Melanoma Res 13 : 2003 (in press)

Growth inhibition of subcutaneous mouse melanoma and induction of natural killer cells by liposome-mediated interferon-beta gene therapy

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Running Title: IFN-beta gene therapy to syngeneic mouse melanoma

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Summary

We investigated the antitumor effect and mechanism of action of cationic liposome-mediated murine IFN-beta gene therapy in mouse B16F1 melanoma cells in vitro and in vivo. Murine IFN-beta gene transfer by cationic liposome resulted in a substantial growth inhibition of B16F1 melanoma cells in culture when compared to PBS- or recombinant murine IFN-beta treatment, and lacZ control gene transfer. A use of video-enhanced contrast-differential interference contrast (VEC-DIC) microscopy revealed that liposome containing the murine IFN-beta gene [lip(pSV2muIFN-beta)], but not recombinant murine IFN-beta, induced dramatic morphologic changes including bleb formation, shrinkage of cells, nuclear condensation and "ballooning" that characterize apoptosis in approximately 30 % of the cells treated. Intratumoral administration of lip(pSV2muIFN-beta) resulted in a 5.5-fold reduction in the mean volume of subcutaneous melanoma in syngeneic mice 15 days after the treatment and eradicated the tumor in 18 % of the mice treated. Immunocytochemical analysis demonstrated that larger number of natural killer cells infiltrated the tumor following lip(pSV2muIFN-beta)-treatment than in the controls. In vivo depletion of NK cells using the anti-asialoGM1 antibody reduced the efficacy of lip(pSV2muIFN-beta) treatment. Taken together, our data demonstrated that cationic liposome-mediated IFN-beta gene therapy could be effective against melanoma by inducing direct cell death and by stimulating NK cells.

Key words: Gene therapy, liposome, interferon-beta, melanoma, apoptosis, and natural killer cells

Introduction

Interferon (IFN)-beta, a type I IFN, possesses multiple factors to inhibit tumor growth both directly by suppressing cell replication (cytostatic effect)[1] and inducing apoptosis (cytocidal effect)[2-4] and indirectly by activating tumoricidal properties of natural killer (NK) cells[5-9] and macrophages[10,11], by stimulating specific cytotoxic T lymphocyte's (CTL) immune response[12-14] and by suppressing tumor angiogenesis.[2, 15, 16] However clinical use of IFN-beta has proven to be minimally useful as an adjuvant treatment for most solid tumors in combination with other anticancer agents and/or irradiation.[17, 18] On the other hand, we have developed an effective IFN-beta gene therapy using cationic liposomes as a gene transfer system. In our previous studies using human, mouse, and rat glioma cell lines, we found that cationic liposome-mediated IFN-beta gene transfer both in vitro and in vivo induced a cytocidal, but not cytostatic, response to cell lines resistant to exogenous IFN-beta.[19-21] In addition, we demonstrated that activation of NK cells plays a role in inducing the antitumor effect by liposome-mediated human IFN-beta gene transfer in nude mice harboring intracranial human glioma xenograft.[22] More recently, we reported that in a syngeneic intracranial mouse glioma model, activation of tumor-specific CTL response participates in tumor regression as well as a direct cytocidal effect of the IFN-beta gene.[23, 24] A region of chromosome 9, surrounding the IFN-beta locus and the IFN-alpha gene cluster on 9p13-p22, has been shown to be frequently deleted or rearranged in a number of human cancers, including glioma, leukemia, non-small-cell lung carcinoma, and melanoma.[25-29] This finding suggested the possibility that our IFN-beta gene therapy

system may be effective in treating not only glioma but also a number of tumors. In this study we evaluated the antitumor effect and immune responses resulting from liposome-mediated IFN-beta gene transfer in a syngeneic (subcutaneous) mouse melanoma.

Materials and methods

Cell line

The murine melanoma cell line, B16F1, was cultured in Dulebecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM nonessential amino acids, 5mM L-glutamine, streptomycin (100 μg/ml) and penicillin (100 units/ml).

Mice

The animals used were 8-12 week-old female B6 mice (SLC, Shizuoka, Japan). They were kept under pathogen-free conditions in the animal facility of Nagoya University. Animal experiments were performed according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals prepared by the office of the Prime Minister of Japan.

Antibodies

The following monoclonal antibodies (mAbs) and polyclonal antibody (pAb) were purchased: rat mAbs to CD4 (KT174; Serotec, Oxford, UK), CD8 (YTS 105.18; Serotec),

F4/80 (CI:A3-1; Serotec) and murine IFN-beta (7F-D3; Yamasa Shoyu, Chiba, Japan) a mouse mAb to NK1.1 (PK136, biotinylated; PharMingen, San Diego, CA), and a rabbit pAb to asialoGM1 (Wako Chemical, Osaka, Japan)

Plasmids

We used plasmids designated pSV2muIFN-beta, pCH110 and pEGFP-C1. The first plasmid contains the SV40 early promoter and the murine IFN-beta coding sequence. The second was constructed by fusion of the E. coli *lacZ* (β-galactosidase) gene to the SV40 early promoter obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The third, containing the cytomegalovirus promoter-immediate early enhancer and the coding sequence of enhanced green fluorescent protein (EGFP), was purchased from Clontech (Palo Alto, CA).

Preparation of liposomes containing a plasmid

Cationic liposomes were prepared with N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), dilauroyl phosphatidylcholine (DLPC), and dioleoyl phosphatidylethanolamine (DOPE) as described in our previous paper.[21] We prepared three kinds of liposomes containing a plasmid: a liposome containing 20 μg pSV2muIFN-beta [lip(pSV2muIFN-beta)], a liposome containing 20 μg pCH110 [lip(pCH110)] and a liposome containing 20 μg pEGFP-C1

[lip(pEGFP-C1)]. The total volume of liposomes containing a plasmid was adjusted to 0.5 ml with phosphate-bufferd saline (PBS) (2 μmol lipid and 40 μg DNA/ml).

Efficiency of liposome-mediated gene transduction

In a 35mm x 10mm petri dish (351008, Becton Dickinson), 8 x 10^4 B16F1 cells were inoculated with 2 ml of medium. At 24 hr after inoculation, 15 μl of lip(pEGFP-C1) was added to the medium and the incubation was continued for another 48 hr. The cells expressing EGFP were observed using a fluorescent microscope. Transduction efficiency was evaluated by count of the number of $EGFP^+$ cells in four high power fields.

VEC-DIC

To study the morphologic changes of B16F1 cells treated with lip(pSV2mulFN-beta), we employed video-enhanced contrast-differential interference contrast (VEC-DIC) microscopy. The cells were examined with an inverted Nomarski microscope equipped with a x63 DIC objective lens and a x25 insertion lens (Axiovert 135; Zeiss, Germany). The cover slip was fixed with petroleum jelly to a square hole cut in the center of a plastic slide. The optimal image was detected with a 0.5-inch CCD camera (ZVS3C57DEC; Sony, Tokyo, Japan), and image contrast was enhanced with a high-speed digital image processor. The processed image was observed on a slightly overscanned video monitor and simultaneously video recorded on a laser disc or in S-VHS format recorder.

Cultured cell growth

Growth inhibition of B16F1 cells transfected with lip(pSV2muIFN-beta) was evaluated using a hemocytometer, counting numbers of viable cells excluding trypan blue. As controls, PBS, recombinant murine IFN-beta (final concentration at 100 IU/ml) and lip(pCH110) were used. Aliquots of 2 x 10^4 B16F1 cells were inoculated in each well of a Falcon plate (No. 3047) with 1.5 ml of medium and incubated at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. At 24 hr after inoculation, 15 μl of lip(pSV2muIFN-beta) or controls were added to the medium and incubation was continued for additional 2 or 5 days and the number of viable cells was counted.

Tumor implantation and treatment

To generate a tumor, a donor B6 mouse was subcutaneously implanted in the right back with 50 μl of a single cell suspension containing 1 x 10^6 B16F1 cells. Tumor volume was calculated using a formula; $ab^2/2$ (a; long axis, b; short axis). When the tumor reached a size of 3 cm^3, recipient B6 mice were subcutaneously transplanted with 8-10 mm^3 of solid tumor from the donor mouse in the hair-shaved right back. Treatment was started on Day 4, when tumors reached a size of 60-110 mm^3. Animals were once injected intratumorally with 75 μl of either PBS (n=9), murine IFN-beta (1000 IU) (n=5), lip(pCH110) (n=5), or lip(pSV2muIFN-beta) (n=11). Tumor sizes were measured every other day for a period of 21 days.

Immunocytochemistry

Tumors were cut with a cryostat microtome at a thickness of 10 μm and mounted on silanized glass slides. After fixing sections in acetone for 15 min at 4° C and blocking with PBS containing 1% skim milk, staining was performed using the following antibodies: anti-CD4 Ab, anti-CD8 Ab, anti-NK1.1 Ab (biotinylated) and anti-F4/80 Ab. The sections were incubated with the primary antibodies for 1 h at room temperature, and then were immersed in 0.3% H_2O_2 in absolute methanol for 15 min to block endogenous peroxidase. All sections but those stained with anti-NK1.1 Ab were incubated with biotinylated secondary antibodies for 30 min at room temperature. Diaminodenzidine was used as a chromogen.

NK cell depletion

Mice were injected intraperitoneally with 25 μl anti-asialoGM1 antibody to deplete NK cells 1 day before and every 7 days after liposomal injection. This treatment was found to completely deplete NK cell populations for the entire duration of the study as determined by fluorescence-activated cell sorting (FACS) analysis (data not shown). NK cell depletion did not affect parent tumor growth (data not shown).

Western blot analysis

Animals were injected intratumorally with 75 μl of lip(pSV2muIFN-beta) or lip(pCH110). After animals were sacrificed 2 days later, the subcutaneous tumors were dissected and

homogenized in 0.05 M tris-HCl buffer (pH 7.0) containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol. Supernatants were collected after removing debris by centrifugation, and these were heated at 99° C for 2 min with Laemmli sample buffer. Total protein (20 μg) was subjected to 4-15 % SDS polyacrlamide gel elecrophoresis and transferred onto a polyvinylidene difluoride membrane and blotted. The blot was washed and incubated with rat anti-murine IFN-beta antibody (1:1000 dilution) at room temperature for 2 hr followed by horseradish peroxidase-labeled goat antibody to rat IgG (1:20000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 hr. The bands were visualized by ECL.

Results

Transduction efficiency of liposome containing a plasmid DNA (Fig. 1)

To evaluate an efficiency of cationic liposome-mediated gene transduction, we observed $EGFP^+$ B16F1 cells 48 hr after transfection with lip(pEGFP-C1). The mean percentage of $EGFP^+$ cells in four high power fields was $30\pm2\%$.

Cytocidal, but not cytostatic, effect of lip(pSV2mulFN-beta) on B16F1 cells

We examined whether cationic liposome-mediated murine IFN-beta gene transfer suppressed the growth of B16F1 cells in vitro. We counted the number of viable cells in culture 2 and 5 days after addition of PBS, recombinant murine IFN-beta, lip(pCH110), or

lip(pSV2muIFN-beta). As shown in Fig. 2, lip(pSV2muIFN-beta) inhibited the cell growth significantly compared to PBS, murine IFN-beta and lip(pCH110). Although murine IFN-beta suppressed the cell growth, the growth inhibitory effect was significantly less than that of lip(pSV2muIFN-beta). To examine whether the growth inhibitory effect of lip(pSV2muIFN-beta) was cytocidal or cytostatic, we observed sequential morphologic changes in B16F1 cells transfected with lip(pSV2muIFN-beta) under the VEC-DIC microscope that allowed us to pursue changes of cellular membrane and intracellular structural elements in a particular living cell over time. The attainable resolution by this approach is the order of 1 μm. As shown in Fig. 3, approximately 30% of B16F1 cells treated with lip(pSV2muIFN-beta) became to develop an abnormally bright nucleoli and a blebbing cellular membrane by 24 hr. By 48 hr these morphologically altered cells shrank and had large membrane outpouchings that we term "ballooning." The sequence is typical for apoptosis.

Liposome-mediated expression of murine IFN-beta in subcutaneous melanoma

Subcutaneous B16F1 tumors were treated with lip(pSV2muIFN-beta) or lip(pCH110). Two days later, the tumors were resected and protein was extracted and analyzed by Western blotting. A 19 KD murine IFN-beta immunoreactive band was present in the lysate of lip(pSV2muIFN-beta)-treated tumor, but not in the lip(pCH110)-treated tumor (Fig. 4).

In vivo treatment of B16F1 subcutaneous tumor with lip(pSV2muIFN-beta)

Treatment was started on day 4, when tumors reached a size of 10-12 mm^3. Animals were injected intratumorally with 75 μl of either PBS, murine IFN-beta (1000 IU), lip(pCH110), or lip(pSV2muIFN-beta). As shown in Fig. 5, tumor growth in the lip(pSV2muIFN-beta)-treated animals was significantly suppressed. Complete eradication of subcutaneous tumors occurred in 18 % (two of eleven) of mice receiving lip(pSV2muIFN-beta). In contrast, treatment of mice with murine IFN-beta or lip(pCH110) did not affect tumor growth and tumors reached 8 cm^3 in 21 days, similar to PBS-treated animals. We performed the above experiments three times and we obtained the same results (data not shown).

Induction of NK cells by lip(pSV2muIFN-beta) treatment

To examine whether liposome-mediated murine IFN-beta expression induced activation of cellular immunity as well as a direct cell death, we immunocytochemically studied infiltration of $CD4^+$and $CD8^+$ T lymphocytes, NK cells and macrophages within the subcutaneous tumors 7 days after treatment. Immunohistchemistry using an antibody to NK cells revealed that the lip(pSV2muIFN-beta)-treated tumors contained a higher number of infiltrating NK cells than that found in PBS-, murine IFN-beta-, and lip(pCH110)-treated tumors (Fig. 6A). In contrast, there was no significant difference in number of $CD4^+$ and $CD8^+$ T lymphocytes and macrophages among these treatment groups (Fig. 6B). Next, to determine whether NK cells participate in the tumor growth inhibition by

lip(pSV2mulFN-beta), we depleted NK cell populations by administrating anti-asialo GM1 antibody. NK cell depletion resulted in a significant decrease in the efficacy of lip(pSV2mulFN-beta) treatment. Tumor growth in NK-depleted animals that received lip(pSV2mulFN-beta) treatment was similar to that found in PBS-treated animals (Fig. 7).

Discussion

The principal finding of the experiments reported in this communication is that liposome-mediated murine IFN-beta gene transfer showed a significant growth inhibitory effect on mouse B16F1 melanoma in vitro and in vivo and that the antitumor mechanism appeared to include a direct cell death, probably involving apoptosis, and an activation of NK cells. While recombinant murine IFN-beta slowed the growth of B16F1 cells in culture, we did not observe cell death under a continuous VEC-DIC microscopy and therefore murine IFN-beta appeared to induce suppression of cell replication (cytostatic effect). On the other hand, liposome-mediated murine IFN-beta gene transfer had a greater growth inhibitory effect in vitro than recombinant murine IFN-beta, and VEC-DIC microscopy revealed that lip(pSV2mulFN-beta) induced dramatic morphologic changes in 30% of the treated cells including nuclear condensation, bleb formation, shrinkage of cells and "ballooning" as we term. These changes characterize apoptosis. Although lip(pSV2mulFN-beta) failed to reduce total number of initially inoculated B16F1 cells, this is probably because liposome-mediated gene transfer was not efficient enough as shown in Fig. 1. Since the percentage of $EGFP^+$ cells following transfection with lip(pEGFP-C1)

was similar to that of dying cells after transfection with lip(pSV2muIFN-beta) that were observed under the VEC-DIC microscope (Figs. 1 and 3), we speculate that the cells transduced with the murine IFN-beta gene underwent cell death (autocrine) and the expressed murine IFN-beta suppressed proliferation of neighboring cells (paracrine).

How does lip(pSV2muIFN-beta), but not recombinant murine IFN-beta, induce cell death? The mechanisms responsible are not known but may include apoptosis, specific alteration or effects on autocrine and paracrine loops by downregulating production of growth factors or growth factor receptors or by influencing their signaling pathways. Autocrine IFN secretion, rather than exogenous IFN, has been found to regulate growth of melanoma cells[30] as well as glioma cells.[31] Our previous studies for over a decade demonstrated that liposome-mediated human IFN-beta gene transfer elicited a much stronger growth inhibitory effect on a number of human glioma cell lines than exogenous human IFN-beta.[20, 21] A 40-fold increase in concentration of human IFN-beta was necessary to obtain an inhibitory effect similar to that seen in human IFN-beta gene transfer. Addition of a neutralizing antibody to human IFN-beta did not cancel the growth inhibition of human glioma cells transfected with human IFN-beta gene.[20] Therefore these results suggested that antitumor mechanisms regulated by liposome-mediated IFN-beta gene transfer might be distinct from those by exogenous IFN-beta.

In the present study in vivo, we demonstrated that treatment of immunocompetent B6 mice with lip(pSV2muIFN-beta), but not control agents including murine IFN-beta, inhibited the growth of subcutaneous B16F1 melanoma significantly and recruited NK cells in inhibiting

the tumor growth. An NK cell depletion study suggested that NK cells may contribute to the antitumor effect in vivo. These results are consistent with our previous study showing that activation of NK cells was important in the efficacy of IFN-beta gene transfer in an experimental glioma that was established in nude mice brains.[22] It was not surprising that larger number of $CD4^+$ and $CD8^+$ T lymphocytes did not infiltrate in the tumor following the lip(pSV2muIFN-beta) treatment when compared to controls because FACS analysis revealed that treatment of cultured B16F1 cells with murine IFN-beta or even lip(pSV2muIFN-beta) failed to upregulate expression of major histocompatibility complex (MHC) class I and II antigens on this poorly immunogenic tumor cell line (data not shown). It is well known that type I-IFNs stimulate NK cell activity against tumorigenicity and viral infection.[7-9] Although NK cells that can kill sensitive targets can be isolated from naïve individuals, this activity is increased by between 20- and 100-fold when NK cells are exposed to type I-IFNs. It has been demonstrated that IFN-beta can reverse NK cell activity that was suppressed by antitumor agents[6] and the transcription factor Interferon regulatory factor-1 (IRF-1) is essential for NK cell function.[5] Why intratumoral injection of murine IFN-beta did not affect the growth of subcutaneous B16F1 tumors is unclear but one explanation for discrepancy between the in vivo effects of murine IFN-beta and lip(pSV2muIFN-beta) may be pharmacokinetic reasons.[32] The half life of IFN-beta in the circulation of patients is less than 5 min and serum concentration of IFN-beta drops to less than 2 units/ml 1 hr after intramuscular or subcutaneous injection with 6×10^6 units.[33] This concentration is far below that required to activate NK cells. Another possibility is

that tumor cells may become fragile by cytocidal effect of lip(pSV2muIFN-beta) and more susceptible to attack by NK cells.

Further studies will be required to define the antitumor mechanisms through liposome-mediated IFN-beta gene transfer. However, the results reported show that it is effective in eradication of melanoma as well as glioma, and thus supports the possible utility of IFN-beta gene therapy with cationic liposome in the treatment of patients with melanoma.

Acknowledgements

This work was supported in part by the Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science, Tokyo.

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Figure legends

Fig. 1. Enhanced green fluorescent protein (EGFP)-expressing mouse B16F1 melanoma cells after transfection with liposome containing an EGFP plasmid (pEGFP-C1). EGFP was expressed in approximately 30% of the cells treated. Fluorescent (left) and confocal (right) photomicrographs were taken from the same field. Original magnification, x2,000.

Fig. 2. Growth inhibition of cultured mouse B16F1 melanoma cells transfected with liposome containing the murine IFN-beta gene. Twenty four hours after aliquots of 2×10^4 B16F1 cells were inoculated in each well, 15 μl of phosphate-buffered saline (PBS), recombinant murine IFN-beta (muIFN-beta), liposome containing the lacZ gene [lip(pCH110)], or liposome containing the murine IFN-beta gene [lip(pSV2muIFN-beta)] were added to the medium. Incubation was continued for additional 2 or 5 days and the number of viable cells was counted using a hemocytometer. *$p<0.05$ compared with PBS and lip(pCH110). **$p<0.05$ compared with muIFN-beta. All values are means $\pm$ SEM.

Fig. 3. Morphologic changes in cultured mouse B16F1 cells transfected with liposome containing the murine IFN-beta gene. Under a video enhanced contrast-differential interference contrast microscope, we observed that approximately 30% of the cells displayed bleb formation and abnormally bright nucleoli by 24 hr after transfection. By 48 hr the same population had shrunk, developed large membrane outpouchings (ballooning). Original magnification, x 2,000.

Fig. 4. Liposome-mediated expression of murine IFN-beta in subcutaneous melanoma. Lane 1-murine IFN-beta standard (1000 IU), 2- lipsome containing the lacZ gene-injected; 3 and 4- liposome containing the murine IFN-beta gene-injected

Fig. 5. Growth inhibition of mouse B16F1 subcutaneous tumors treated with liposome containing the murine IFN-beta gene [lip(pSV2mulFN-beta)]. Animals were injected intratumorally with 75 μl of either phosphate-buffered saline (PBS), recombinant murine IFN-beta (mulFN-beta; 1000 IU), lacZ control [lip(pCH110)], or lip(pSV2mulFN-beta). Tumor sizes were measured for a period of 21 days. *$p<0.05$ compared with PBS, mulFN-beta, or lip(pCH110). All values are means ± SEM.

Fig. 6. Immunocytochemistry for immune cells infiltrating mouse B16F1 subcutaneous tumors. A; NK cells. B; CD4, CD8, and macrophages. Representative photomicrographs from tumors 7 days after treatment with liposome containing the murine IFN-beta [lip(pSV2mulFN-beta)] or phosphate-buffered saline (PBS). Natural killer cells, CD4 T and CD8 T lymphocytes, and macrophages were stained with antibodies to NK1.1, mouse CD4, mouse CD8, and F4/80, respectively. The tissues were counterstained with hematoxyline. Immunocytochemistry in the PBS-treated tumors was similar to those in the tumors treated with recombinant murine IFN-beta and liposome containing the lacZ gene (data not shown).

Fig. 7. The effect of in vivo natural killer (NK) cell depletion on the growth of subcutaneous tumors treated with liposome containing the murine IFN-beta gene [lip(pSV2mulFN-beta)]. NK cells were depleted by injecting the mice intraperitoneally with 25 μl anti-asialoGM1 antibody 1 day before and every 7 days after lip(pSV2mulFN-beta) injection. The NK cell-depleted (NK^-) animals were injected intratumorally with 75 μl of lip(pSV2mulFN-beta). Tumor sizes were measured and compared with NK cell-undepleted (NK^+) animals treated with lip(pSV2mulFN-beta) or PBS (the same data shown in Fig. 5 are used.). *$p<0.05$ compared with the PBS-treated group and the lip(pSV2mulFN-beta) treated NK^- group. There was no statistical significance between these two groups at any time. All values are means ± SEM.