

Gene Therapy for Murine Renal Cell Carcinoma Using Genetically Engineered Tumor Cells to Secrete Interleukin-12

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ABSTRACT

To determine the possibility of gene therapy for renal cell carcinoma (RCC) using interleukin-12 (IL-12), we prepared genetically engineered murine RCC cells (Renca) which secrete IL-12 and evaluated the usefulness of these cells as a tumor vaccine. The IL-12 gene was transduced using MFG retroviral vector. The *in vitro* characteristics of transfectants—i.e., cell proliferation and expression of surface antigens—were then examined. *In vivo* tumorigenicity was assessed by subcutaneously injecting each type of cell in syngenic BALB/c mice. For the challenge experiments, the mice rejecting previously injected Renca IL-12 cells were rechallenged with parental cells. To determine the antitumor effect at remote sites, mice were injected with parental cells into the left flank, and then either Renca IL-12 or parental cells were inoculated into the opposite site on day 0 or 1. The transfected cells can secrete 146.7ng/ml/10⁶cells/48 hr of IL-12, as confirmed here by bioassay. The *in vitro* characteristics of the transfectants were not altered, but *in vivo* tumorigenicity was significantly reduced. Of the 21 mice that rejected Renca IL-12 cells, 9 failed to develop tumors after the challenge with parental cells. In the mice treated with Renca IL-12 as a vaccine, both number and tumor volume of the mice that developed tumors at remote sites were reduced. IL-12 secreting Renca cells conferred both protective immunity to parental cells and delay of tumor growth at remote sites, indicating that IL-12 secreting Renca cells are a feasible candidate for use in gene therapy of RCC.

Key words: IL-12, Gene therapy, Murine renal cell carcinoma

Approximately 25% of patients with renal cell carcinoma (RCC) have metastatic disease at initial presentation, and nearly 20% of patients who undergo curative surgery for RCC nonetheless develop advanced disease. The prognosis of advanced RCC remains highly unfavorable because the traditional therapies, including surgery, radiation, hormonal therapy, and chemotherapy, have not altered the natural history of this disease^{11,13,14}. For this reason, biological modifier therapy combined with surgical removal of both the metastases and the primary tumor has been conducted. While interferons (IFNs) and interleukine-2 (IL-2) are currently the most promising agents for such treatment of advanced RCC, their efficacy remains inadequate,²³ and there is thus need of alternative agents or modalities.

IL-12 is an important regulator of cell-mediated immune responses because of its effects on NK cells and T lymphocytes. In mouse models, sys-

temic administration of recombinant IL-12 revealed no serious side effects at doses that led to significant antitumor responses^{2,15,22}. However, systemic administration of IL-12 in mice and squirrel monkeys has been shown to cause significant toxicity^{6,17,21}. In humans, phase I trials of systemic IL-12 treatment of RCC have been completed^{4,10}. Phase II trials were temporarily suspended due to apparent toxicity, but this toxicity has since been reduced due to an improvement of administration protocol, and the trials have been resumed¹. Since IL-12 is secreted only by "professional" antigen presenting cells (i.e., macrophages and B-cells), it is possible that local secretion of IL-12 at the tumor site might induce an immune response similar to that occurring during immune response to bacterial pathogens. Thus, IL-12 may be a suitable cytokine for gene therapy as part of a tumor vaccine¹⁹. To examine the effectiveness of gene therapy using IL-12 for treatment of RCC, we prepared

genetically engineered murine RCC which secrete IL-12, then characterized these cells and evaluated their antitumor effect in a murine model of RCC.

MATERIALS AND METHODS

Animals and tumor cells.

Six-week-old female BALB/c mice were purchased from Charles River Japan Inc. (Kanagawa, Japan). The animals were kept under constant pathogen-free conditions and fed the commercial pellet diet CE-2 (CLEA, Tokyo, Japan). All experiments were conducted in accordance with the University of Hiroshima Animal Care Guidelines.

Renca, a BALB/c mouse renal cell adenocarcinoma of spontaneous origin¹², was generously provided by Dr. Fujioka (Iwate Medical School, Morioka, Japan) and maintained in Dulbecco's Modified Essential Medium (DMEM) (GIBCO BRL, Life Technologies, Inc., Rockville, MD) supplemented with 15% Fetal Bovine Serum (FBS) (GIBCO BRL).

Recombinant retroviral vectors and transfection to tumor cells.

DNA sequences encoding the p35 or p40 of murine IL-12 were inserted into the retroviral vector MFG,⁵ and the resulting constructs were introduced into ψ CRIP cells to generate recombinant viruses with an amphotropic host range. Renca cells were sequentially exposed to viral supernatants, including p35 or p40 of IL-12, and the transduced cells were characterized by measuring the efficiency of infection and the secretion of gene products, as follows:

Briefly, 5×10^5 Renca cells were inoculated into a 100 mm Petri dish and incubated until the logarithmic phase. These cells were transfected with 5 ml of retroviral supernatants (MFG-p35 of mIL-12 or MFG-lac Z) in the presence of 8 μ g/ml of polybrene (Sigma Chemical Co., St. Louis, MO) for 24 hr. The transfected cells were incubated in DMEM supplemented with 15% FBS for another 2 days (Renca IL-12 p35 or Renca lacZ).

The efficiency of infection was estimated by the expression of lac Z as a reporter gene using X-gal staining⁸. Virus titer (cfu/ml) was measured by the formula: (number of infectious cells) \times (percentage of X-gal staining positive cells)/(volume of viral supernatant). After confirming that the infection was sufficiently efficient, Renca IL-12 p35 cells were sequentially transfected with the mIL-12 p40 gene using the same methods as used for Renca IL-12.

The biological activity of IL-12 produced by transfectants was estimated by the proliferative response of 4-day human phytohemagglutinin-activated lymphocytes (PHA), as described previously¹⁸. Results were extrapolated from a standard curve using a defined dose of recombinant mouse IL-12 (Pharmingen, San Diego, CA). Production of IL-12 was also confirmed immunologically using

ELISA (Interest-12XTM; Genzyme, Cambridge, MA).

Cell proliferation assay.

2×10^4 parental Renca cells, Renca cells transfected by either lacZ gene or IL-12 gene were inoculated into a 35 mm Petri dish containing DMEM supplemented with 15% FBS. On days 1, 2, 3 and 5, the total cells in each dish were enumerated.

Immunofluorescent flow-cytometry analysis.

The alteration of surface antigens, including major histocompatibility complex (MHC) class I antigen, class II antigen and B7-1, in Renca cells after gene transfection was analyzed. FITC-conjugated anti-MHC class I (H-2K^d) and class II (I-A^d) monoclonal antibody were purchased from Meiji Nyugyo Co., Ltd. (Tokyo, Japan), and FITC-conjugated anti-B7-1 monoclonal antibody was purchased from Sumitomo Denko (Yokohama, Japan). Each cell type was incubated with the respective antibody in phosphate-buffered saline (PBS) supplemented with 5% FBS for 45 min at 4°C. After washing the cells three times with PBS, 10,000 cells of each group were analyzed by flow cytometry (EPICS Elite Flow Cytometer; Coulter, Hialeah, FL).

Animal studies.

A total of 1×10^5 , 1×10^6 or 1×10^7 Renca IL-12 viable cells suspended in 0.1 ml Hanks' balanced salt solution (HBSS) (GIBCO BRL) were inoculated subcutaneously (s.c.) into syngenic 6-week-old female BALB/c mice, and the perpendicular diameter of each tumor was measured twice a week using vernier calipers. Renca IL-12 cells were also inoculated admixed with parental cells in various ratios (1:0, 1:1, or 1:9).

For the challenge experiments, the mice that had rejected the previously injected Renca IL-12 cells were injected with 1×10^6 parental cells in the opposite flank. Further, to determine the antitumor effects at remote sites, 1×10^5 parental cells were injected s.c. into the left flank of mice and either Renca IL-12 (1×10^5 or 1×10^6) or parental cells were inoculated into the opposite flank at the same time or a day later. Tumor incidence and volume were monitored twice a week. The tumor volume was estimated by the formula: $1/4 \times (\text{long diameter}) \times (\text{short diameter})^2$. Mice bearing a tumor more than 5 mm in short diameter were considered to be positive.

Statistical analysis

Statistical analysis of the cell proliferation assay and animal studies was performed using Fisher's PLSD-test and Scheffe's F-test, and StatView J. 4.11 statistical analysis software was used for the intergroup comparisons (Abacus Concepts, Inc., Berkeley, CA). The differences were considered statistically significant at $p < 0.05$.

RESULTS

Efficiency of infection and secretion of IL-12 by transfectants.

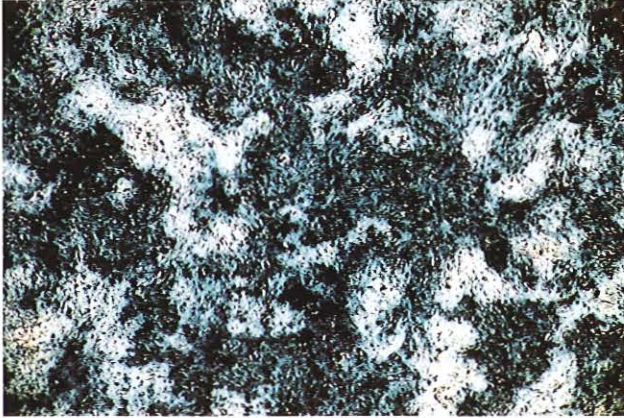


Fig. 1. X-gal staining of Renca lac Z cells.

The efficiency of infection was estimated by the expression of lac Z in Renca cells transfected with MFG-lac Z as a reporter gene (Renca lac Z). Renca cells expressing lac Z turned blue by X-gal staining. Eighty-six percent of Renca lac Z cells expressed lac Z, suggesting that the efficiency of infection was adequate in this series. (Original magnification $\times 100$.)

The efficiency of infection was estimated by the expression of lac Z in Renca cells transfected with MFG-lacZ as a reporter gene (Renca lac Z). Eighty-six percent of Renca lacZ expressed lac Z products, as confirmed by X-gal staining, suggesting that the efficiency of infection was adequate in this series (Fig. 1). The viral titer was 0.86×10^6 cfu/ml.

In fact, Renca IL-12 cells, which were transfected

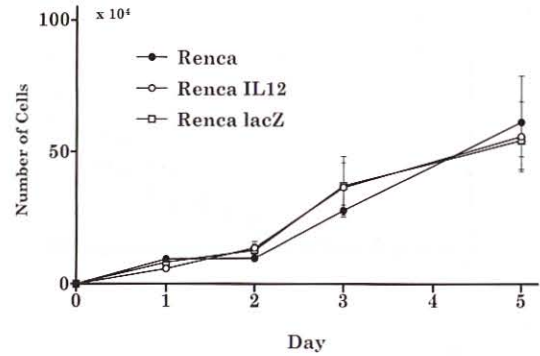


Fig. 2. *In vitro* growth of Renca transfected cells.

To determine the direct effect of transduction of the IL-12 gene or secretion of the biologically active form of m-IL-12 on Renca cell proliferation, 2×10^4 Renca cells were inoculated into a 35 mm Petri dish. Total cells in each dish were enumerated on days 1, 2, 3 and 5. The *in vitro* growth of each cell was not altered by gene transduction or secretion of IL-12.

by MFG-mIL-12-p35 and MFG-mIL-12-p40 sequentially, could secrete $146.7 \text{ ng}/10^6 \text{ cell}/48 \text{ hr}$ of biologically active IL-12, as measured by the proliferative response of 4 day human PHA-activated lymphocytes. In addition, IL-12 secretion was confirmed immunologically by ELISA ($129.33 \text{ ng}/10^6 \text{ cell}/48 \text{ hr}$). ***In vitro characteristics of Renca cells after gene transduction.***

To determine the direct effect on *in vitro* characteristics by the transfection of IL-12 gene or the

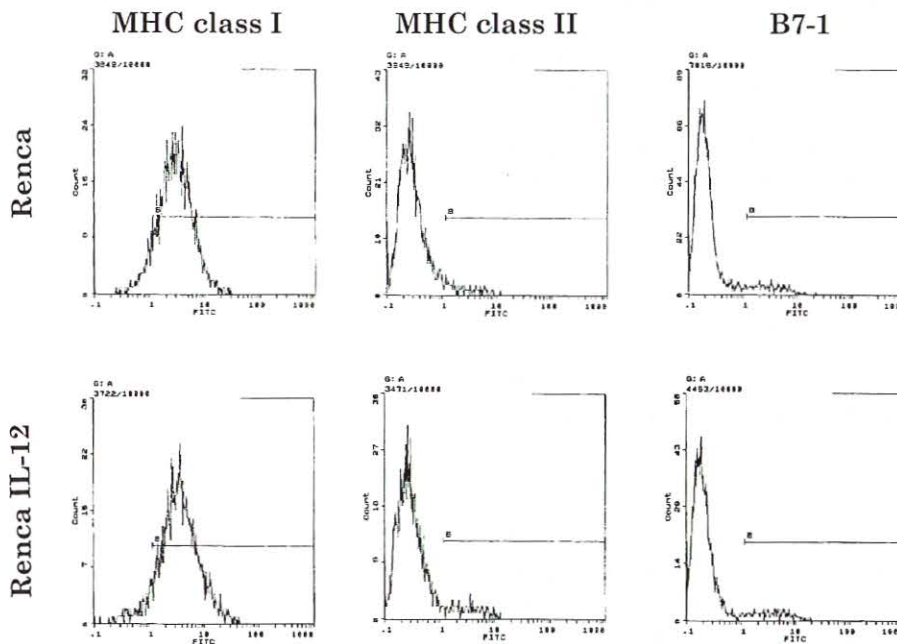


Fig. 3. Expression of surface antigens in Renca transfected cells.

To ascertain whether gene transfection changes the surface antigens of Renca cells, we analyzed the expression of MHC class I antigen, class II antigen and B7-1 by flow cytometry. MHC class I molecules were expressed in parental cells and transfected cells, but MHC class II molecules and B7-1 molecules were not detected in Renca parental cells and transfected cells. There was no alteration of surface antigens after gene transfer.

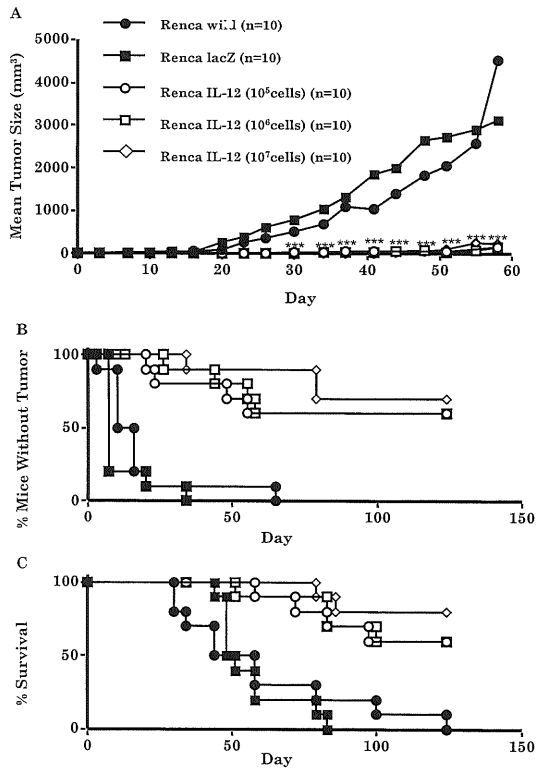


Fig. 4. *In vivo* tumorigenicity of Renca transfectants. To examine the tumorigenicity of Renca cells after gene transduction, parental Renca cells (10^6 cells), Renca lac Z cells (10^6 cells) and Renca IL-12 cells (10^5 , 10^6 or 10^7 cells) were injected s.c. into the right flank of syngenic BALB/c mice. Each group consisted of 10 mice.

The tumor volume of Renca IL-12 cells was reduced significantly compared with those of parental cells and Renca lac Z cells. *, $p < 0.01$ versus control (A). All mice injected with parental Renca cells developed tumors within 65 days, and those injected with Renca lac Z cells within 37 days. In the mice inoculated with Renca IL-12 cells, however, 6 of 10 mice injected with 10^5 Renca IL-12 cells, 6 of 10 mice injected with 10^6 cells, and 7 of 10 mice injected with 10^7 cells escaped tumor formation until day 130, when these experiments closed (B). Six of 10 mice injected with 10^5 Renca IL-12 cells, 6 of 10 mice with injected 10^6 cells, and 8 of 10 mice injected with 10^7 cells were alive until day 130, whereas all mice injected with parental Renca cells died within 122 days, and those injected with Renca lacZ cells died within 80 days (C).

secretion of bioactive form of mIL-12, the activity of cell proliferation and the expression of surface antigens were examined. As shown in Fig. 2, there were no significant differences in cell proliferation among parental Renca cells and Renca cells transfected by the lacZ or the IL-12 gene.

As shown in Fig. 3, a high basal expression of MHC class I molecules was found in both parental cells and transfectants, but MHC class II molecules and B7-1 molecules were not detected. There

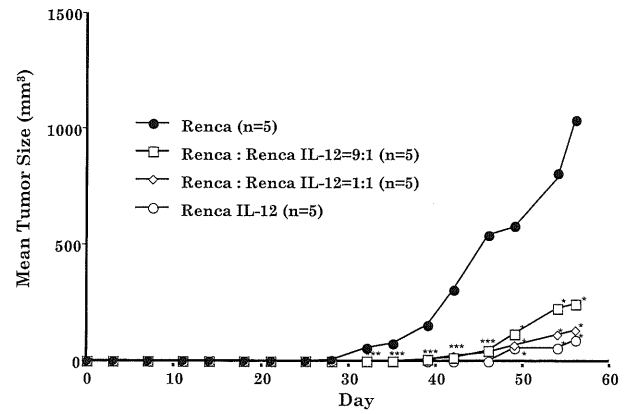


Fig. 5. *In vivo* tumorigenicity of Renca IL-12 admixed with parental cells.

To examine the *in vivo* tumorigenicity when Renca IL-12 cells were admixed with parental Renca cells in the various ratios (1:0, 1:1, or 1:9), a total of 1×10^6 viable cells were inoculated into mice. The tumor volumes in each group on day 56 were significantly smaller than those induced by the parental cells alone. *, $p < 0.01$ versus control.

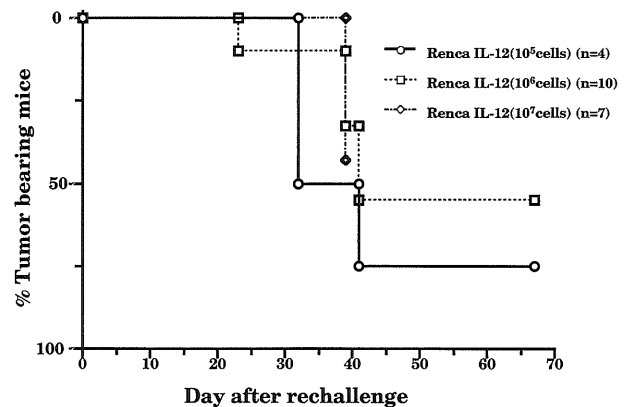


Fig. 6. Acquisition of protective antitumor immunity to parental cells.

To examine whether the mice acquired protective immunity to parental cells, those mice that rejected Renca IL-12 cells were inoculated with 1×10^6 parental Renca cells into the opposite flank. One of 4 mice (25%) that had previously rejected 1×10^5 Renca IL-12 cells, 4 of 10 mice (40%) that had rejected 1×10^6 Renca IL-12 cells, and 4 of 7 mice (57.1%) that had rejected 1×10^7 Renca IL-12 cells did not develop any tumors.

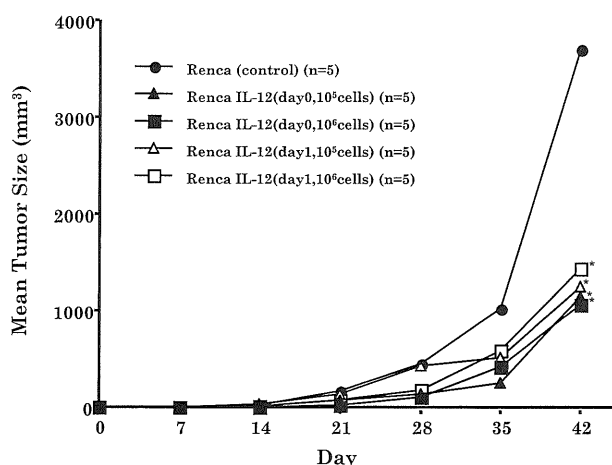
was no change of surface antigens after gene transfer. Therefore, neither transduction of the IL-12 gene nor secretion of the bioactive form of IL-12 had any direct effect on the *in vitro* characteristics of transfectants, including the activity of cell proliferation and the expression of surface antigens.

***In vivo* tumorigenicity of Renca cells after gene transduction.**

To examine the tumorigenicity of Renca cells after gene transfection, parental Renca cells,

Table 1. Tumor incidence after vaccination

Breeding period (day)	Tumor incidence					
	7	14	21	28	35	42
vaccination at once						
Renca wild (control)	0/5	2/5	5/5			
Renca IL-12 (10^5 cells)	0/5	3/5	4/5	4/5	4/5	4/5
Renca IL-12 (10^6 cells)	0/5	0/5	2/5	4/5	4/5	4/5
vaccination one day later						
Renca IL-12 (10^5 cells)	0/5	2/5	3/5	5/5		
Renca IL-12 (10^6 cells)	0/5	2/5	4/5	4/5	5/5	

**Fig. 7.** Antitumor effects at distant sites.

To determine the antitumor effects on the parental Renca cells at a distant site, 1×10^6 parental cells were inoculated s.c. into the left flank. At the same day or the next day, 1×10^5 or 1×10^6 Renca IL-12 cells or 1×10^5 parental cells were injected into the opposite flank. On day 42, the volume of tumors observed in mice treated with parental Renca cells as a control was significantly larger than that of those treated with Renca IL-12 as a vaccine. *, $p < 0.05$ versus control.

Renca lac Z cells, or Renca IL-12 cells were inoculated s.c. into BALB/c mice. Tumor volumes in mice injected with Renca IL-12 cells were significantly smaller than those observed in mice injected with parental Renca or Renca lac Z cells (Fig. 4A).

All mice injected with parental Renca cells developed tumors within 65 days, and those with Renca lac Z cells did so within 37 days. In the mice inoculated with Renca IL-12 cells, however, 6 of 10 mice injected with 10^5 of Renca IL-12 cells, 6 of 10 mice injected with 10^6 cells, and 7 of 10 mice injected with 10^7 cells escaped tumor formation until day 130, when these experiments closed (Fig. 4B).

The survival of mice inoculated with Renca IL-12 was also improved. Six of 10 mice injected with 10^5 Renca IL-12 cells, 6 of 10 mice injected with 10^6 cells, and 8 of 10 mice injected with 10^7 cells

were alive until day 130, whereas all mice injected with parental Renca cells died within 122 days, and those injected with Renca lacZ cells died within 80 days (Fig. 4C).

Renca IL-12 cells were also inoculated admixed with parental cells in various ratios. As shown in Fig. 5, the mean tumor volumes in each group treated with Renca IL-12 cells were significantly smaller than those of parental Renca cells alone on 56 days. Even if a considerable number of cells were not transfected with the IL-12 gene, adequate secretion of IL-12 could maintain the antitumor effect.

Anti-tumor effect of Renca cells after gene transduction.

To determine whether Renca IL-12 cells can confer protective immunity to parental cells, the mice that had rejected the previously injected Renca IL-12 cells were inoculated with 1×10^6 parental cells into the opposite flank. One of 4 mice (25%) that had previously rejected 1×10^5 Renca IL-12 cells, 4 of 10 mice (40%) that had rejected 1×10^6 Renca IL-12 cells, and 4 of 7 mice (57.1%) that had rejected 1×10^7 Renca IL-12 cells did not develop any tumors (Fig. 6). These mice were determined to have acquired protective anti-tumor immunity to Renca parental cells.

To determine the anti-tumor effect at remote sites, 1×10^5 parental cells injected s.c. into the left flank of mice and either Renca IL-12 (1×10^5 or 1×10^6) or parental cells were inoculated into the opposite flank on day 0 or day 1. All mice treated with parental Renca cells as a control developed tumors within 21 days, whereas 1 out of 5 mice (20%) treated with 1×10^5 and 1×10^6 Renca IL-12 cells on day 0 rejected tumors in the opposite flank (Table 1). In addition, in the mice treated with 1×10^5 and 1×10^6 Renca IL-12 cells on day 1, tumor formation was delayed at the remote sites (Table 1). As shown in Fig. 7, the tumor volume at day 42 in control mice treated with parental Renca cells was significantly larger than tumor volumes in mice treated with Renca IL-12 cells on day 0 or in those treated with Renca IL-12 on day 1.

Although Renca IL-12 cells could not achieve complete tumor regression as a vaccine, they induced the delay of both tumor formation and tumor growth compared with the parental cells as a control.

DISCUSSION

IL-12, formerly termed natural killer cell stimulatory factor (NKSF)⁹ or cytotoxic lymphocyte maturation factor (CLMF)¹⁸, is a disulfide-linked heterodimeric cytokine composed of a 35 kDa light chain (p35) and a 40 kDa heavy chain (p40). To produce biologically active IL-12, simultaneous expression of both the p35 and p40 genes within the same cell is necessary^{9,18}. Gubler et al pointed out a troublesome possibility that the sequential

transfection by retroviral vector might deteriorate the efficacy of transduction⁷. To avoid such reduced efficacy, Zitvogel et al created a vector with an internal ribosome entry site (IRES) that could express more than one gene at a time. They succeeded in acquiring transfectants that could produce an adequate amount of bioactive IL-12 (133 ng/10⁶ cells/48 hr)²⁴. Although we transduced p35 and p40 genes into Renca cells separately in the present study, we were able to obtain transfectants which, like Zitvogel's, secreted sufficient amounts of IL-12. These excellent results might have been related to the simple structure of the MFG vector, which could have led to both a high efficacy of infection and a high expression of each gene product.

Jaffee et al reported that, in the absence of selection, they achieved a mean transduction efficacy of 60% in primary human tumor explants using the retroviral vector MFG in conjunction with short-time culture techniques, and they developed a safe autologous tumor vaccine for use in clinical trials. They also reported that the immunization potential was markedly more dependent on the number of vaccinating cells than on the percentage of purified cells⁹. Our efficacy of gene transfer into Renca cells was as high as Jaffee's efficacy of gene transfer into primary human explants. In fact, our admixed experiment showed no difference when Renca IL-12 was admixed with parental cells at various ratios. We therefore used Renca IL-12 as a tumor vaccine without cell selection, in the same manner as Jaffee.

The mechanisms underlying the antitumor activity of IL-12 are likely to be complex and have not been fully elucidated. In the present study, Renca IL-12 secreting sufficient IL-12 reduced the tumorigenicity in syngeneic BALB/c mice without any significant changes in the characteristics of Renca IL-12 cells compared with parental cells *in vitro*. This reduced tumorigenicity *in vivo* is probably mediated through the effects of IL-12 on the host immune system, because IL-12 did not directly inhibit the growth of malignant cells *in vitro*, and gene transfer did not induce the alteration of surface antigens, including MHC class I, MHC class II and B7-1 molecules.

In the present study, 9 of 21 animals that were rechallenged following regression of the initial tumor demonstrated long-term immunity. The same antitumor immune response has been reported in a murine sarcoma cell line, MCA 207, by Tahara et al²⁰. These authors provided a precise analysis of the anti-tumor mechanism and concluded that NK cells and IFN- γ play important roles in the development of early-phase antitumor responses, but that T cells, especially CD4+ and CD8+, play the major role in the subsequent events leading to long-term protective immunity of

parental cells. Because our present animal studies were conducted in a similar manner and gave similar results, we anticipated that a similar mechanism of antitumor immuno-responses would be operative in our animals.

However, 12 animals succumbed to the rechallenge in the present study. Nastala et al also found that a fraction of animals cured of tumors by IL-12 did not possess long-term immunity, suggesting that the antitumoral effect of IL-12 may also include non-T-cell-mediated events¹⁵. Voest et al have demonstrated that IL-12 exhibits antiangiogenic activities that can account for some antitumor activity²². On the other hand, Bramson et al considered that this observation indicated a wane of immunity over the long term because of the latency of residual tumor cells within the body. Thus, they considered that one possible way of maintaining long-term immunity is by boosting the host with irradiated tumor cells, periodically providing a regular source of tumor antigen⁹.

CD4+ T cells, CD8+ T cells, NK cells, macrophages or other cell types may participate in antitumor responses induced by IL-12. This provides a rationale for gene therapy using tumor cells genetically engineered to secrete IL-12 so that a specific T cell response can be obtained against nontransduced metastatic cells present at the time of vaccination. Our study demonstrated that Renca IL-12 could work as a tumor vaccine that induces eradication of parental Renca cells at a distant site. This phenomenon was thought to be led by the cell-mediated immune responses.

These results indicate that our system is very simple and effective. IL-12 gene therapy using the MFG vector system is likely to be a feasible strategy for the treatment of advanced cases of RCC in which other effective modalities are lacking, and should be further investigated as a novel strategy.

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REFERENCES

1. Berg, W.J., Bukowski, R., Thompson, J.A., Nemunaitis, J., Murphy, B., Ellerhorst, J. and Motzer, R.J. 1998. A randomized phase II trial of

- recombinant human interleukin-12 (IL-12) versus interferon alpha-2a (IFN) in advanced renal cell carcinoma (RCC). *Proc. ASCO*. **17**: 318a.
2. **Brunda, M.J., Luistro, L., Warriar, R.R., Wright, R.B., Hubbard, B.R., Murphy, M., Wolf, S.F. and Gately, M.K.** 1993. Antitumor and antimetastatic activity of Interleukine12 against murine tumors. *J. Exp. Med.* **178**: 1223–1230.
 3. **Bramson, J.L., Hitt, M., Addison, C.L., Muller, W.J., Gauldie, J. and Graham, F.L.** 1996. Direct intratumoral injection of an adenovirus expressing interleukin-12 induces regression and long-lasting immunity: That is associated with highly localized expression of Interleukin-12. *Hum. Gene Ther.* **7**: 1995–2002.
 4. **Coen, J.** 1995. IL-12 deaths: explanation and puzzle. *Science* **270**: 908.
 5. **Dranoff, G., Jaffe, E., Lazenby A., Golumbek, P., Levisky H., Brose, K., Jackson, V., Hamada, H., Pardoll, D. and Mulligan, R.** 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA*. **90**: 3539–3543.
 6. **Gately, M.K., Warriar, R.R., Honasoge, S., Carvajal, D.M., Sarmiento, U., Hubbard, B.R. and Murphy, M.** 1994. Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocytes activity and induces production of IFN- γ *in vivo*. *Int. Immunol.* **6**: 157–167.
 7. **Gubler, U., Chua, A.O., Schoenhaut, D.S., Dwyer, C.M., McComa, W., Motyka, R., Nabavi, N., Wolitzky, A.G., Quinn, P.M., Familletti, P.C. and Gately, M.K.** 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA*. **88**: 4143–4147.
 8. **Jaffe, E.M., Dranoff, G., Cohen, L.K., Hauda, M.K., Clift, S., Marshall, F.F., Mulligan, R.C. and Pardoll, D.M.** 1993. High efficacy gene transfer into primary human tumor explants without cell selection. *Cancer Res.* **53**: 2221–2226.
 9. **Kobayashi, M., Fitz, L., Ryan, M., Hewick, R.M., Clark, S.C., Chang, S., Loudon, R., Scherman, F., Perussia, B. and Trinchieri, G.** 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* **170**: 827–845.
 10. **Motzer, R.J., Rakhit, A., Schwartz, L.H., Olencki, T., Malone, T.M., Sandstrom, K., Nadeau, R., Parmar, H. and Bukowski, R.** 1998. Phase I trial of subcutaneous recombinant human Interleukin-12 in patients with advanced renal cell carcinoma. *Clinical Cancer Res.* **4**: 1183–1191.
 11. **Mulders, P., Figlin, R., deKernion, B.J., Wiltrout, R., Linehan, M., Parkinson, D., deWolf, W. and Belidegrum, A.** 1997. Renal cell carcinoma: recent progress and future directions. *Cancer Res.* **57**: 5189–5195.
 12. **Murphy, G.P. and Hrushesky, W.J.** 1973. A murine renal cell carcinoma. *J. Natl. Cancer Inst.* **50**: 1013–1025.
 13. **Nakamoto, T., Mitani, S., Usui, A., Ikemoto, H., Usui, T. and Nakano, H.** 1990. Surgical treatment of renal cell carcinoma I.: Results of curative nephrectomy for renal cell carcinoma without distant metastases. *Nishinohon J. Urol.* **52**: 701–704.
 14. **Nakamoto, T., Mitani, S., Usui, A., Ikemoto, H., Usui, T. and Nakano, H.** 1990. Surgical treatment of renal cell carcinoma II.: Results of adjunctive nephrectomy for renal cell carcinoma with distant metastases. *Nishinohon J. Urol.* **52**: 705–708.
 15. **Nastala, C.L., Edington, H., Storkus, W., Mckinney, T.G., Brunda, M., Gately, M.K., Wolf, S.F., Schreiber, R. and Lotze, M.T.** 1994. Recombinant IL-12 administration induces tumor regression in association with IFN- γ . *J. Immunol.* **153**: 1697–1706.
 16. **Robertson, M.J. and Ritz, J.** 1996 Interleukin-12: Basic biology and potential application in cancer treatment. *The Oncologist.* **1**: 93–102.
 17. **Sarmiento, U.M., Riley, J.H., Knaack, P.A., Lipman, J.M., Becker, J.M., Gately, M.K., Chizzonite, R. and Anderson, T.D.** 1994. Biologic effects of recombinant human interleukin-12 in squirrel monkeys (*Sciureus saimiri*). *Lab. Invest.* **71**: 862–873.
 18. **Stern, A.S., Podlaski, F.J., Hulmes, J.D., Pan, Y.C.E., Quinn, P.M., Wolitzky, A.G., Familletti, P.C., Stremlo, D.L., Truitt, T., Chizzonite, R. and Gately, M.K.** 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA*. **87**: 6808–6812.
 19. **Tahara, H., Zeh III, H.J., Storkus, W.J., Pap, I., Watkins, S.C., Gubler, U., Wolf, S.F., Robbins, P.D. and Lotze, M.T.** 1994. Fibroblasts genetically engineered to secrete Interleukin 12 can suppress tumor growth and induce antitumor immunity to a murine melanoma *in vivo*. *Cancer Res.* **54**: 182–189.
 20. **Tahara, H., Zitvogel, L., Storkus, W.J., Zeh III, H.J., McKinney, T.G., Schreiber, R.D., Gubler, U., Robbins, P.D. and Lotze, M.T.** 1995. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. *J. Immunol.* **154**: 6466–6474.
 21. **Tare, N.S., Bowen, S., Warriar, R.W., Carvajal, D.M., Benjamin, W.R., Riley, J.H., Anderson, T.D. and Gately, M.K.** 1995. Administration of recombinant interleukin-12 to mice suppresses hematopoiesis in bone marrow but enhances hematopoiesis in the spleen. *J. Interferon Cytokine Res.* **15**: 377–383.
 22. **Voest, E.E., Kenyan, B., O'Reilly, M.S., Truitt, G., D'Amato, R.J. and Folkman, J.** 1995. Inhibition of angiogenesis *in vivo* by Interleukin 12. *J. Natl. Cancer Inst.* **87**: 581–586.
 23. **Wirth, M.P.** 1993. Immunotherapy for metastatic renal cell carcinoma. *Urol. Clin. North Am.* **20**: 283–295.
 24. **Zitvogel, L., Tahara, H., Cai, Q., Storkus, W.J., Muller, G., Wolf, S.F., Gately, M., Robbins, P.D. and Lotze, M.T.** 1994. Construction and characterization of retroviral vectors expressing biologically active human interleukin-12. *Hum. Gene Ther.* **5**: 1493–1506.