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Rejection of Mouse Renal Cell Carcinoma Elicited by Local Secretion of Interleukin-2

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We introduced the interleukin-2 (IL-2) gene into mouse renal cell carcinoma (RenCa) in order to examine the mechanism of tumor rejection. IL-2 gene-transfected RenCa (RenCa/IL-2Hi) exhibited marked retardation of tumor growth when implanted in a syngeneic host. Growth retardation of RenCa/IL-2Hi was also observed in athymic nude mice even after depletion of natural killer (NK) cells by treatment with anti-asialo GM1 antibody. Histological analysis of RenCa/IL-2Hi tumors disclosed non-specific inflammatory changes in syngeneic hosts. Co-injection of Bacillus Calmette Guerin with RenCa/IL-2Hi considerably enhanced the anti-tumor effects. Taken together, these findings strongly suggest that *in situ* IL-2 production leads to tumor rejection through non-specific inflammatory responses without participation of T cells and NK cells. On the other hand, the syngeneic mice that had rejected RenCa/IL-2Hi acquired immunity against parental RenCa, suggesting possible participation of memory T cells in the second rejection of the tumor.

Key words: Renal cell carcinoma — Interleukin-2 — Tumor rejection — Inflammation

Interleukin-2 (IL-2) is one of the most important cytokines studied in tumor immunology, since it activates T lymphocytes, such as lymphokine-activated killer cells and cytotoxic T cells (CTL), which are known to play roles as effector cells against cancer.[1,2] There are, however, problems with the clinical use of IL-2. First, it is difficult to maintain the appropriate level of IL-2 at the tumor site due to the short lifetime of IL-2 in the body. Second, adverse effects occur when huge amounts of IL-2 are systemically administered.[3] One of the ways to avoid these problems is to introduce the IL-2 gene into tumor cells, which then themselves secrete IL-2 continuously at the tumor site.

Although several studies have been made of introduction of the IL-2 gene into cancer cells, the mechanism of the anti-tumor effect of IL-2 remains controversial.[4–11] In a previous study, we reported that IL-2 secretion by a mouse melanoma cell line (B16F10) elicited a non-specific inflammatory response which leads to inhibition of tumor growth.[4] Here we present evidence that introduction of the IL-2 gene into a mouse renal cell carcinoma (RenCa) resulted in inhibition of tumor growth even in the absence of T cells and NK cells. We also report that the IL-2-mediated tumor suppression was augmented by co-injection of Bacillus Calmette Guerin (BCG), which is known to activate macrophages and to induce strong delayed-type hypersensitivity (DTH) responses.

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MATERIALS AND METHODS

Tumor cell lines RenCa, a mouse renal cell carcinoma of Balb/c origin, was kindly provided by Dr. Ko Okumura (Juntendo University, Tokyo) and maintained in Eagle's MEM supplemented with 5% fetal bovine serum.

Expression plasmid and transfection to tumor cells The expression plasmid used in this study was previously described.[12] In pCEXV3/IL-2, a 0.6kb DNA fragment encoding human IL-2 was inserted into the *Sma*I site of pCEXV3. Standard calcium phosphate-mediated transfection was performed to introduce the gene into the cell line.[13] For drug selection, pSV2neo was co-transfected. Briefly, 2×10^5 RenCa cells were plated in a 6 cm dish one day before transfection. Nine μg of purified pCEXV3/IL-2 or pCEXV3 (as a control) was co-transfected with 1 μg of purified pSV2neo. Drug selection was begun 3 days after transfection. The concentration of Geneticin (Sigma, St. Louis, MO) was 1 mg/ml. After 2 weeks of drug selection, colonies were picked up with cloning cylinders and expanded to cell lines.

IL-2 assays Parental or IL-2 gene-transfected RenCa cells (3×10^5) were plated in 6-well plates (9.6 cm^2/well) with 1.5 ml of media, and supernatants were collected 24 h later. IL-2 activity was measured using IL-2-dependent human primary lymphoblasts in a proliferation assay, as previously described.[14]

Animal studies Athymic nude mice (Balb/c *nu/nu*, female, 6–8 weeks old) and control mice (Balb/c +/+, female, 6–8 weeks old) were purchased from CLEA

Japan, Inc. (Tokyo). In every animal experiment for measurement of the tumor growth, each group consisted of five mice. Tumor cells were trypsinized and washed twice in phosphate-buffered saline (PBS), and 10^6 cells in 0.1 ml were injected subcutaneously in the flank of each mouse, or 5×10^6 cells in 0.5 ml were injected intraperitoneally. The growth of subcutaneous tumor was measured in millimeters using a caliper. The longest surface length (a) and the width perpendicular to it (b) were measured, and tumor size was reported as the product of a and b. Tumors were checked at least twice per week. When the cells were injected intraperitoneally, mice were observed every three days and checked for clinical signs of ascites. For histological evaluation, tissue at the site of tumor cell inoculation was fixed in formalin solution, embedded in paraffin, sectioned at every 4 microns and stained with hematoxylin and eosin (HE).

Immunological treatment *in vivo* A total of 50 μl of anti-asialo GM1 antiserum (Wako, Osaka) diluted with 150 μl of PBS was injected intraperitoneally at day -1 and every 5 days thereafter. Depletion of NK cells was confirmed by 4 h ^{51}Cr-release assays with YAC cells as targets and spleen cells as effector cells.

The strain of BCG was Tokyo 172, obtained from the Japan BCG Laboratory (Tokyo). Two mg of BCG and 5×10^6 of parental or IL-2-secreting RenCa cells were mixed in 0.5 ml of PBS, and injected intraperitoneally into each mouse. The concentration of BCG was equivalent to that used in human intravesical BCG therapy (80 mg/40 ml).

RESULTS

***In vitro* studies of RenCa sublines** RenCa was transfected with pCEXV3/IL-2 or pCEXV3 as a control. Several transfected clones were picked up, and the IL-2 activities of their culture supernatants were measured. The clone with the highest IL-2 secretion, RenCa/IL-2 Hi, secreted 20 U/ml of IL-2 per 10^6 cells per 24 h. RenCa/IL-2Mo secreted a modest amount of IL-2, 4 U/ml, under the same conditions. Neither parental RenCa cells nor RenCa cells transfected with pCEXV3 (RenCa/Con) produced a detectable amount of IL-2.

To compare *in vitro* proliferation of the transfectants, 5,000 cells of each cell line per well were seeded in 12-well plates (3.8 cm^2/well), and cell growth was monitored. There was no significant difference in cell proliferation *in vitro* among RenCa/IL-2Hi, RenCa/IL-2Mo, RenCa/Con and parental RenCa cells (data not shown).

***In vivo* studies of RenCa sublines** To examine the effect of IL-2 secretion on tumor growth *in vivo*, 10^6 cells of RenCa/IL-2Hi, RenCa/IL-2Mo, RenCa/Con or parental RenCa were injected into the right flank of syngeneic

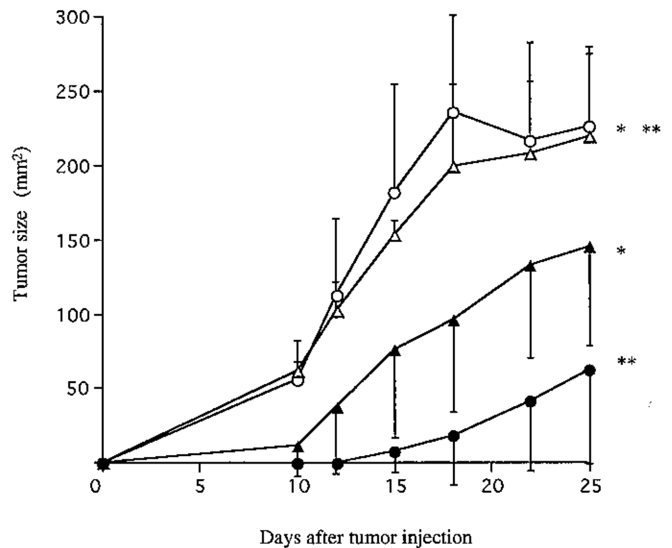

Fig. 1. Tumor growth in Balb/c mice of RenCa parental cell line (○ RenCa), control vector-transfected cell line (△ RenCa/Con) or clones of RenCa transfected IL-2 (● RenCa/IL-2Hi, ▲ RenCa/IL-2Mo). Mice were subcutaneously injected with 10^6 cells in the right flank on day 0. Tumor size was measured as the product of the greatest diameter and the perpendicular diameter. Bars represent standard deviations of tumor size with 5 mice per group. * Not significant, ** $P<0.05$ (Student's t test)

Table I. Tumorigenicity of Parental and IL-2-producing RenCa Cells

No. of inoculated cells[a]	No. of mice with tumor/No. of mice injected (%)[b]	
	RenCa	RenCa/IL-2Hi
10^5	5/5 (100)	2/5 (40)
10^4	5/5 (100)	1/5 (20)
10^3	3/5 (60)	1/5 (20)

a) Tumor growth was observed in Balb/c +/+ mice subcutaneously injected in the right flank.
b) Absence of tumor was judged until 11 weeks after injection; all the tumor-bearing mice had developed tumors by 6 weeks.

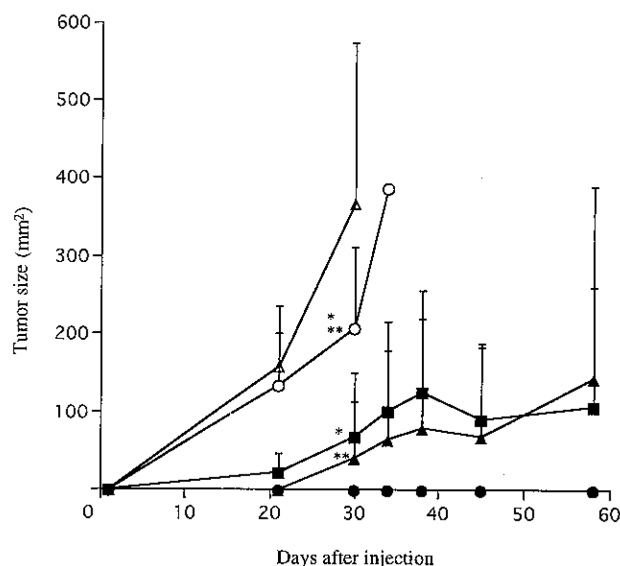

Fig. 2. Tumor growth of parental RenCa cells in Balb/c mice pre-injected with control PBS (△), 10^3 cells of parental RenCa (○) or 10^5 (●), 10^4 (▲) or 10^3 (■) cells of RenCa/IL-2Hi. Balb/c mice were injected with cells of one of these lines in the right flank. Surviving mice were re-challenged with injection of 10^6 parental RenCa cells in the left flank 11 weeks after the initial injection. Tumor measurements were performed as described in the legend to Fig. 1 and in "Materials and Methods." Bars represent standard deviations of tumor size, with 5 mice per group. * $P<0.1$, ** $P<0.05$ (Student's t test).

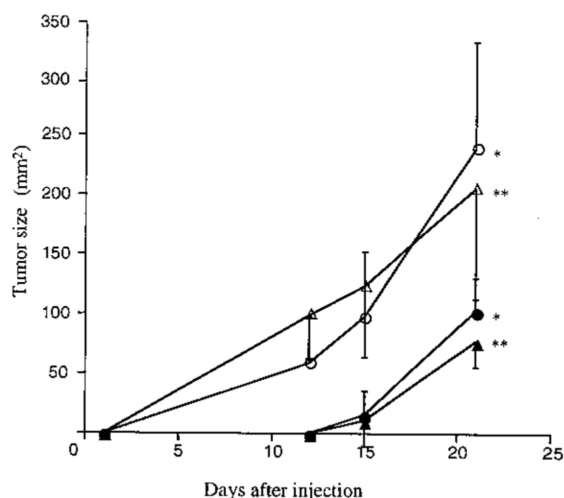

Fig. 3. Tumor growth of parental RenCa or RenCa/IL-2Hi in Balb/c *nu/nu* mice with or without NK cell depletion. For *in vivo* NK cell depletion, 50 μl of anti-asialo GM1 antiserum diluted with 150 μl of PBS was injected intraperitoneally at day −1 and repeated every 5 days. Mice were subcutaneously injected with 10^6 cells in the right flank on day 0. Tumor measurements were performed as described in the legend to Fig. 1 and in "Materials and Methods." Bars represent standard deviations of tumor size, with 5 mice per group. ○, parental RenCa in *nu/nu* mice; △, parental RenCa in NK-depleted *nu/nu* mice; ●, RenCa/IL-2Hi in *nu/nu* mice; ▲, RenCa/IL-2Hi in NK-depleted *nu/nu* mice. * $P<0.05$, ** $P<0.05$ (Student's t test).

+/+ Balb/c mice. RenCa/Con and parental RenCa exhibited almost the same degree of tumor growth. On the other hand, RenCa/IL-2Hi grew significantly more slowly than the above two groups (Fig. 1, $P<0.05$). Although the degree of inhibition of tumor growth in RenCa/IL-2Mo was not statistically significant, onset of tumor development was delayed and the rate of subsequent tumor growth was slower (Fig. 1).

In the next experiment, various numbers (10^5, 10^4, 10^3) of RenCa/IL-2Hi or parental RenCa cells were subcutaneously injected. Table I shows the rate of tumor development in each group. Even injection of only 10^4 parental RenCa cells resulted in tumor development in all mice. However, tumor formation was observed in only 40% and 20% of the mice injected with 10^5 and 10^4 RenCa/IL-2Hi cells, respectively. All the surviving mice were re-challenged with 10^6 cells of parental RenCa. Although tumors developed normally in the mice that had rejected 10^3 cells of parental RenCa, there was a tendency toward retardation of tumor growth in mice that had rejected the same number of RenCa/IL-2Hi cells (Fig. 2, $P<0.1$). Mice that had rejected 10^4 cells of RenCa/IL-2Hi exhibited significantly decreased tumor growth compared with control mice (Fig. 2, $P<0.05$). None of the mice that had rejected 10^5 cells of RenCa/IL-2Hi developed a tumor.

Rejection of RenCa/IL-2Hi tumors in athymic nude mice To study the anti-tumor effect of IL-2-secreting cells, 10^6 parental RenCa cells or RenCa/IL-2Hi cells were subcutaneously injected into *nu/nu* mice. Since IL-2 is a cytokine which mainly activates T cells, it was expected that no anti-tumor effect would be found in *nu/nu* mice. Unexpectedly, tumor rejection was observed with RenCa/IL-2Hi even in *nu/nu* mice (Fig. 3, $P<0.05$). Moreover, tumor growth of RenCa/IL-2Hi in *nu/nu* mice was still effectively retarded even after depletion of asialo-GM1 positive cells, which includes NK cell populations (Fig. 3, $P<0.05$).

Histological evidence of inflammatory response at sites of tumor rejection To study the effector cells further, histological examination was performed. Parental RenCa is a relatively immunogenic tumor, and infiltration of a few mononuclear inflammatory cells was noted at the parental RenCa tumor site 14 days after injection (Fig. 4A). However, infiltration of these cells was more prom-

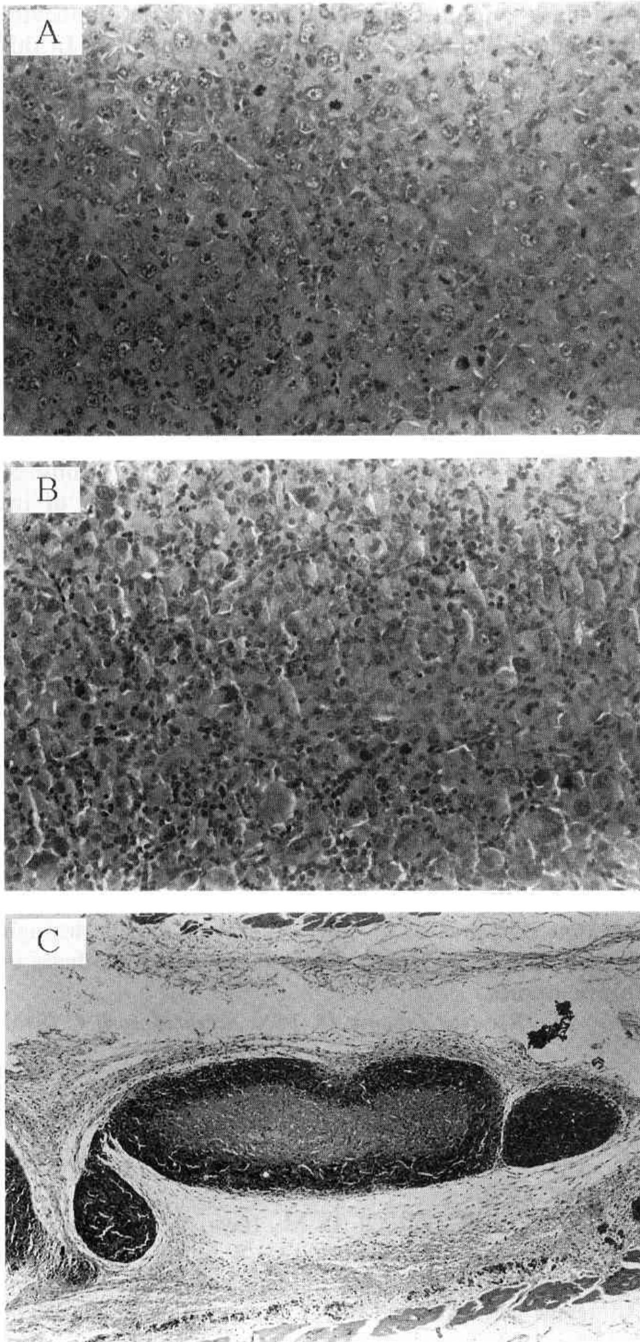

Fig. 4. Histological analyses of injection sites of parental or IL-2-transfected RenCa tumor. (A) Parental RenCa 10 days after injection, with little infiltration of inflammatory cells (×200). (B) RenCa/IL-2Hi 10 days after injection demonstrating severe infiltration of inflammatory cells (lymphocytes, macrophages and neutrophils) (×200). (C) RenCa/IL-2Hi 14 days after injection, demonstrating central necrosis of the tumor site (×40).

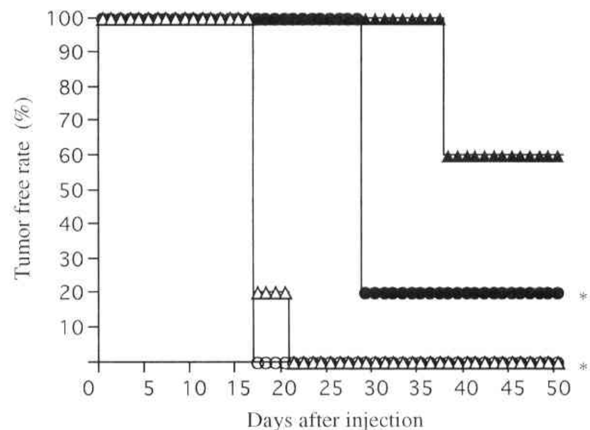

Fig. 5. Tumor growth of parental RenCa or RenCa/IL-2Hi in Balb/c mice with or without BCG co-injection. Mice were injected intraperitoneally with 5×10^6 cells in 0.5 ml of PBS. For BCG co-administration, 2 mg (dry weight) of BCG in 0.5 ml of PBS was co-injected intraperitoneally. Mice were observed to see whether ascites was present every 3 days after injection. ○, parental RenCa; △, parental RenCa with BCG co-administration; ●, RenCa/IL-2Hi; ▲, RenCa/IL-2Hi with BCG co-administration. * $P<0.01$, ** $P<0.05$ (Wilcox test).

inent at the RenCa/IL-2Hi tumor site (Fig. 4B). Moreover, central necrosis was observed at the RenCa/IL-2Hi tumor site (Fig. 4C).

Synergism of BCG and local secretion of IL-2 When the cells were injected intraperitoneally, RenCa/IL-2Hi exhibited inhibition of tumor growth compared with parental RenCa (Fig. 5, $P<0.01$). The tumor growth of RenCa/IL-2Hi was more strongly inhibited by co-injection of BCG (Fig. 5, $P<0.05$), whereas the tumor growth of parental RenCa was not affected by BCG.

DISCUSSION

As techniques for the introduction of genes into mammalian cells have developed, various kinds of cytokine genes (including IL-2, IL-4, IL-6, IL-7, IL-12, G-CSF, tumor necrosis factor-α and interferon-γ) have been introduced into cancer cells in order to induce their rejection by the immune system (reviewed by Columbo *et al.*[15]). The gene for IL-2 is representative in this respect, since IL-2 activates T lymphocytes and NK cells, which are considered important effector cells in the immunological response to cancer.[1,2] We previously reported that introduction of the IL-2 gene into a murine melanoma cell line inhibited tumor growth in mice.[4]

In accordance with previous results, including ours, we demonstrated in the present study that introduction of the IL-2 gene into a mouse renal cell carcinoma cell line

RenCa inhibited tumor growth in mice (Fig. 1 and Table I). We also showed that mice that had rejected RenCa/IL-2Hi acquired immunity against parental RenCa (Fig. 2). Given that those mice that had rejected parental RenCa could not acquire this immunity, the magnitude or type of immune response to RenCa/IL-2Hi appears to differ from that to parental RenCa. This observation implies the possibility of participation of memory cells (mainly T cells) in the second rejection, and therefore argues for the possible use of IL-2-secreting cells as an anti-cancer vaccine.

Although many researchers have studied the anti-tumor effect of IL-2 gene transfer to cancer cells, the mechanism of this anti-tumor effect remains controversial.[4–11] The first study made of the introduction of the IL-2 gene into cancer cells showed that local secretion of IL-2 directly activated CTL and killed cancer cells in an MHC class I-restricted manner.[5] On the other hand, Cavallo *et al.* showed that rejection of an IL-2-producing mouse mammary tumor involved neutrophils, with $CD8^+$ T cells playing only a minor role, and $CD4^+$ T cells and NK cells none at all.[11] The difference between these results might be attributable to the difference in cell lines used. Since the degrees of immunogenicity and sensitivity to CTL-mediated cellular injury vary with different cell lines, the principal mechanisms of tumor rejection would naturally differ depending on the cell line as well. Our observations that rejection of mouse renal cell carcinoma RenCa and malignant melanoma by locally secreted IL-2 did not require T cells or NK cells (Fig. 3 and Ref. 4) are comparable with earlier findings.[4, 11] It is not clear how IL-2 secreted by tumor cells elicits inflammatory responses in the absence of T cells and NK cells, but IL-2 might trigger the release of secondary cytokines and/or directly activate monocytes and neutrophils that express IL-2 receptors.[16, 17] In this connection, we previously observed that the anti-tumor effect against melanoma was enhanced by systemically administered macrophage colony-stimulating factor.[4] This result suggests an important role of macrophages for inhibition of tumor growth in our system.

We also demonstrated that co-administration of BCG with IL-2-secreting cells enhanced the anti-tumor effects. BCG activates macrophages to induce a strong DTH response. BCG is also known to have anti-tumor effects in some cases (e.g., BCG instillation therapy for bladder cancer). Although the exact mechanism of the anti-tumor effect of BCG remains unknown, it probably involves nonspecific inflammatory changes rather than a tumor-specific immune response. Histological study of RenCa/IL-2Hi tumors demonstrated infiltration of inflammatory cells such as macrophages, neutrophils and lymphocytes, as well as central necrosis of the tumor. It is thus likely that local secretion of IL-2 elicits an inflammatory response and leads to tumor rejection.

Another possible effector cell is the $\gamma\delta$ T cell. *Nu/nu* mice do not have $CD4^+$, $CD8^+$ or $\alpha\beta$ T cells, but they do have $\gamma\delta$ T cells.[18] There is no study which has yet clearly shown that $\gamma\delta$ T cells have anti-tumor activity. We demonstrated that antibody titer against BCG 65 kDa heat shock protein increased in patients who had undergone BCG instillation therapy.[19] Also, it is noteworthy that BCG 65 kDa heat shock protein is a ligand of $\gamma\delta$ T cells.[20, 21] Given these findings, it is reasonable to speculate that IL-2 and BCG exert synergistic effects through the function of $\gamma\delta$ T cells. We will test this hypothesis in future studies.

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