) on day 0. IL-1 (0.2 μg/0.1 ml/mouse × 3) was injected into the right tumor (double grafted tumor system) or injected subcutaneously into the right flank (single-tumor system). A 21-day observation period followed. Significant difference from the double-tumor control (*a*) or the single-tumor control (*b*): ** $P<0.01$; NS, not significant.

Table III. Anti-Meth-A Fibrosarcoma Effect of TNF and IL-2 in the "Double Grafted Tumor System"

Group	Right tumor (1×10^6 cells)		Left tumor (2×10^5 cells)	
	Tumor-free /tested	Tumor weight (g±SD)	Tumor-free /tested	Tumor weight (g±SD)
Control	0/10	5.2±0.94	0/10	3.4±0.58
TNF, 3×10^3 U×3 days 5, 6, 7	5/10*	0.3±0.17**	0/10	3.1±0.44
IL-2, 10^4 U×10				
days 1–10	0/10	2.1±1.00**	0/10	2.9±0.65
days 6–15	0/10	4.5±0.89	0/10	3.2±0.55
days 10–19	0/10	4.3±1.07*	0/10	3.0±0.75

Significant difference from the control group: * $P<0.05$, ** $P<0.01$.

Table IV. Characterization of Several Cytokines in the "Double Grafted Tumor System" and "Single Tumor System"

Cytokines	Dose	Double tumor		Single tumor	Second challenge
		Right	Left	Left	
IL-1	0.2 μg×3	++	++	−	++
IFN	10^5 U×8	++	++	+	++
TNF	3×10^3 U×3	++	−	ND	ND
IL-2	10^4 U×10	±	−	ND	ND

++, tumor rejection; +, growth inhibition; ND, not done.

inhibitory effect at all in the single tumor system. These results show that IL-1 itself does not directly affect the opposite flank and that intratumoral IL-1 immunotherapy in the primary region does affect tumor growth in the metastatic region.

Antitumor effect of four different cytokines in the double grafted tumor system The effect of recombinant IFNαA/D has been reported previously.[13] Recombinant tumor necrosis factor (rTNF, 3000 U×3) was effective only on the treated, right tumor, having no effect on the distant, left tumor in the double grafted tumor system. Recombinant interleukin-2 (rIL-2, 10^4 U×10) was effective on neither the right tumor nor the left tumor in this system as shown in Table III. These results show that there are major differences of antitumor mechanism among cytokines, as summarized in Table IV.

Table V. Adoptive Immunotherapy with IL-1 Immunized Spleen Cells

Group	Tumor-free /tested	Tumor weight (g±SD)	Tumor diameter (mm±SD)
Control	0/6	5.5±1.38	25.6±2.24
CY+control	0/6	$3.3\pm0.72^{a)**}$	$21.5\pm1.98^{a)**}$
CY+normal cell	0/6	$3.6\pm1.35^{b)NS}$	$21.0\pm3.72^{b)NS}$
CY+IL-1(21d) cell	3/6	$1.3\pm0.82^{b)**}$	$13.3\pm5.54^{b)NS}$
CY+IL-1(14d) cell	3/6	$1.1\pm1.19^{b)**}$	$12.0\pm7.50^{b)NS}$
CY+IL-1(7d) cell	2/6	$0.6\pm0.40^{b)**}$	$10.9\pm2.72^{b)**}$

IL-1 immunized spleen cells were taken from mice 7, 14 or 21 days after tumor inoculation. Recipients received intradermal inoculation of Meth-A sarcoma cells in the flank on day 0. Cyclophosphamide (CY; 2 mg/0.2 ml/mouse) was injected intravenously one hour prior to injection of immunized spleen cells. Spleen cells (2×10^7 cells/0.1 ml/mouse) were injected into the tumor on day 3, and a 21-day observation period followed. Significant difference from control (*a*) or CY control (*b*): ** $P<0.01$; NS, not significant.

Table VI. Effect of Various Pretreatments on the Capacity of Spleen Cells to Suppress the Growth of Meth-A Tumors in Intratumoral Adoptive Transfer

Group	Tumor-free /tested	Tumor weight (g±SD)	Tumor diameter (mm±SD)
Control	0/10	1.15±0.72	24.07±2.33
CY+control cells	0/7	2.57±0.22	20.77±0.87
CY+whole (IL-1) cells	5/6*	0.30	8.50
CY+ā-Thy1.2+C (IL-1) cells	1/6	1.37±1.18	15.00±5.70
CY+G-10 (IL-1) cells	3/6	1.00±0.40**	13.57±1.99**

(CY), cyclophosphamide (2 mg/mouse) was injected intravenously one hour before spleen cells were injected into the Meth-A tumor on day 3. (IL-1), IL-1 treated mice 14 days after tumor inoculation; (whole), whole spleen cells; (ā-Thy-1+C), anti-Thy-1 monoclonal antibody and complement treated spleen cells; (G-10), G-10 column-passed spleen cells. Significant difference from CY+control cells: * $P<0.05$, ** $P<0.01$.

Adoptive transfer of IL-1β immunized spleen cells The next step was to analyze the mechanisms by which the cytotoxic effect observed in the left, non-treated tumor of the double grafted tumor system depended upon IL-1 treatment of the right tumor. Immunized spleen cells were obtained from BALB/c mice which had been administered IL-1 intratumorally. After the injection of 2 mg of cyclophosphamide intravenously one hour prior to the injection of spleen cells in order to obviate the function of suppressor T cells,[18] 2×10^7 IL-1 immunized spleen cells were injected into the tumor on day 3. This procedure resulted in the inhibition of the growth of the tumor, and the occurrence of many tumor-free mice (Table V).

Next, characterization of the effector cell subpopulation that showed antitumor activity in the adoptive transfer was conducted with spleen cells obtained from mice 14 days after tumor inoculation. IL-1 (0.2 μg/mouse) was intratumorally injected on days 3, 4 and 5. As shown in Table VI, antitumor activity was abolished by Thy-1 monoclonal antibody treatment. When the adherent cells were removed by G-10 treatment, the antitumor activity was observed. These two experimental results clearly showed that it was G-10 column-passed T cells that were responsible for antitumor activity *in vivo*. These results suggest that the intratumoral administration of IL-1 might first induce cytotoxic cells in the primary region, and the cells later move to and accumulate in the metastatic region, inhibiting the growth of the specific tumor.

DISCUSSION

The pathogenesis of metastasis involves many complicated biological processes, and its outcome depends on the interaction of tumor cells with their host and is influenced by various factors at each metastatic step. The role of host defence in the control of metastasis is not well understood. Recently, studies of immunotherapy for animal and human cancers have suggested that it might be possible to eradicate or prevent micrometastases by immunological modulation of the host defense mechanism. The present report showed that intratumoral immunotherapy with IL-1 causes complete rejection of primary Meth-A tumors and growth inhibition of metastatic tumors in syngeneic BALB/c mice (Table II). These cured animals are able to reject specifically rechallenged Meth-A cells (Table I) and induce cytotoxic cells against Meth-A cells as evidenced by adoptive transfer experiments (Table V). That is, this report has shown for the first time that using our "double grafted tumor system," intratumoral IL-1 immunotherapy in the primary region causes a regression of metastatic tumors.

Biological response modifiers such as PSK and OK432 are known to induce tumor immunity against a rechallenge by the same tumor cells in mice cured after intratumoral treatment. These mice reject the growth of a second tumor, inoculated in either the footpad or the peritoneal cavity. This may suggest that the local immune reaction in the dermal tissues around the tumor leads to a systemic immunity to tumor cells. Against this background, we developed the double grafted tumor system, which may both raise a local immune response and generate an easily detectable systemic tumor immunity in the same animal within a limited experimental period. In parallel, the growth of a left distant tumor of the same inoculum size (2×10^5) was examined by the "single tumor system," where Meth-A cells were inoculated in the left flank, and drugs were subcutaneously given in the right flank. By comparing the results of these two systems, we could distinguish any host-mediated activity of agents from their direct action. We found that there were four types of mode of action among cytokines (Table IV).

IL-1 type: Effective on the left tumor in the double grafted tumor system, but ineffective in the single tumor system. This suggests that the antitumor effect of IL-1 on the distant tumor is not direct, but is host-mediated. PSK, a *Coriolus* preparation, belongs to this type.[13-15]

IFN type[13]: Effective on the distant tumor in the double grafted tumor system. IFNαA/D exhibited slight growth inhibition in the single tumor system. However, the antitumor effect of IFNαA/D was more effective on the distant tumor in the double grafted tumor system than on the tumor in the single tumor system. This shows that IFN can act on both the immune system and also directly on the tumor target cells.

TNF type: Effective only on the treated right tumor, with no effect on the distant, left tumor in this system. This suggests that the antitumor effect of TNF is direct. OK-432, a *Streptococcus* preparation, belongs to this type.[13]

IL-2 type: Although Morikawa *et al.*[19] have reported that the intratumoral injection of IL-2 with a sustained release vehicle into a rat fibrosarcoma showed antitumor activity, it was effective neither on the treated right tumor nor on the distant left tumor in the murine double grafted tumor system. This means that IL-2 has no antitumor effect in this system. Inhibition of the growth of the left-side tumor after IL-1 treatment in the double grafted tumor system was first detected on day 8 after tumor implantation. Furthermore, the cure of the left-side tumor was in many cases accompanied with the cure of the right-treated tumor. For effector cell analysis, we used intratumoral adoptive transfer into 3-day Meth-A bearing recipient mice pretreated with 2 mg of cyclophosphamide intravenously. The effector cell phenotype in spleen cells obtained from mice treated with IL-1 proved to be G-10 column-passed T cells (Table VI). These T cells migrate via the arteries to the spleen, where effector T cells are probably generated, further migrating via the veins to tumor sites and there demonstrating effector activity.[20] The lethal dose 50 (LD_{50}) of IL-1β by the subcutaneous route is 3 mg/kg in BALB/c mice. However, intratumoral therapy increases the effectiveness of drugs at high concentration, while ensuring minimal side effects to the host. The biological activity of IL-1β was observed in the serum of Meth-A bearing mice 30 min after intratumoral administration (100 μg/kg) and lasted for 4 h. Therefore following our animal experiments, a clinical study in which primary gastric, colon and breast cancers will be removed surgically after intratumoral administration of IL-1 should be of interest. Further histological and adoptive transfer experiments on the subpopulation of cytotoxic cells in the metastatic region are now being carried out.

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