Postoperative immuno-gene therapy of murine bladder tumor by *in vivo* administration of retroviruses expressing mouse interferon-γ

Ai-Li Shiau,[1] Chih-Yun Lin,[1] Tzong-Shin Tzai,[2] and Chao-Liang Wu[3]

[1]Departments of Microbiology,[2]Urology, and [3]Biochemistry, National Cheng Kung University Medical College, Tainan, Taiwan.

The murine MBT-2 bladder tumor model in syngeneic C_3H/HeN mice was used to investigate the feasibility of gene therapy based on the delivery of interferon-γ (IFN-γ) *in vivo* by retroviral vectors. We constructed a recombinant retroviral vector pRUFneo/IFN-γ, which was transfected into a retroviral packaging cell line ψCRE, to produce ψCRE/pRUFneo/IFN-γ cells. The expressions of the *neo* and IFN-γ genes were verified by reverse transcription-polymerase chain reaction and IFN-γ was detected in the culture supernatant from ψCRE/pRUFneo/IFN-γ cells. After receiving MBT-2 cells admixed with retroviral pRUFneoIFN-γ supernatant, C_3H/HeN mice exhibited lower tumor incidence, lower tumor mass, and higher survival rate, as well as higher antitumor responses compared to those injected with MBT-2 cells admixed with control retroviral supernatant. Moreover, the retroviral pRUFneoIFN-γ supernatant was able to suppress the growth of rechallenged tumors in postoperated mice. Although the IFN-γ protein secreted from ψCRE/pRUFneo/IFN-γ cells partly contributes to the antitumor effect of retroviral pRUFneoIFN-γ supernatant, the retroviruses carrying the IFN-γ gene transduced MBT-2 cells *in vivo,* which may result in enhancing local IFN-γ production from tumor cells. Because bladder is suitable for the intravesical instillation of therapeutic agents, *in vivo* administration of retroviral vectors encoding IFN-γ may be explored for the treatment of bladder cancer. **Cancer Gene Therapy (2001) 8, 73–81**

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Interferon-γ (IFN-γ) is a pleiotropic cytokine produced by activated T lymphocytes and natural killer (NK) cells, which exerts various biological functions. It plays important roles in the induction of cytolytic activity in cytotoxic T lymphocytes and lymphokine-activated killer (LAK) cells. IFN-γ also induces major histocompatibility complex class I and II expressions in target tissues, primes macrophages for tumor killing, and enhances NK cytotoxicity.[1] IFN-γ is also an antiangiogenic factor *via* the induction of angiogenesis inhibitory chemokines, IP-10[2] and Mig[3] and may up-regulate Fas[4] and caspase I expressions,[5] thus leading to cell apoptosis. Moreover, IFN-γ affects cell cycle and growth-factor-induced cell proliferation *via* the activation of STAT1.[6] IFN-γ has been shown to induce strong antitumor responses, which may be attributable to its combined immunomodulatory, antiangiogenic, and antiproliferative activities.

The most common form of bladder cancer is found to be superficial transitional cell carcinoma, which accounts for 75% to 80% of cases, whereas the invasive type with poor prognosis contains 20% of cases. One of the biological characteristics of superficial bladder cancer is its frequent recurrence after surgical resection, which may become invasive.[7] However, when combined with chemotherapy, its recurrence rate decreased to 30% to 40% in comparison to 60% to 80% with surgery alone. Moreover, intravesical instillation with bacillus Calmette-Guerin (BCG) after surgery may further decrease the recurrence rate. Although BCG is highly effective, approximately 30% of patients with carcinoma *in situ* or rapidly recurring papillary tumors will not respond to treatment. Therefore, another approach to BCG using a number of cytokines, such as interferon-α-2b, interleukin-2 (IL-2), and IFN-γ, either singly or in combination has been employed for immunotherapy of bladder cancer.[8–11]

Because IFN-γ has been used to transduce tumor cells *ex vivo* or *in vivo* for tumor therapy in various animal models,[12–16] we exploited the murine MBT-2 bladder tumor model to investigate the feasibility of gene therapy based on the delivery of IFN-γ *in vivo* by retroviral vectors. In this report, we showed that retroviruses encoding mouse IFN-γ may suppress MBT-2 tumor growth in syngeneic C_3H/HeN mice, which is concomitant to the increase of cytotoxic activities in the mice. Moreover, the incidence of tumor outgrowth was inhibited in the postoperative tumor-rechallenge study. Although the IFN-γ protein secreted from the packaging cells partly contributes to the antitumor effect of retroviruses carrying the IFN-γ gene, the retro-

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Address correspondence and reprint requests to Dr. Chao-Liang Wu, Department of Biochemistry, National Cheng Kung University Medical College, 1 Dashiue Road, Tainan 701, Taiwan.
E-mail address: wumolbio@mail.ncku.edu.tw

viruses transduced MBT-2 cells *in vivo*, as demonstrated by PCR analysis, resulting in enhancing local IFN-γ production from tumor cells. In conclusion, gene therapy by *in vivo* retrovirus-mediated IFN-γ gene transfer may be an effective approach in the treatment of bladder cancer. Because bladder is suitable for intravesical instillation of therapeutic agents, the complication of recurrence after surgical resection in superficial transitional cell carcinoma of bladder may be also approached by exploring the treatment of *in vivo* administration of retroviruses encoding IFN-γ in combination with surgery. Alternatively, other viral vector systems such as adenoviral or adeno-associated viral vectors may also be tested in the postoperative bladder tumor model.

MATERIALS AND METHODS

Construction of retroviral vectors

A 0.7-kb DNA fragment containing the mouse IFN-γ cDNA was excised from the plasmid pms10 (ATCC)[17] by digestion with *Dpn*I and *Eco*RI, and cloned into the *Sma*I/*Eco*RI sites of pBluescriptIISK(+) to generate pBluescript-IFN-γ. The plasmid pRUFneo is a retroviral vector derived from the genome of murine Moloney leukemia virus (MuMoLV) and contains the bacterial neomycin resistance (*neo*) gene that is used as a selectable marker. The retroviral vector pRUFneoIFN-γ was constructed by inserting the *Bse*RI–*Hin*dIII fragment that encompasses the coding region of the mouse IFN-γ into the *Hpa*I/*Hin*dIII sites of pRUFneo.[18]

Cells and mice

The ψCRE ecotropic retroviral packaging cell line, containing the packaging signal for MuMoLV, was cultured in complete medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 2 mM L-glutamine, and 50 μg/mL gentamicin.[19] MBT-2 murine bladder tumor, YAC-1 mouse T-cell lymphoma, and P815 mastocytoma cells were grown in RPMI1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/mL gentamicin. Female C_3H/HeNCrj mice at the age of 4 to 6 weeks were obtained from the Animal Center of the National Cheng Kung University Medical College and maintained under isothermal conditions with regular photoperiods.

Generation of packaging clones and detection of mRNAs specifying neo and IFN-γ by reverse transcription-polymerase chain reaction (RT-PCR) in transfected cells

Retroviral vector constructs were converted into corresponding viruses by transfection of 40 μg of pRUFneoIFN-γ or pRUFneo into ψCRE cells by electroporation. At 24 hours following transfection the cells were selected in complete medium containing 0.75 mg/mL G418 (Gibco BRL, Rockville, MD). Drug-resistant clones were isolated from the pRUFneoIFN-γ–transfected and pRUFneo-transfected cells, which were designated ψCRE/pRUFneoIFN-γ and ψCRE/pRUFneo cells, respectively, and expanded to cell lines.

Cell clones that stably transfected with retroviral vectors were harvested, and the total cellular RNA was isolated using RNA isolation kit (Maxim Biotech, San Francisco, CA). The RNA samples (5 μg) were transcribed into cDNA in a 50-μL RT reaction mixture containing 50 mM Tris–HCl (pH 8.3), 30 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM each of deoxynucleotide triphosphate, 5 U MuMoLV RT (NEB, Beverly, MA), and 2 μM each of 18mer and 10mer oligo(dT) primer. The reaction mix was incubated at 37°C for 1 hour. The cDNA products (100 ng) were then amplified by PCR to detect the transcripts of neomycin phosphotransferase, IFN-γ, and β-actin, as a quantitative control, in the presence of 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, 0.4 mM each of deoxynucleotide triphosphate, 1 U Vent DNA polymerase (NEB), and 1 μM specific primers. PCR conditions were 35 cycles of 1 minute at 94°C, 45 seconds at 60°C, and 1 minute at 72°C. The specific primers used for mouse IFN-γ were 5′-TGA ACG CTA CAC ACT GCA TCT TGG and 5′-CGA CTC CTT TTC CGC TTC CTG AG, for *neo* were 5′-GCT GTC CTC GAC GTT GTC AC and 5′-CTC TTC GTC CAG ATC ATC CTG, as well as for β-actin were 5′-TGG AAT CCT GTG GCA TCC ATG AAA C and 5′-TAA AAC GCA GCT CAG TAA CAG TCC G.

Determination of retrovirus titers and detection of transgene in viral RNA

ψCRE/pRUFneoIFN-γ or ψCRE/pRUFneo cells were plated at 2×10^5 cells per well with 1 mL of complete medium in 24-well culture dishes, and the viral supernatant harvested 24 hours later. At the same time, 5×10^3 NIH3T3 cells per well were seeded onto 24-well dishes. After 1 day, serial dilutions of 0.2 mL of the 24-hour cell-free viral supernatant were added to the NIH3T3 cells for a 2-hour adsorption period followed by feeding with 1 mL of fresh medium containing 8 μg/mL of polybrene. Two days after, the cells were refed with complete medium supplemented with G418 (0.75 mg/mL) and cultured for 2 weeks. Cells were then fixed and stained with crystal violet and the G418-resistant clones were counted. Retrovirus titers in the culture supernatant of packaging cells measured by the NIH3T3 assay are expressed as colony-forming units per milliliter (cfu/ml).

Viral supernatants were collected from ψCRE/pRUFneoIFN-γ or ψCRE/pRUFneo cells (2.4×10^6) cultured in 10 mL of complete medium in 10-cm dishes after 48 hours. Viruses were concentrated by low-speed centrifugation.[20] After removing cell debris, the supernatants were filtered through a 0.22-μm filter and centrifuged at $2900\times g$ for 20 hours at 4°C. The supernatant was carefully removed, and the invisible pellet was resuspended in 0.5 mL of serum-free Dulbecco's modified Eagle's medium and was used for purifying viral RNA with the viral RNA miniprep system (Viogene, Sunnyvale, CA) according to the manufacturer's instructions. Following RT with oligo(dT) primers, cDNA was

synthesized. The IFN-γ gene product was detected by RT-PCR with the aforementioned procedures.

Measurement of IFN-γ secretion

Supernatants from ψCRE/pRUFneoIFN-γ, ψCRE/pRUFneo, or parental ψCRE cells (8×10^5) cultured in 1 mL of complete medium in 6-cm dishes were collected after 24 hours and assayed for mouse IFN-γ using the Duoset ELISA kit (Genzyme, Cambridge, MA). The IFN-γ titers are expressed as picograms secreted from 10^6 cells during a 24-hour culture period.

Generation of recombinant retroviral stocks

The clones of ψCRE/pRUFneoIFN-γ or ψCRE/pRUFneo cells with similar *neo* gene expression, determined by RT-PCR analysis of the viral supernatants, were plated at 2.4×10^6 cells per 10-cm culture dish with 3.5 mL of complete medium and subsequently cultured at 37°C. The culture supernatant was collected 24 hours later, filtered through a 0.22 μm-filter, and used immediately for animal studies.

Experimental protocols for animal studies

To assess the antitumor efficacy of IFN-γ immuno-gene therapy for primary tumors, groups of 10 or 13 mice were injected subcutaneously (s.c.) with MBT-2 cells (5×10^5 or 10^5) along with the retroviral supernatant (0.2 or 0.4 mL) collected from the 24-hour conditioned medium of 2.4×10^6 of either ψCRE/pRUFneoIFN-γ or ψCRE/pRUFneo cells grown in 3.5 mL of culture medium. Groups of mice that were injected with MBT-2 cells (5×10^5) admixed with recombinant IFN-γ (400 pg), culture medium, or PBS were included in some experiments.

A postoperative tumor-rechallenge model was also established to mimic a status of postoperative residual tumors due to inadequate tumor resections or micrometastases.[21] Mice were injected s.c. on the right flank with 10^6 MBT-2 cells on day 0 and palpable tumors were resected on day 18. Mice that had undergone tumor resections were placed in groups of 15 and rechallenged s.c. on the left flank using 2×10^6 MBT-2 cells admixed with retroviral supernatant (0.4 mL) from the ψCRE/pRUFneo or the ψCRE/pRUFneoIFN-γ culture medium as described, or with recombinant mouse IFN-γ (390 pg) at a level equivalent to the concentration of IFN-γ present in the ψCRE/pRUFneoIFN-γ culture medium.

All mice were monitored for tumor growth and survival for 60 to 90 days after tumor injection. Palpable tumors were measured twice a week in two perpendicular axes with a tissue caliper and the tumor volume was calculated as: $(\text{length of tumor})\times(\text{width of tumor})^2\times0.45$.

Detection of the neo sequence in mouse tumors by PCR

Genomic DNA from tumors removed at day 12 from mice that had received MBT-2 cells along with the ψCRE/pRUFneoIFN-γ or ψCRE/pRUFneo retroviral supernatant was prepared using the DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The genomic DNA was subjected to analysis of the *neo* sequence in tumor tissues by PCR. The primers used were 5′-GGT GGA GAG GCT ATT CGG CTA TGA and 5′-ATC CTG ATC GAC AAG ACC GGC TTC, which amplify a 440-bp fragment of the *neo* gene. PCR conditions were 40 cycles of 1 minute at 95°C, 1.5 minutes at 69°C, and 1.5 minutes at 72°C.

Cytotoxicity assays in vitro

The NK activity was assessed using freshly isolated mouse spleen cells as effector cells and YAC-1 cells as target cells. For the LAK assay, splenocytes were cultured in the presence of 2000 U/mL of human recombinant IL-2 (R&D Systems, Minneapolis, MN) for 4 days followed by incubation with ^{51}Cr-labeled MBT-2 or P815 target cells. To perform the ^{51}Cr-release assay, target cells (5×10^6) were incubated in 1 mL RPMI1640 complete medium with 200

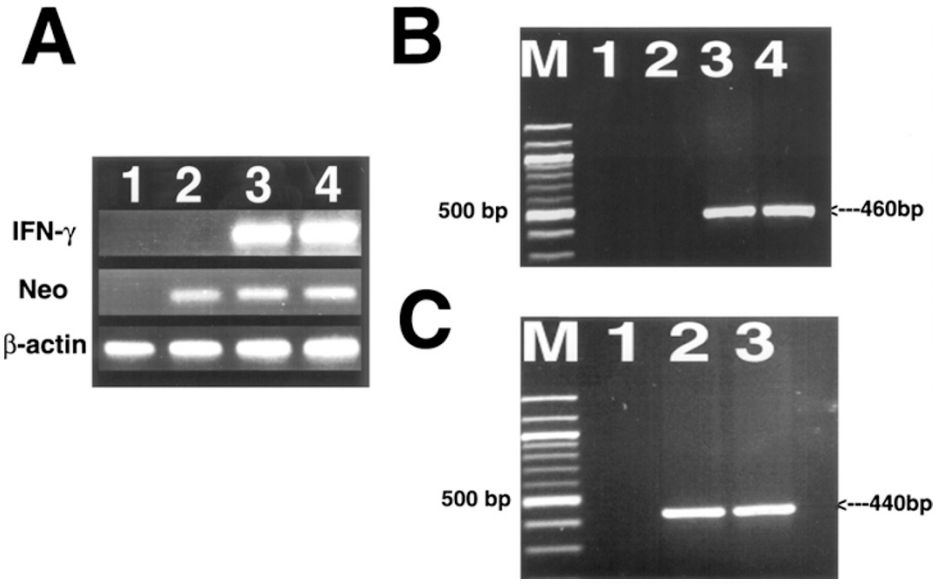

Figure 1. (A) Detection of IFN-γ, neo, and β-actin transcripts by RT-PCR in ψCRE, ψCRE/pRUFneo, or ψCRE/pRUFneoIFN-γ cells. Lane 1, ψCRE; lane 2, ψCRE/pRUFneo; lane 3, ψCRE/pRUFneoIFN-γ no. 7; lane 4, ψCRE/pRUFneoIFN-γ no. 8. **(B)** Detection of IFN-γ transgene by RT-PCR in viral RNA purified from culture supernatants of ψCRE, ψCRE/pRUFneo, or ψCRE/pRUFneoIFN-γ cells. M, 100-bp marker; lane 1, ψCRE; lane 2, ψCRE/pRUFneo; lane 3, ψCRE/pRUFneoIFN-γ no. 7; lane 4, ψCRE/PRUFneoIFN-γ no. 8. **(C)** Detection of neo transgene by PCR in tumors at day 12 from mice injected with MBT-2 cells admixed with ψCRE/pRUFneoIFN-γ supernatant, ψCRE/pRUFneo supernatant, or PBS. Lane 1, PBS; lane 2, ψCRE/pRUFneo; lane 3, ψCRE/pRUFneoIFN-γ.

μCi of $Na^{51}CrO_4$ (Amersham, Aylesbury, UK) at 37°C for 90 minutes, washed three times, resuspended at 1×10^5 cells/mL in RPMI1640 medium containing 5×10^{-5} M of β-mercaptoethanol, and aliquots of 100 μL were added with 100 μL of various concentrations of mouse spleen cells in the same medium to achieve the desired effector to target ratios. For spontaneous and total ^{51}Cr release, 100 μL of culture medium and 0.2% sodium dodecyl sulfate were substituted for effector cells, respectively. The cultures were incubated for 4 hours (NK assay) or 7 hours (LAK assay) at 37°C followed by centrifuging microtiter plates at $200\times g$ for 10 minutes, and removing 100 μL of supernatant from each well for isotope counting on a gamma counter (LKB 1282 Compu Gamma; Pharmacia, Finland). Data were presented as: % specific cytotoxicity = 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)].

Statistical analysis

Tumor volumes were compared using an unpaired, two-tailed Student's *t* test. The survival analysis was performed using the Kaplan-Meier survival curve and the log-rank test. Any *P* value less than .05 is regarded as statistically significant.

RESULTS

Construction of recombinant retroviruses and characterization of retroviral packaging cells transfected with mouse IFN-γ gene

Retroviral vectors were converted into the corresponding viruses by transfection of pRUFneoIFN-γ or pRUFneo into ψCRE cells, followed by selection with G418 to isolate the drug-resistant clones. Cell clones stably transfected with pRUFneoIFN-γ or pRUFneo were obtained. RNA was isolated from some of the clones for RT-PCR analysis with specific primers for *neo* and mouse IFN-γ genes. In ψCRE/pRUFneoIFN-γ clones, mRNAs specifying the *neo* and IFN-γ gene products were detectable, whereas only the *neo* signal was detected in ψCRE/pRUFneo clones, and neither were present in parental ψCRE cells (Fig 1A). The titers of the IFN-γ released from various ψCRE/pRUFneoIFN-γ cell clones ranged from 987 to 2396 pg from 10^6 cells during 24-hour culture, whereas none were detectable in the supernatant from either ψCRE/pRUFneo or parental ψ CRE cells as determined by ELISA (Table 1). The retroviral titer in the ψCRE/pRUFneo supernatant determined by counting G418-resistant colonies of transduced NIH3T3 cells was 10^5 cfu/mL. Based on this titration method, the retroviral titers in the ψCRE/pRUFneoIFN-γ supernatant could not be determined because no G418-resistant colonies were isolated. Nevertheless, the analysis of viral RNA purified from ψCRE/pRUFneoIFN-γ supernatant indicated that the IFN-γ transgene was present in retroviral RNA (Fig 1B). The IFN-γ sequence of the PCR product was also confirmed by DNA sequencing (not shown). To simplify the production of the same amount of retroviruses for *in vivo* experiments, we chose one of each cell clone from

Table 1. IFN-γ Secretion From ψCRE Cells Transduced With pRUFneoIFN-γ or pRUFneo Vector

Clone	IFN-γ (pg/24 hours/10^6 cells)
ψCRE	0
ψCRE/pRUFneo	0
ψCRE/pRUFneoIFN-γ no.7	1721
ψCRE/pRUFneoIFN-γ no.8	2396
ψCRE/pRUFneoIFN-γ no.10	1641
ψCRE/pRUFneoIFN-γ no.32	1334
ψCRE/pRUFneoIFN-γ no.33	987

The culture supernatants from various cell clones (8×10^5) in 6-cm plates containing 1 ml culture medium were collected after a 24-hour incubation period and assayed for mouse IFN-γ by ELISA.

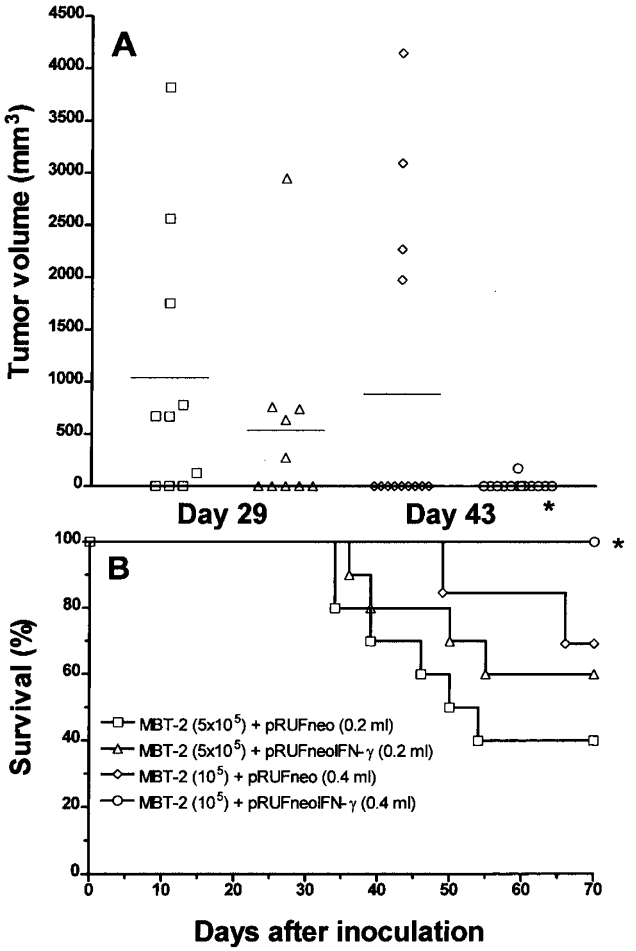

Figure 2. Effect of retroviral pRUFneoIFN-γ supernatant on tumor growth and survival in C_3H/HeN mice. Groups of 10 or 13 mice were injected s.c. with MBT-2 cells (5×10^5 or 10^5) admixed with retroviral pRUFneoIFN-γ or pRUFneo supernatant. **(A)** The tumor volumes at days 29 and 43 postinoculation in each mouse are shown and the horizontal bar denotes the mean tumor volume in each group. **(B)** The survival curves at day 70 in each group are shown. An asterisk indicates that the tumor volume and survival time are significantly different compared with the control by Student's *t* test ($P<.05$) and log-rank test ($P<.05$), respectively.

ψCRE/pRUFneoIFN-γ and ψCRE/pRUFneo cells that had similar *neo* expression levels determined by RT-PCR for producing recombinant retroviruses in animal studies.

Tumors transduced with pRUFneoIFN-γ or pRUFneo retroviral vectors contained the neo gene

Day-12 tumors excised from mice injected with MBT-2 admixed with retroviral supernatant were used to prepare genomic DNA for PCR analysis. Figure 1C shows that a 440-bp fragment of the *neo* sequence was present in the tumor tissues from mice that had been injected with either pRUFneoIFN-γ or pRUFneo retroviral supernatant, but not from the control mice. This result suggests that *in vivo* retroviral delivery leads to transduction of the tumors with the transgene, which may result in the antitumor effect observed in mice treated with the pRUFneoIFN-γ retroviral supernatant.

Retroviral supernatant from ψCRE/pRUFneoIFN-γ cells inhibited tumor growth in mice inoculated with MBT-2 cells

Tumors grew in 70% of mice inoculated s.c. with 5×10^5 MBT-2 cells admixed with 0.2 mL of retroviral pRUFneo supernatant, and in 31% of mice inoculated with 10^5 MBT-2 cells admixed with 0.4 mL of the same supernatant. Conversely, those injected with MBT-2 cells admixed with retroviral pRUFneoIFN-γ supernatant exhibited lower tumor size (Fig 2A), lower tumor incidence (Table 2), and longer survival time (Fig 2B) compared with their control counterpart. In animals co-injected with 10^5 MBT-2 cells and 0.4 mL of retroviral pRUFneoIFN-γ supernatant, tumor mass became palpable at 41 days postinoculation, in comparison to 25 days in mice treated with the control retroviral supernatant. Furthermore, the retroviral pRUFneoIFN-γ supernatant significantly decreased the tumor volume and boosted the survival time in these mice. In a separate experiment shown in Figure 3, no significant differences were seen in the tumor incidence, tumor growth, and survival among three control groups of mice, which concomitantly received 5×10^5 MBT-2 cells with pRUFneo retroviruses, culture medium, or PBS, suggesting that retroviruses *per se* had no effect on tumor growth. In contrast, treatment of retroviral pRUFneoIFN-γ supernatant in mice resulted in a significant reduction in tumor size ($P = .027$) and tumor incidence (Fig 3A). Moreover, the treatment also resulted in significant prolongation of survival of the mice compared with control mice that were treated with pRUFneo supernatant ($P = .018$) (Fig 3B). Because the ψCRE/pRUFneoIFN-γ supernatant contained 390 pg of IFN-γ protein, we tested if the same amount of

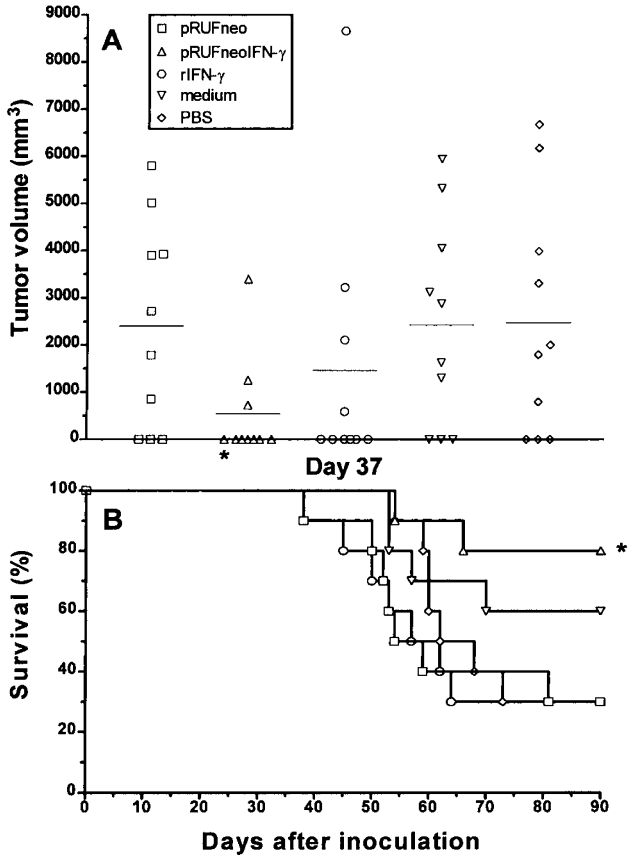

Figure 3. Effect of retroviral pRUFneoIFN-γ supernatant or IFN-γ protein on tumor growth in C_3H/HeN mice. Groups of 10 mice were injected s.c. with MBT-2 cells (5×10^5) admixed with pRUFneo supernatant, pRUFneoIFN-γ supernatant, recombinant IFN-γ (400 pg), culture medium, or PBS. **(A)** The tumor volume at day 37 postinoculation in each mouse is shown and the horizontal bar denotes the mean tumor volume in each group. An asterisk indicates that the tumor volume is significantly different compared with pRUFneo-treated group by Student's *t* test ($P < .05$). **(B)** The survival curves at day 90 in each group are shown.

Table 2. Incidence of Tumor Growth in C_3H/HeN Mice Inoculated With MBT-2 Cells Admixed With the Culture Supernatant From ψCRE/pRUFneo or ψCRE/pRUFneoIFN-γ Cells

Cell numbers of MBT-2 inoculated	Treatment	Amount (mL)	Tumor incidence
5×10^5	ψCRE/pRUFneo	0.2	7/10 (70%)
5×10^5	ψCRE/pRUFneoIFN-γ	0.2	5/10 (50%)
1×10^5	ψCRE/pRUFneo	0.4	4/13 (31%)
1×10^5	ψCRE/pRUFneoIFN-γ	0.4	1/13 (8%)

On day 0, MBT-2 cells admixed with the culture supernatant of ψCRE/pRUFneo or ψCRE/pRUFneoIFN-γ no.7 were injected s.c. into groups of 6-week-old C_3H/HeN mice. The incidence rate of tumor growth at 70 days postinjection is represented as: (numbers of tumor-bearing mice)/(total numbers in each group).

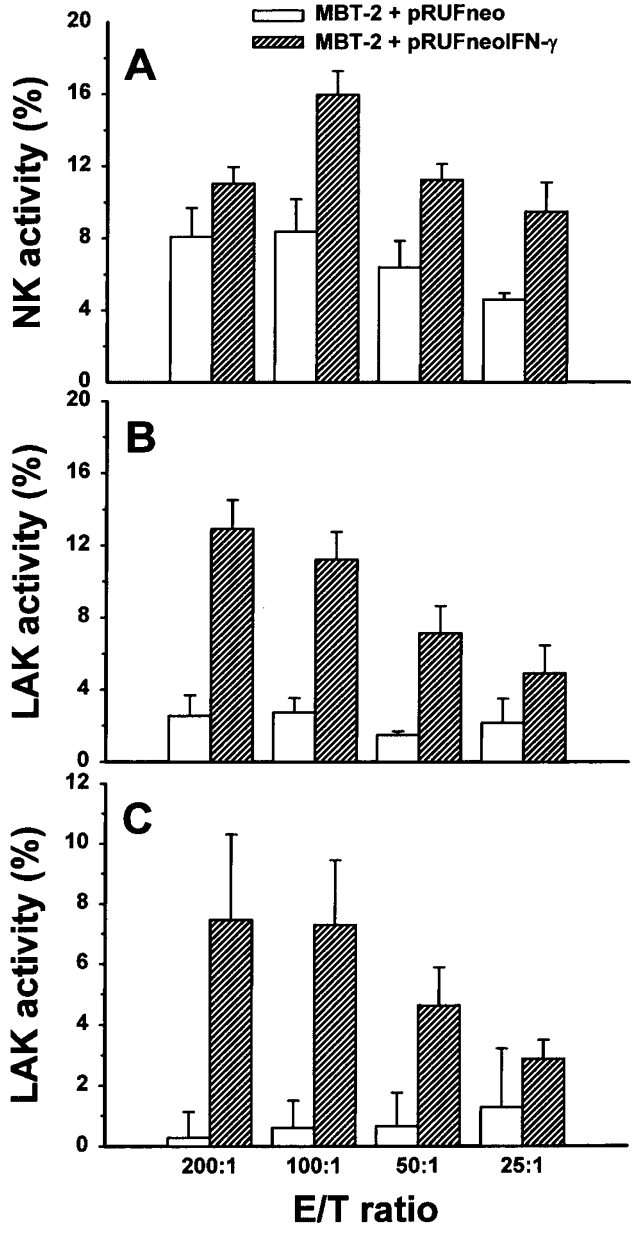

Figure 4. Induction of the activities of **(A)** NK, **(B)** LAK against MBT-2 targets, and **(C)** LAK against P815 targets in mice injected with MBT-2 cells admixed with retroviral pRUFneo IFN-γ supernatant. C_3H/HeN mice were injected s.c. with MBT-2 cells along with retroviral pRUFneoIFN-γ or pRUFneo supernatant. Eighteen days later, pooled spleens from four mice were aseptically removed and the cytotoxicity by the splenocytes was determined by the ^{51}Cr-release assay. E/T ratio denotes effector cell to target cell ratio.

recombinant IFN-γ protein exerted similar antitumor efficacy. Although the mean tumor volume was smaller and the survival was increased in the IFN-γ–treated group compared with the pRUFneo-treated group, the differences were not statistically significant ($P = .409$ in tumor size and $P = .143$ in survival). Taken together, coadministration of retroviruses encoding IFN-γ suppressed tumor growth and prolonged survival time in mice particularly after inoculation of smaller amounts of tumor cells and larger amounts of viral supernatant. Moreover, IFN-γ *per se* present within the ψCRE/pRUFneoIFN-γ supernatant may only contribute in part to inhibiting tumor growth.

Induction of cytotoxic activities in mice inoculated with MBT-2 cells admixed with the retroviral supernatant from ψCRE/pRUFneoIFN-γ cells

To observe whether the inhibitory effects of retroviruses encoding IFN-γ on MBT-2 tumor growth are correlated with the induction of cytotoxic effector cells, spleen cells were isolated 18 days after treatment and NK and LAK activities were determined using the standard cytotoxicity assay. Mice treated with retroviruses encoding IFN-γ

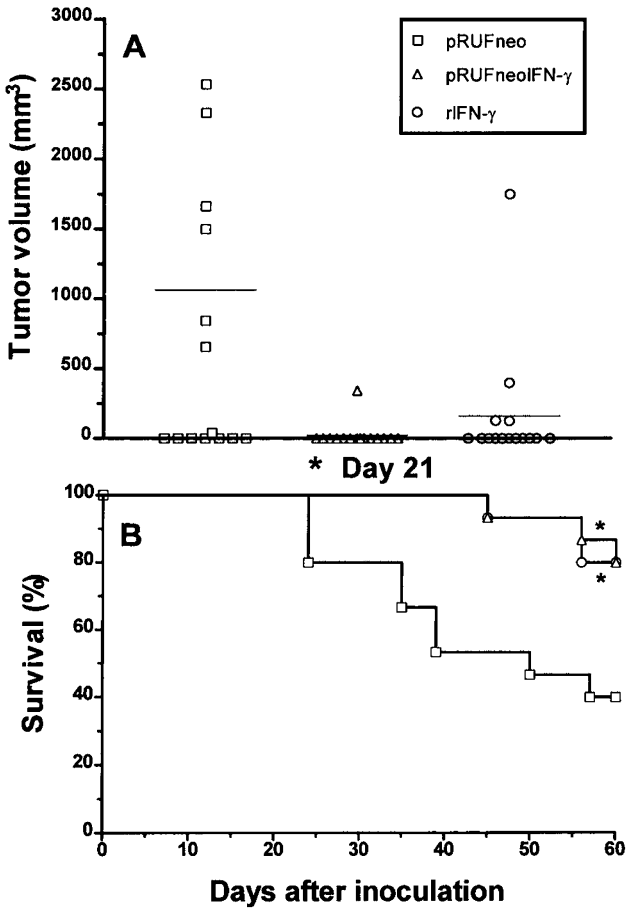

Figure 5. Effect of retroviral pRUFneoIFN-γ supernatant on the outgrowth of rechallenged tumor cells in postoperated C_3H/HeN mice. Mice were inoculated s.c. on the right flank with 10^6 MBT-2 cells on day 0 and palpable tumors were resected on day 18. Mice that had undergone tumor resections were placed in groups of 15 and rechallenged s.c. on the left flank using 2×10^6 MBT-2 cells admixed with retroviral pRUFneoIFN-γ or pRUFneo supernatant (0.4 mL), or with recombinant mouse IFN-γ (390 pg). **(A)** The tumor volume in each mouse is shown and the horizontal bar denotes the mean tumor volume in each group. **(B)** The survival curves at day 60 in each group are shown. An asterisk indicates that the tumor volume and survival time are significantly different compared with the control by Student's *t* test ($P < .05$) and log-rank test ($P < .05$), respectively.

induced higher levels of NK (Fig 4A) and LAK (Fig 4B *and* C) activities compared with those treated with control retroviruses. In particular, the LAK activity was more enhanced than the NK activity.

Retroviral supernatant from ψCRE/pRUFneoIFN-γ cells inhibited tumor outgrowth in the postoperative model for bladder tumor

In the postoperative tumor-rechallenge model to mimic a status of postoperative residual tumors, Figure 5A *and* B shows that the retroviral supernatant encoding IFN-γ significantly inhibited the growth of rechallenged tumors ($P = .027$) and increased the survival ($P = .014$) in mice that had undergone tumor resections followed by rechallenge of MBT-2 cells. The IFN-γ protein also significantly prolonged the survival time ($P = .014$) (Fig 5B), but inhibited tumor growth to a substantially lesser degree ($P = .06$) (Fig 5A) in mice that were injected with MBT-2 cells and 390 pg of the recombinant IFN-γ protein. Taken together, the retroviral supernatant encoding IFN-γ has a higher antitumor effect than recombinant IFN-γ protein in the postoperative tumor-rechallenge model.

DISCUSSION

The administration of IFN-γ to experimental animals has been shown to elicit antitumor activities.[22] However, these activities require repeated administration or continuous perfusion of IFN-γ to sustain due to its short half-life *in vivo*.[23] Therefore, using IFN-γ gene–modified cells as *in vivo* IFN-γ producers seemed to be an alternative approach to the limitations of systemic administrations. It has been demonstrated that the transduction of tumor cells with IFN-γ gene abrogated tumorigenicity through the activation of host-mediated antitumor responses.[13,24] By immunizing IFN-γ–modified tumor cells s.c., a localized cellular response was induced, which was accompanied by an effective systemic reaction and a long-lasting memory response.[12,25] Various mechanisms for IFN-γ–induced antitumor immunity have been suggested. In the case of Restifo et al,[26] an enhanced antigen presentation was considered due to the increase in major histocompatibility complex class I antigen when murine methylcholanthrene-induced sarcomas were transduced with IFN-γ gene, whereas Porgador et al[27] suggested cytotoxic T lymphocyte induction as the primary mechanism. In the bladder cancer model, Connor et al[28] reported that although IFN-γ was more effective than IL-2 in reducing the tumorigenicity of MBT-2 cells, irradiated IFN-γ–secreting MBT-2 cells exerted only a limited therapeutic effect in the orthotopic tumor model. However, bladder tumor regression and long-term immunological memory were observed in mice treated with irradiated IL-2–secreting or GM-CSF–secreting MBT-2 cells.[29]

In the work described here, recombinant retroviruses carrying the mouse IFN-γ gene were used directly for *in vivo* gene therapy of the murine MBT-2 bladder tumor. Our results show that retroviral pRUFneoIFN-γ supernatant coadministered with MBT-2 cells inhibited tumor growth in mice with efficacy of the supernatant being dose-dependent (Table 2). The proportion of mice that developed tumors decreased as the ratio of recombinant viruses to tumor cells increased. In mice treated with the retroviral pRUFneoIFN-γ supernatant, any tumors that did develop were much smaller than those treated with the control retroviral pRUFneo supernatant (Fig 2A). Furthermore, the survival rate reached 90% to 100% in the pRUFneoIFN-γ group compared with 60% to 65% in the control group at 60 to 70 days postinoculation. Mice treated with retroviral pRUFneoIFN-γ supernatant induced higher levels of NK and LAK activities compared to those treated with control retroviruses (Fig 4), and the enhanced induction of these cytotoxic activities may contribute in part to the antitumor responses found. Because IFN-γ may play an important role in the induction of cytolytic LAK activities (Fig 4B *and* C), immuno-gene therapy using retroviral supernatant carrying the IFN-γ gene may be an effective therapeutic approach for the treatment of bladder cancer.

Lauret et al[30] reported that failure to produce retroviral vectors expressing murine interferon-β (IFN-β) by murine ψ-2 packaging cells was due to their very high sensitivity to the autocrine murine IFN-β encoded by the vector. According to their data, production of retroviral vector was reduced by about four $\log_{10}$ units compared with the control clones transformed by vectors coding for inactive murine IFN-β. Furthermore, low autocrine IFN-β synthesis or exposure of cells to exogenous IFN-β may block retroviral infection by preventing virus from getting inside the cells.[31] In this study, the titer of the recombinant retroviruses expressing IFN-γ was not obtainable by determining G418-resistant colonies of transfected mouse NIH3T3 fibroblasts, whereas that of retroviruses containing the control vector pRUFneo could be titrated, which reached 10^5 cfu/mL. The anti–IFN-γ antibody treatment was unable to rescue the pRUFneoIFN-γ retroviral titer determined in NIH3T3 cells (data not shown). We speculate that the failure of determining the titer of retroviruses expressing IFN-γ may result from the inhibitory effect of murine IFN-γ on the production of retroviral particles by the ψCRE packaging cell line and the infection of NIH3T3 cells. Because the IFN-γ expression was detected in the pRUFneoIFN-γ supernatant by RT-PCR analysis, the retroviral particles were indeed produced from the ψCRE/pRUFneoIFN-γ cells. Moreover, IFN-γ protein was also secreted from the packaging cells. Thus, the ψCRE/pRUFneoIFN-γ supernatant that we used for animal injection was composed of both retroviruses carrying the IFN-γ gene and the IFN-γ *per se*. Our result shows that combination of immunotherapy and gene therapy was superior to IFN-γ immunotherapy alone in the post-operative animal model (Fig 5A and B).

Surgery alone usually fails to treat cancer at moderate to advanced stages due to local recurrence and/or distant metastasis. Therefore, surgery is often combined with chemotherapy or immunotherapy in cancer treatment. Theoretically, the perioperative period is optimal for adjunct anticancer therapy, because at this critical stage the tumor

burden is at its minimum and the tumor-induced immunosuppression is temporarily terminated. In addition, a certain degree of host-specific antitumor immunity may have been induced before tumor resection. We have previously used the postoperative tumor-rechallenge model mimicking a status of postoperative residual tumors to investigate the effect of immunotherapy in combination with surgery on the antitumor immunity of a tumor-bearing host.[21,32] The murine MBT-2 tumor is immunogenic in the syngeneic C_3H/He mice, because relatively low tumor incidence (66.7%) was found in C_3H/He mice injected subcutaneously with 10^6 of the tumor cells, whereas immunologically deprived animals were more susceptible to the challenge of inoculated tumor cells.[21] It has also been suggested that the immunogenicity of MBT-2 tumor is partially mediated by tumor suppression by autocrine IFN-β.[33] Therefore, in this study the effect of the postoperative treatment of recombinant retroviruses expressing IFN-γ or IFN-γ protein *per se* on host antitumor immunity was examined by s.c. reimplantation of a large amount of tumor cells (2×10^6) to mice, 24 hours after surgical removal of the primary tumor initially implanted with 10^6 tumor cells. In this work, we demonstrate that tumor outgrowth from rechallenged tumor cells can be markedly suppressed in mice treated with retroviral pRUFneoIFN-γ supernatant when administered ipsilateral to the side of rechallenge site. Recombinant IFN-γ was shown to be moderately effective in preventing tumor outgrowth within the same model. Taken together, IFN-γ may be a promising agent for the prevention of local recurrence and/or metastasis in patients with bladder cancer.

In the work described here, the antitumor effects of the retroviral pRUFneoIFN-γ supernatant were attributed to both the recombinant retroviruses carrying the IFN-γ gene and the IFN-γ protein *per se*, because the retroviral pRUFneoIFN-γ supernatant is more efficacious than IFN-γ protein in reducing tumor growth and promoting survival time in mice bearing bladder tumor. Practically, a local inoculation of either the retroviruses encoding IFN-γ and/or the IFN-γ protein in the vicinity of the tumor region may be beneficial in inhibiting tumor growth. In view of the previously reported low efficiency of retroviral gene transfer *in vivo*, our result showing the efficacy of retroviral pRUFneoIFN-γ supernatant in suppressing the growth of murine bladder tumors was interesting. In our study, pRUFneoIFN-γ or control retroviral supernatant was mixed with MBT-2 cells for s.c. injection into mice, which may have been sufficient for the retroviruses to transduce MBT-2 cells *in vivo*, because retroviruses have an inherent advantage in targeting rapidly dividing tumor cell populations. Indeed, PCR analysis of the *neo* signal in the tumors infected *in vivo* by recombinant retroviruses also suggested that *in vivo* gene transfer by the retroviral vectors have occurred (Fig 1C). Furthermore, the inhibitory effect of the IFN-γ protein present in the retroviral supernatant on the transduction efficiency of retroviruses may be minimal *in vivo* compared with that occurring *in vitro*, as the IFN-γ protein within the viral supernatant may be diluted out *in vivo*. Other reports also support this view. The intratracheal administration of retroviral antisense K-*ras* supernatant[34] or retroviral wild-type p53 supernatant[35] was able to prevent the growth of human lung cancer cells implanted orthotopically in *nu/nu* mice. Vile et al[36] also demonstrated that multiple systemic administrations of retroviral supernatant carrying the herpes simplex thymidine kinase gene were effective in rendering recently established lung metastases sensitive to ganciclovir. Furthermore, the integration of provirus has been observed in some metastasis-bearing lungs and spleens by PCR analysis.

In conclusion, the direct administration of retroviral supernatant carrying mouse IFN-γ gene suppressed tumor growth and prolonged survival time in mice with primary s.c. MBT-2 tumors. Interestingly, this retroviral supernatant also inhibited the growth of rechallenged tumor cells in postoperated mice, which mimics the condition of presenting residual tumors encountered in patients with cancer due to inadequate tumor resections or micrometastases. Therefore, this study provides some implications for the prevention and therapy of bladder cancer.

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