Increase in the Capability of Interleukin 2 Gene-Transduced Renal Cell Carcinoma Cells to Induce Cytotoxic Lymphocytes

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Received for publication April 15, 1994

Summary: The immunogenicity of human cancer cells transfected with interleukin 2 (IL-2) gene, a potent vaccine candidate, has not yet been fully investigated. Human renal cell carcinoma (RCC) cells transduced with human IL-2 gene (RCC-IL-2) were investigated *in vitro* for the capability to induce lymphokine-activated killer (LAK) or cytotoxic T lymphocyte (CTL) activity in peripheral blood mononuclear cells (PBMC) or tumor-infiltrating lymphocyte (TIL). The RCC-IL-2 cells stimulated PBMC to demonstrate LAK activity, and also stimulated autologous TILs to proliferate and exhibit cytotoxicity relatively restricted to autologous tumor cells. In contrast, both parental RCC and RCC transduced with neomycin gene alone failed to induce these activities. These results indicate that RCC-IL-2 cells are more potent than the other RCC cells with regard to inducing cytotoxic lymphocytes against autologous tumor cells.

Key words: interleukin 2 gene — renal cell carcinoma — gene transduction — immunogenicity — cytotoxic T lymphocytes

Introduction

A large number of studies using animal models provide evidence that tumor cells transfected with cytokine genes are more immunogenic than control tumor cells (Tepper et al. 1989; Ferron et al. 1990; Blankenstein et al. 1991; Mullen et al. 1992). In contrast, few studies have investigated the immunogenicity of human cancer cells transfected with cytokine

genes (Gastl et al. 1992; Schendel et al. 1993; Uchiyama et al. 1993). RCC cells are one of the most appropriate tumor cells among various human cancer cells to evaluate immunogenicity; because RCC is partly sensitive to immunotherapy, but resistant to conventional chemo- or radiotherapy (Harris, 1983; Lockburn and DeVere, 1984; Rosenberg et al. 1987).

In this study the capability of RCC-IL-2 to induce cytotoxicity in PBMC or

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TILs was investigated. RCC-IL-2 cells stimulated autologous TILs to exhibit cytotoxicity that was relatively restricted to autologous tumor cells.

Materials and Methods

Tumor cells and gene transduction

A human RCC cell clone (RC30 #14) from a parental RC30 line (RCC-Pa cells) reported in a previous manuscript (Hayakawa et al. 1992) was used for IL-2 gene transduction. The other tumor cells were RC 7 and RC 100 tumor cell lines, RPM melanoma cells that were resistant to natural killer (NK) activity but susceptible to LAK activity, and K562 NK sensitive tumor cells (Hayakawa et al. 1992). The Xho 1 site or Not 1 site was attached to the 17th residue or the 532nd residue of human IL-2 cDNA (Taniguchi et al. 1983), respectively, which was ligated to pBCMGS Neo that had been digested by Xho 1 and Not 1 (Karasuyama et al. 1989). pBCMGS Neo IL-2 or pBCMGS Neo alone was transformed into competent cells (DH5 α) followed by bacterial growth, colony selection and large-scale plasmid preparation using the CIRCLEPREP KIT (BIO 101, Inc., La Jolla, CA). RC30 #14 cells growing in petri dishes (10 cm diameter) were washed three times with serum-free DMEM followed by the addition of 3 ml of 10 μ g/ml lipofectin (BRL, Gaithersburg, MD). The cells were incubated for 60 min at 37°C followed by the addition of 20 μ g pBCMGS Neo or pBCMGS Neo IL-2. The cells were incubated for 8 hs at 37°C followed by the addition of 3 ml DMEM supplemented with 20% FCS. Three days later, selection

by geneticin (GIBCO Lab. Grand Island, New York) began at a concentration of 400 mg/ml with a gradual increase up to 800 mg/ml. One month later, geneticin-resistant tumor cells were tested for their ability to produce IL-2. Only IL-2-producing tumor cells were used for cloning at 0.3 cells/well, as reported previously (Hayakawa et al. 1992).

Cytokine assays and RCC-IL-2 cells

2×10^5 tumor cells were incubated in 2 ml of culture medium in a 24-well culture plate at 37 °C for 24 hs. The supernatants were collected and cytocentrifuged, and the IL-2 activity in the cell-free supernatant was measured by both a bioassay using an IL-2-dependent NK8 clone as reported previously (Salmeron et al. 1992) and an IL-2 ELISA Kit (Genzyme, Cambridge, MA) (Hayakawa et al. 1991). Recombinant human IL-2 (Shionogi, Japan) (Hayakawa et al. 1992) with a specific activity of 1 pg= 10^{-2} units was used as a standard. Five tumor cell clones producing >10 units/ml (>1 ng/ml) IL-2 per 10^5 cells were established, and one clone RC30 #14-IL-2 producing clone #20 (termed RCC-IL-2) was used throughout these studies. An RCC cell clone (RC30 #14) transduced with the neomycin gene alone was also used as a control (RCC-Neo cells). These supernatants were also tested for their IL-4 and IL-6 activity using the IL-4 ELISA kit (Genzyme) and the IL-6 ELISA kit (R & D System, Minneapolis, MN), respectively. IL-4 activity was not detectable from any of the cells tested under these culture conditions, while 10 to 20 pg/ml IL-6 per 10^5 cells was produced by RCC-Pa, RCC-Neo or RCC-IL-2 cells.

Reverse transcriptase (RT)-polymerase chain reaction (PCR)

Total RNA was isolated from RCC-Pa or RCC-IL-2 cells using RNAzolTMB (Biotecx Lab. Inc., Houston, TX), and a cDNA preparation was performed using the SuperscriptTM preamplification system (BRL). The PCR primers used for this study were β -actin primers (sense primer: 5'-CTACAATGAGCTGCGTGTGG, antisense primer: 5'-CGGTGAGGATCTTCATGAGG) (Iijima et al. 1985), IL-2 primers (sense primer: 5'-ATGTACAGGATGCAACTCCTGTCTT, antisense primer: 5'-GTTAGTGTTGAGATGATGCTTTGAC) (Taniguchi et al. 1983), IL-4 primers (sense primer: 5'-TCGACACCTATTAATGGGTCTCACC, anti-

sense primer: 5'-ATTAAATATTCAGCTCGAACAACCTT) (Yokota et al. 1986) and IL-6 primers (sense primer: ATGAACTCCTTCTCCACAAGCGC, antisense primer: GAA-GAGCCCTCAGGCTGGACTG) (Hirano et al. 1986). PCR was performed using cDNA (0.4 μ g per assay), oligonucleotide primers, dNTPs, Taq DNA polymerase in a DNA thermal cycler (MJ Research, Inc., MA) for 35 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec). RT-PCR amplified mRNA was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. RCC-IL-2 cells expressed IL-2, IL-4 and IL-6 at the mRNA level, whereas RCC-Pa cells expressed only IL-4 and IL-6 (Fig. 1). RCC-IL-2 cells did not express either interferon ($\text{IFN}\gamma$) or tumor necrosis factor ($\text{TNF}\alpha$) at the mRNA level (data not shown).

Fig. 1. Cytokine mRNA expression in RCC-Pa or RCC-IL-2 cells. mRNA was isolated from 1×10^6 cells of RCC-Pa or RCC-IL-2 cells. Four-tenths μ g of mRNA was provided for the analysis of β -actin, IL-2, IL-4 or IL-6 expression.

Lymphocytes and mixed lymphocyte-tumor cell culture (MLTC)

PBMC from healthy volunteers or TILs from the RC30 patient were prepared as reported previously (Itoh et al. 1988). Briefly, the RCC tumor was minced with scissors followed by treatment with collagenase (2 mg/ml) and type IV DNase (0.4 mg/ml) (Sigma Chemical Co., Mo., USA) for 60 min at room temperature. Following digestion, the cells were applied on a Ficoll-Hypaque gradient to obtain viable cells. Freshly isolated TILs were cryopreserved at -130°C , and thawed on the morning of experiments and were provided for experiments as uncultured TILs. PBMC or TILs were incubated with irradiated (5,000 cGy) RCC-IL-2, parental RC30 #14 clone (RCC-Pa) or RC30 #14 clone transduced with pBCMGS Neo alone (RCC-Neo) at an effector to stim-

ulator ratio of 10:1 in the presence of 0, 2, 20 or 200 U/ml human recombinant IL-2 kindly provided by Shionogi Res. Lab. (Osaka, Japan) as reported previously (Hayakawa et al. 1992). Stimulation of TILs with tumor cells was repeated every 7 days.

Surface antigen analysis

TILs were incubated with FITC-conjugated anti-CD3, -CD4 or -CD8mAb (Becton Dickinson, CA, USA) for 30 min at 4 °C. After washing, the antigenic expression of these cells was analyzed by flow cytometry (EPICS-C, Coulter, FL, USA).

Cytotoxicity and proliferation assays

A 4-h ^{51}Cr -release assay was used to measure cytotoxicity (Itoh et al. 1988).

Cytotoxicity was also measured in the presence of 10 $\mu\text{g/ml}$ anti-CD3mAb or normal mouse IgG (Becton Dickinson). Proliferation was measured by the ^3H -TdR uptake assay and also by the enumeration of the fold increase of lymphocytes as compared to the original number of cells used for the culture (Itoh et al. 1988).

Results and Discussion

Effect of X-ray radiation on RCC-IL-2 cells

RCC-IL-2 cells were exposed to X-rays at various doses up to 20,000 cGy and their viability and capability to produce IL-2 were measured 24 hs after the irradiation. Non-irradiated RCC-IL-2 cells

TABLE 1.
Susceptibility of RCC-IL-2 cells to NK or LAK activity^a

Effector cells	Incubation Time (HR)	Specific Cytotoxicity (LU/10 ⁶) VS		
		RCC-Pa	RCC-Neo	RCC-IL-2
PBMC	4	<0.1	<0.1	1.0 ^b
PBMC	12	0.1	0.1	4.5 ^b
PBMC	24	1.0	0.4	10.0 ^b
IL-2 activated PBMC	4	67	90	250 ^b

a: PBMC from healthy donors (n=5) were used as NK cell sources. Those activated with 200 U/ml exogenous IL-2 for 3 days were used as LAK cell sources. Tumor cells were trypsinized, ^{51}Cr -labeled, washed and incubated for 18hs in wells of a 96-well flat microplate (5,000 cells/well). After washing, effector cells were added to the wells in triplicate at effector to target cell ratios of 40:1, 20:1, and 10:1. Spontaneous release did not exceed 20% of maximum release by a 4 h assay or 30% by 12- and 24-h assays. The values represent means (n=5) of lytic units (LU) per 10⁶ effector cells. One LU represents numbers of effector cells to lyse 30% of 10⁴ target cells.

b: p<0.01 as compared to the others (Student's two-tailed t-test).

(1×10^5 cells) produced 20 to 60 U/ml (2 to 6 ng/ml) IL-2 in the supernatants. Irradiated RCC-IL-2 cells (3,000 to 10,000 cGy) were generally viable (>70%) and they produced 10 to 45 U/ml IL-2 (1 to 4.5 ng/ml/ 10^5 cells). These results indicate that RCC-IL-2 cells were relatively resistant to X-ray radiation. Therefore, RCC cells were exposed to X rays at 5,000 cGy when used as stimulator cells in these experiments.

Susceptibility to NK or LAK activity

Freshly isolated PBMC or IL-2-activated PBMC were used to measure NK or LAK activity, respectively (Table 1). NK activity (LU/ 10^6 cells) against RCC-Pa, RCC-Neo, or RCC-IL-2 cells was respectively <0.1, <0.1 or 1 in a 4-h ^{51}Cr -release assay, 0.1, 0.1 or 4.5 in a 12-h

assay and 1, 0.4 or 10.0 in a 24-h assay (Table 1). LAK activity against RCC-IL-2 cells (250 LU) in a 4-h ^{51}Cr -release assay was three to four times higher than that against either RCC-Pa (67 LU) or RCC-Neo cells (90 LU) (Table 1). These results indicate that the transduction of the IL-2 gene to human RCC cells increased their susceptibility to NK or LAK activity. The increased susceptibility would be primarily due to IL-2 produced by the RCC-IL-2 because IL-2 is a potent NK or LAK cell activator (Itoh et al. 1985 a, b).

Capability to induce LAK activity

PBMC from healthy donors were incubated for four days with irradiated (5,000 cGy) RCC-IL-2 or other cell types in the presence or absence of exogenous IL-2 (20 U/ml) followed by measurements of

TABLE 2.
Capability of inducing LAK cells^a

Donor (PBMC)	Exogenous IL-2	Proliferation (cpm, $\times 10^{-3}$) and LAK activity (LU) (in parenthesis) in response to			
		medium	RCC-Pa	RCC-Neo	RCC-IL-2
1	0	2.9 $\pm$ 0.1 (0.2)	10.2 $\pm$ 3.9 (0.2)	4.6 $\pm$ 2.3 (<0.1)	31.3 $\pm$ 8.7 ^b (>500)
	20 U/ml	18.0 $\pm$ 5.4 (3.4)	23.7 $\pm$ 6.1 (3.4)	9.0 $\pm$ 2.0 (2.2)	30.2 $\pm$ 1.2 ^b (>500)
2	0	0.8 $\pm$ 0.3 (0.2)	0.3 $\pm$ 0.6 (0.2)	3.2 $\pm$ 1.4 (<0.1)	33.9 $\pm$ 5.1 ^b (>500)
	20 U/ml	9.2 $\pm$ 2.3 (8.3)	33.2 $\pm$ 9.2 (8.3)	11.7 $\pm$ 8.4 (7.4)	34.5 $\pm$ 7.6 ^b (>500)

a: PBMC from healthy donors were incubated for 4 days with medium alone, irradiated (5,000 cGy) RCC-Pa, RCC-Neo or RCC-IL-2 cells at an effector to stimulator ratio of 10: 1 in the presence or absence of 20 U/ml exogenous IL-2 in the culture. Experiments were performed in six different individuals with consistent results. Two cases are shown in the table. ^3H -TdR (0.5 μCi /well) was added to wells (10^5 PBMC/0.2 ml/well) of 96-well flat bottom microplates, 6 hs before harvesting. Proliferation of irradiated tumor cells (10^4 cells/ well) alone was <500 cpm. The values represent mean $\pm$ SD of cpm from triplicate determinations. To measure cytotoxicity, PBMC were harvested from wells (2×10^6 PBMC/well) of 24-well culture plates, washed and tested for cytotoxicity against RPM melanoma cells. LAK activity represents LU / 10^6 effector cells.

b: Significantly higher ($p < 0.05$) than the others (Student's two-tailed t-test).

their proliferation and LAK activity against RPM melanoma cells (Table 2). RCC-IL-2 cells induced both the highest proliferative response and the highest LAK activity in the absence of exogenous IL-2. Addition of 20 U/ml (Table 2) or 200 U/ml (data not shown) exogenous IL-2 failed to augment these responses. These results indicate that RCC-IL-2 cells by themselves were sufficient for LAK cell induction and could substitute for exogenous IL-2 under these short term culture conditions. These results could also be partially explained by the capability of RCC-IL-2 cells to produce IL-2, which in turn could induce LAK activity in PBMC (Itoh et al. 1985 *a, b*).

PBMC from healthy donors were stimulated with irradiated (5,000 cGy) RCC-Pa or RCC-IL-2 every 7 days for up to 5 weeks in the presence of 0, 2, 20 or 200 U/ml exogenous IL-2. The PBMC increased in number for up to 3 weeks in a culture with exogenous IL-2 alone or IL-2 plus RCC-Pa cells, but not with RCC-IL-2 cells even in the presence of exogenous IL-2 (Table 3). Cytotoxicity was tested only once at 3 days after the second stimulation (Table 3). The PBMC that were stimulated with RCC-Pa demonstrated higher cytotoxicity to RC30 cells and lower cytotoxicity to either RC7 or melanoma tumor cells (RPM). The PBMC cultured with IL-2 alone failed to lyse RC30 or RC7 cells, but demonstrated lower levels of lysis to RPM. Cytotoxicity in PBMC that were stimulated with RCC-IL-2 was not tested because of the limited numbers of the effector cells. These results indicate that RCC-IL-2 cells markedly inhibited the IL-2-dependent proliferation of PBMC during prolonged incubation periods.

Autologous PBMC for RCC-IL-2 cells were not available because the patient died of cancer several months after surgery. Allogeneic PBMC whose HLA-A locus was matched to an RC30 patient were also very difficult to obtain because of a unique HLA-A type (A-30 and Aw 34). Therefore, PBMC from six different healthy donors were used as effectors in these studies. In all individuals, the PBMC that were stimulated with RCC-IL-2 demonstrated significant DNA synthesis in a 4 day assay in association with LAK activity (Table 2). These PBMC, however, did not increase in number under all the culture conditions employed here, which included i) incubation with IL-2 alone (data not shown); ii) restimulation with RCC-IL-2 with or without various doses of exogenous IL-2 in the culture (Table 3); iii) restimulation with RCC-Pa or RCC-Neo with or without exogenous IL-2 (data not shown). RCC-Pa (Table 3) as well as RCC-Neo (data not shown) induced proliferation of allogeneic PBMC with a cytotoxicity that was relatively restricted to RC30 cells, suggesting that RC30 cells possessed the capability to induce allospecific CTLs by a standard MLTC. Either transduction of a pBCMGS Neo gene into RCC-Pa or the following selection with G418 might not be involved in this failure because RCC-Neo induced the proliferation of PBMC. The failure of RCC-IL-2 cells to induce the actual increase in number of PBMC might be largely due to the unresponsiveness to IL-2 in PBMC after the initial contact with RCC-IL-2. It will be important to test whether this is the case for autologous PBMC and to investigate the mechanisms involved in this failure.

TABLE 3.
Inability of RCC-IL-2 cells to induce proliferation of PBMC^a

Stimulators	Exo. IL-2 (U/ml)	Proliferation ($\times 10^6$ cells)				Cytotoxicity (LU/ 10^6 cells)		
		1	2	3	5 (wks)	RC-30	RC-7	RPM
None	0	0.5	0.9	0.2	<0.1	-(ND) ^b	—	—
	2	0.5	0.9	0.2	<0.1	—	—	—
	20	0.7	2.6	0.5	<0.1	0.1	0.1	1.7
	200	0.8	10.1	10.5	1.8	0.5	0.5	2.5
RCC-Pa	0	0.5	1.3	0.3	<0.1	—	—	—
	2	0.8	1.5	0.4	<0.1	—	—	—
	20	1.1	1.7	0.6	<0.1	33.3	0.5	5.6
	200	1.4	16.8	14.0	6.0	42.7	1.0	10.5
RCC-IL-2	0	0.4	0.5	0.5	<0.1	—	—	—
	2	0.4	1.0	0.4	<0.1	—	—	—
	20	0.4	0.3	0.5	<0.1	—	—	—
	200	0.5	0.7	0.7	<0.1	—	—	—

a: PBMC from six healthy donors were incubated with medium alone, irradiated (5,000 rad) RCC-Pa, RCC-Neo or RCC-IL-2 cells at an effector (1×10^6 PBMC/well) to stimulator cell ratio (1×10^5 tumor cells/well) of 10:1 in wells of 24 well culture plates in the presence of 0, 2, 20 or 200 U/ml exogenous IL-2 in the culture. PBMC were harvested, washed and stimulated with the same stimulators every 7 days for up to 42 days at an effector to stimulator cell ratio of 10:1. Experiments were performed in six different donors with consistent results and one representative group of data is shown in this table. Results with RCC-Pa cells were almost the same as those with RCC-Neo cells (data not shown).

b. ND: not determined.

Capability to induce CTL activity in TILs

Uncultured TILs from the RC30 patient were stimulated with irradiated (5,000 cGy) RCC-IL-2, RCC-Pa or RCC-Neo cells every 7 days for up to 4 weeks in the presence of 0, 2 or 20 U/ml exogenous IL-2 in the culture. Their proliferation and cytotoxicity against five different tumor cells, the autologous RC30 tumor cell line (RC30-14), allogeneic RC7 and RC 100 tumor cell lines, the melanoma cell line (RPM), and K562 tumor cells, were measured (Fig. 2).

TILs increased in number by 3.5 times 1 week after the stimulation with RCC-IL-2 cells regardless of the presence or absence of exogenous IL-2 in the culture (Fig. 2a). Exogenous IL-2 facilitated the proliferation of TILs at 2 weeks in the culture. In contrast, TILs failed to proliferate with any of the other culture conditions (data not shown). TILs stimulated by RCC-IL-2 cells in the presence of 2 U/ml exogenous IL-2 were tested for surface phenotypes and their cytotoxicity was measured 3 days after the third

Fig. 2. Capability of inducing CTL activity. Uncultured TILs (5×10^5 TIL/2 ml/well) were incubated with medium alone, irradiated (5,000 cGy) RCC-IL-2, RCC-Pa or RCC-Neo cells in the presence of 0, 2, 20 U/ml exogenous IL-2 for up to 4 wks. Stimulation with irradiated tumor cells was repeated every 7 days. a: TIL proliferation in culture with RCC-IL-2 cells was measured by the enumeration of viable numbers of TILs in the absence (○) or presence of 2 U/ml (●) or 20 U/ml (△) of exogenous IL-2. TIL did not proliferate with the other culture conditions and those results are not shown. b: TIL stimulated by RCC-IL-2 cells in the presence of 2 U/ml exogenous IL-2 was tested for cytotoxicity at day 17 of the culture against five different tumor cells by a 4-h ^{51}Cr -release assay at three different effector to target cell ratios. The values represent LU/10⁶ effector cells.

stimulation (17 days after the initiation of the culture). These activated TILs consisted of 98% CD3⁺, 42% CD4⁺ and 51% CD8⁺ T cells, and had the highest cytotoxicity to autologous RC30 tumor cells (HLA-A30, Aw34, B5<w4>, Bw57, Cw7, Cw8, DR2, DQw1), modest levels of lysis against allogeneic RC7 tumor cells (HLA-A2, -A3, B35<w6>, B44<w4>, C4, DR8, DRw13, DQw1, DQw4), no cytotoxicity to allogeneic RC100 tumor cells (HLA-A1, A-25, B6, Bw50, Cw4, DR8), a low level of lysis to RPM melanoma tumor cells or

K562 tumor cells (Fig. 2b). Addition of anti-CD3mAb, but not normal mouse IgG, to the cytotoxicity assay abrogated the cytotoxicity to autologous tumor cells (data not shown). TILs stimulated by RCC-IL-2 cells in the presence of 20 U/ml exogenous IL-2 demonstrated a similar pattern of cytotoxicity (35 LU vs RC30-14, 8 LU vs RC7, <0.1 LU vs RC100, 3 LU vs RPM and 6 LU vs K562). Thus RCC-IL-2 cells possessed the capability to induce CTL activity in TILs relatively specific to autologous tumor cells in the presence

of exogenous IL-2. HLA restriction involved in this cytotoxicity was not clear from this limited information partly because of the unique HLA-A antigens (A30 and Aw34) in the RC30 patient.

RCC-TILs usually proliferated well in culture with exogenous IL-2 alone, and displayed MHC-nonrestricted cytotoxicity (Belldegrun et al. 1988; Finke et al. 1990; Kim et al. 1990). IL-2 alone or IL-2 plus RCC-Pa or RCC-Neo cells failed to induce TIL proliferation in these studies. Low doses (2 and 20 U/ml) of exogenous IL-2 were used in these studies, and could be at least partly responsible for the failure, because higher doses (>200 U/ml) of exogenous IL-2 induced proliferation of TILs from the RC30 patient and these IL-2-activated TILs demonstrated MHC-nonrestricted cytotoxicity (data not shown). Frozen samples instead of freshly isolated TILs were used for the study, and could also be in part responsible for the failure, because IL-2-induced proliferation of TILs using frozen samples was more difficult to obtain than with fresh samples.

Both IL-4 and IL-6 expressed by RCC-IL-2 cells at mRNA levels (Fig. 1) would not be primarily involved in the capability of RCC-IL-2 cells to induce CTL activity since RCC-Pa cells as well as RCC-Neo cells (data not shown) also expressed both IL-4 and IL-6 at mRNA levels. More than half of RCCs expressed IL-6 gene, as judged by Northern blot analysis (Takenaka et al. 1991), and the serum level of IL-6 was detectable in 48 of 138 patients with metastatic RCC (Blay et al. 1992). RC30-14 clones also produced detectable levels of IL-6, regardless of IL-2 gene transduction. IL-4 expression at the mRNA level in RCC clones has not

been reported in the literature. The biological roles of IL-4 and/or IL-6 in the immunogenicity of human RCCs need to be fully investigated.

In summary, these results indicate that human RCC cells transduced with an IL-2 gene could be more potent to induce cytotoxic lymphocytes only in TILs.

Acknowledgments: The authors would like to thank Dr. H. Karasuyama (Tokyo Univ., Japan) for providing the pBCMGs Neo vector, and Mrs. T. Iwamaru for preparation of the manuscript. This work was supported in part by a Grant-in-Aid of the Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health and Welfare and the Ishibashi Research Fund, Japan.

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