

Adenovirally delivered IFN- β exerts antitumor effects through transient T-lymphocyte depletion and Ag-specific T-cell proliferation

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Abstract. Type I interferons (IFNs), including IFN- β , are known to enhance antigen (Ag) presentation and to promote the expansion, survival and effector function of CD8⁺ cytotoxic lymphocytes (CTL) during viral infections. Furthermore, IFN- β is a potent candidate for antitumor drugs; however, recombinant IFN- β is too unstable for use in tumor therapy *in vivo*. In this study, we therefore examined the efficacy and mechanism of exogenous IFN- β as a biomolecule for tumor therapy, using adenovirus encoding IFN- β (Ad-IFN β) as a therapeutic agent in a mouse model. Ag104L^d and 4T1 tumor cells exposed to Ad-IFN β showed growth retardation and cell death *in vitro*, and tumor growth as well as tumor metastasis was inhibited *in vivo*. The Ad-IFN β -mediated antitumor effect was dependent on CD8⁺ T cells *in vivo*, rather than on a direct cytotoxic effect of Ad-IFN β . Transient T lymphocyte depletion was observed in tumor tissue after intratumoral injection with Ad-IFN β . Despite the T lymphocyte depletion, the proliferation of Ag-specific CD8⁺ T cells was increased in Ad-IFN β -treated mice compared to control virus-treated mice. These results suggest that IFN- β might contribute to the inhibition of tumor growth by depleting Ag-nonspecific T lymphocytes and enhancing proliferation of Ag-specific CD8⁺ T cells.

Introduction

While therapeutic vaccination against tumors is an important approach to cancer therapy, the efficacy of such vaccination in some human tumor trials against certain tumors has been

disappointing (1,2). Type I interferon (IFN) is a candidate for overcoming some obstacles in tumor therapy. Type I IFN is commonly expressed in many cell types in response to TLR (Toll-like receptor) triggering, and is most abundantly produced by plasmacytoid dendritic cells (pDCs) in response to viral and other infections (3-8). Type I IFN acts as a bridge between the innate and adaptive immune systems, as it promotes the activation, expansion, survival, and memory differentiation of T lymphocytes through a variety of mechanisms, including enhanced antigen (Ag) presentation, co-stimulation and cross-priming (9-15).

IFN- β is a cytokine of type I IFN and the IFN- β protein was first used clinically as an anticancer drug in human glioma (16,17). Although the treatment showed a growth-inhibitory effect on malignant glioma and melanoma, tumor regression was observed in only 10-30 and 15-20% of the glioma or melanoma patients treated with IFN- β , respectively (16-18). The reason for the insufficient results could be the low stability of recombinant IFN- β *in vivo*. Numerous animal studies have pursued IFN- β gene therapy as a potential tumor therapy to overcome this impediment of the recombinant protein (19-23).

In this study, we investigated the immune effects of adenovirus-mediated IFN- β (Ad-IFN β) gene therapy in tumor-bearing mice. Ad-IFN β therapy induced regression of primary tumor and prevented tumor metastasis. We found that the tumor regression was dependent on CD8⁺ T cells, but that tumor-infiltrated lymphocytes were depleted transiently after treatment and Ag-specific T cells proliferated. This suggests that Ad-IFN β induces tumor regression through depletion of Ag-nonspecific or regulatory T cells and simultaneous induction of proliferation Ag-specific CD8⁺ T cells.

Materials and methods

Mice. B6C3F1, F1 cross between C57BL/6 and C3H mice, and Balb/c mice were purchased from Charles River. 2C TCR-transgenic mice with *Rag1*^{-/-}-B6 background (2C mice) were used for the survey of antigen (L^d)-specific T cell proliferations. For all experiments, the mice were between the ages

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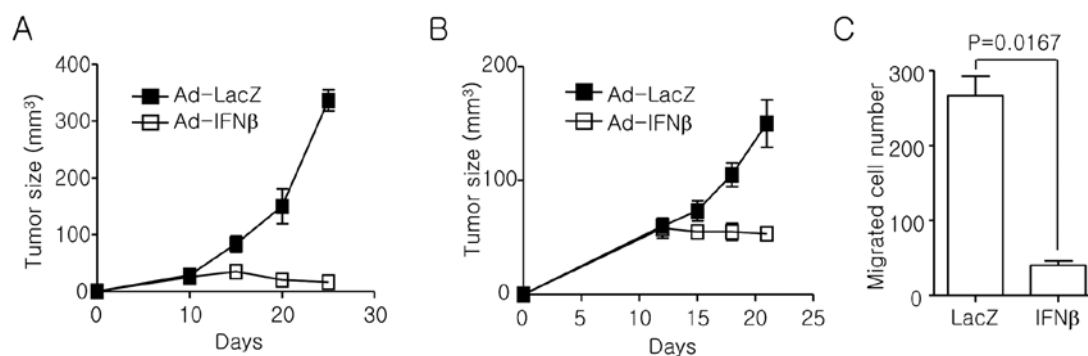


Figure 1. Antitumor effect of Ad-IFN β . L^d-overexpressing Ag104 (Ag104L^d) cells (A) or 4T1 cells (B) were injected subcutaneously into the back of B6C3F1 or Balb/c mice, respectively (n=5). Ad-IFN β (2×10^{10}) was injected intratumorally on Days 9 and 11 after tumor injection. Each tumor size was calculated on the indicated days. For surgical excision of primary 4T1 tumors, mice were anesthetized and tumors were resected with sterilized instruments on Day 20 after tumor injection. Mice in which primary tumors recurred at the site of the surgical excision were eliminated from the experiments. A colonogenic assay was used to evaluate metastases by 4T1 tumors on Day 8 after the surgery (C). These experiments were repeated at least twice.

of 6-10 weeks and were used in accordance with the animal experimental guidelines set by the Animal Ethics Committee of Gyeongsang National University.

Generation of adenovirus-expressing IFN- β . To generate Ad-IFN β , we used the method described by Burnette *et al.* (24). To construct recombinant Ad-mIFN- β , murine IFN- β cDNA was amplified by PCR and cloned into the *NotI/EcoRV* sites of pAdenoVator-CMV5(CuO) under CMV5 promoter. pAdenoVator-mIFN- β was linearized by *PacI* digestion and electroporated into electrocompetent cells BJ5183 at 2.5 kV for recombination with the adenoviral backbone plasmid pAdenoVator δ E1/E3. The hybrid cosmids were selected on Kanamycin LB agar plate. *PacI* digestion was used to further identify recombinant cosmid containing the mIFN- β insert. The Ad-mIFN- β DNA was linearized by *PacI* digestion, and the mixture of *PacI* digestion without further purification was transfected into 293 cells for recombinant adenovirus production. The Ad-mIFN- β is referred to as Ad-IFN β . Ad-LacZ was used as a control of Ad-IFN β .

Establishment of tumor and adenovirus treatments. Ag104L^d cells, an Ag104 fibrosarcoma cell line expressing murine H-2L^d, were trypsinized, washed with media, and injected (cell number, 1×10^6) subcutaneously in the back of mice. Tumor size was determined at 3 to 4 days interval. Tumor volumes were measured along 3 orthogonal axes (a, b, and c) and calculated as tumor volume = $abc/2$. The tumor nodules were inoculated with 1×10^{10} of adenovirus (Ad)-IFN- β or Ad-lacZ virus intratumorally. For antibody-mediated cell depletion, 200 μ g per mouse anti-CD4 (GK1.5), anti-NK1.1 (PK136) or anti-CD8 (YTS.169.4.2) antibody was given to mice intraperitoneally on Days 9, 11 and 13 after primary tumor inoculation.

Evaluation of metastases by colonogenic assay. 4T1 cells (5×10^4) were inoculated subcutaneously into the area around the tail base on BALB/c mice. Ad-IFN β (2×10^{10}) was injected intratumorally on Day 9 and 11 after tumor injection. For surgical excision of primary 4T1 tumors, mice were anesthetized, and tumors were resected with sterilized instruments on Day 20 after tumor injection. Wounds were closed with metallic clips. All mice survived surgery. Mice in which

primary tumors recurred at the site of the surgical excision were eliminated from the experiments. A colonogenic assay was used to evaluate metastases by 4T1 tumors on Day 8 after surgery. Lungs were collected from the mice and chopped before being dissociated in DMEM supplemented with 10% FCS containing 1.5 mg/ml collagenase type D (Sigma-Aldrich) (collagenase D solution) for 30 min in 37°C shaking incubator at 178 rpm speed. Organs were then plated at various dilutions in the DMEM supplemented with 10% FCS and 60 μ M 6-thioguanine. Individual colonies representing micro-metastases were counted after 5-10 days.

Measurement of cytokines in the tumor mass. Solid tumor tissues were weighed and homogenized, and the amount of cytokines in the supernatants was then quantified using the cytometric bead array kit (BD Biosciences, Pharmingen) on a FACSCalibur cytometer equipped with CellQuestPro and CBA software (BD Biosciences), according to the manufacturer's instruction.

Adoptive transfer of T cells. A total of 2×10^6 T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE). For reconstitution of recipients, T cells were sorted using the CD8⁺ T Cell Isolation kit and the automated Magnetic Cell Sorting (Miltenyi Biotec).

Flow cytometric analysis. Single-cell suspensions of cells were isolated and incubated with anti-CD16/32 (anti-Fc γ III/II receptor, clone 2.4G2) for 20 min at room temperature and subsequently stained with conjugated antibodies. The cells were labeled with FITC-conjugated 1B2 mAb (specifically identifies 2C T cells in 2C mice), FITC-conjugated anti-mouse anti-CD3 mAb (identifies pan-T cells) (Pharmingen, San Diego, CA), PE-conjugated anti-mouse anti-CD4 mAb (Pharmingen), and cychrome-conjugated anti-mouse anti-CD8 mAb (Pharmingen). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (TreeStar, Inc.).

Statistical analysis. Data were statistically analyzed using an unpaired Student's 2-tailed t-test. Data are presented as the means, and error bars represent standard deviations (SDs).

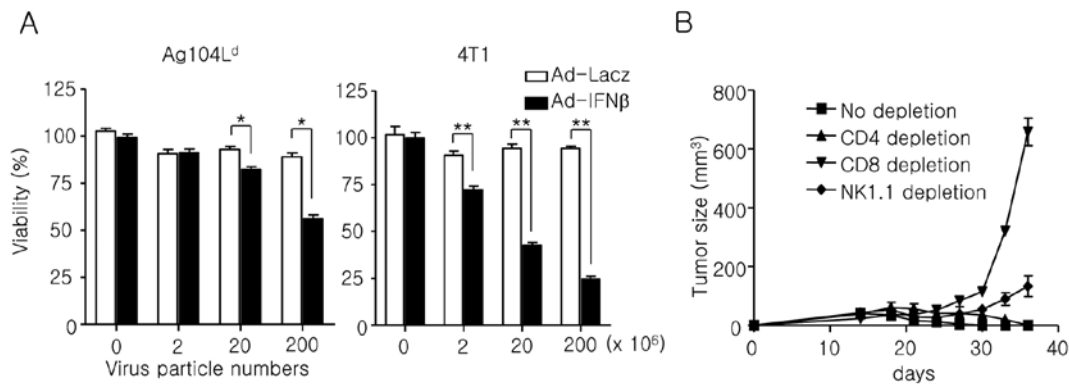


Figure 2. Ad-IFN β induces tumor regression dependent on CD8⁺ T cells as well as apoptosis of tumor cells. Ag104L^d or 4T1 cells were cultured at a density of 5×10^4 cells per well in flat bottomed 96-well plates and treated with indicated particle numbers of Ad-LacZ or Ad-IFN β for 24 h. CellTiter 96[®] Aqueous One Solution Reagent was added to each well according to the manufacturer's instructions. After 4 h in culture, cell viability was determined by measuring the absorbance at 490 nm using a 550 BioRad plate-reader (A). * $P < 0.02$ and ** $P < 0.005$. For antibody-mediated cell depletion, 200 μ g per mouse anti-CD4 (GK1.5), anti-NK1.1 (PK136) or anti-CD8 (YTS.169.4.2) antibody was given to mice intraperitoneally on Days 9, 11 and 13 after primary tumor inoculation. Ad-IFN β was injected intratumorally on Days 12 and 15 after primary tumor inoculation (B).

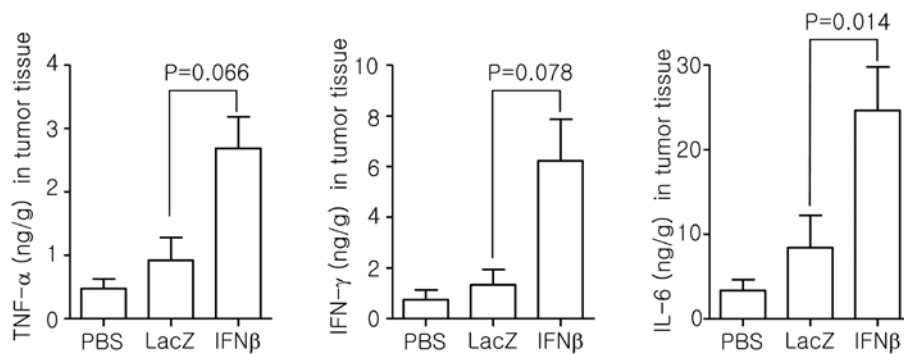


Figure 3. Cytokines induced by Ad-IFN β in tumor mass. Ag104L^d tumor was established for 2 weeks and Ad-IFN β was injected into B6C3F1 mice intratumorally. On Day 3 after the injection, the tumor mass was removed and weighed. The tumor mass was homogenized at 1 g/1 ml PBS containing protease inhibitors. The amount of cytokines in the supernatants was quantified using the cytometric bead array kit.

Results

IFN- β -mediated gene therapy reduces established tumors. To test the efficiency of IFN- β -mediated tumor therapy, Ad-IFN β particles were injected into established Ag104L^d or 4T1 tumor intratumorally 2 times on Days 9 and 12 after tumor inoculation. The treatment reduced Ag104L^d tumor size almost completely, and halted 4T1 tumor growth (Fig. 1A and B). Calculation of the number of metastatic cancer cells in the lungs of 4T1-bearing mice showed that the cell number was significantly decreased in the Ad-IFN β -treated group, compared to the Ad-LacZ-treated group (Fig. 1C).

Therapeutic effect of Ad-IFN β is dependent on CD8⁺ T cells. It is known that IFN β has direct cytotoxic effects on tumor cells. To explore whether Ad-IFN β also induces the cytotoxicity, Ag104L^d or 4T1 cells were treated with indicated number of Ad-IFN β for 24 h *in vitro*. Although 4T1 cells were more susceptible to cytotoxicity by Ad-IFN β than were Ag104L^d cells, significant cytotoxicity was observed in both of them (Fig. 2A). To determine whether the effect on tumor regression noted *in vivo* could be attributed more to such direct cytotoxicity of Ad-IFN β , or to a possible immune response induced by Ad-IFN β , Ad-IFN β was injected intratumorally after lympho-

cyte depletion and the tumor size was monitored. Although NK cells have a partial function in Ad-IFN β -mediated tumor therapy, CD8⁺ T, but not CD4⁺ T or NK cells, were essential in affecting substantial tumor reduction by Ad-IFN β (Fig. 2B). Taken together, we conclude that CD8⁺ T cell activation by Ad-IFN β is more important than direct cytotoxicity in tumor regression by Ad-IFN β .

Ad-IFN β is sufficient to modulate the immunological tumor environment. To investigate the modulation of the immunological tumor environment by Ad-IFN β , we injected Ad-IFN β into the established Ag104L^d tumor intratumorally on Day 14 and surgically removed the tumor tissues on Day 3 after the Ad-IFN β injection. The immunological tumor environment was determined by estimating the level of certain cytokines, i.e., TNF- α , IFN- γ , and IL-6, in the tissue using a CBA assay. Compared to PBS or Ad-lacZ treatment, Ad-IFN β treatment induced a steep increase in the amount of these cytokines within the tumor environment; this increase was statistically significant for IL-6 (Fig. 3). Ad-IFN β stimulates antigen presenting cells (APC) to produce TNF- α and IL-6, and induces communication between APC and NK cells to drive positive regulation loop producing higher inflammatory cytokines and IFN- γ .

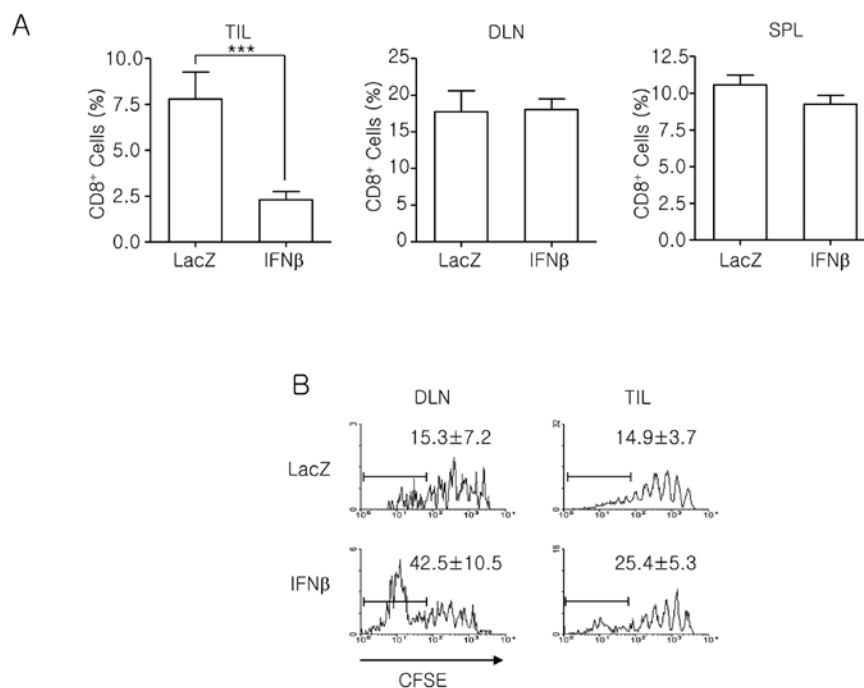


Figure 4. Transient decrease of CD8⁺ T cells and proliferation of 2C CD8⁺ T cells. Following Ad-IFN β treatment, tumor-infiltrated cells were harvested and stained with anti-CD8 Ab (A). ***P<0.02. This experiment was repeated at least 3 times. To analyze Ag-specific CD8⁺ T cells, CD8⁺ T cells were isolated from 2C transgenic mice and labeled with CFSE, and then transferred into tumor-bearing mice, as described in Materials and methods. The percentage of CD8⁺ T cells was calculated on gated CD45⁺ cells. Tumor infiltrated cells were stained with anti-IB2 Ab for 2C transgenic T cell and anti-CD8 Ab (B). The intensity of CFSE on gated CD8⁺IB2⁺ cells was analyzed as antigen-specific T cell proliferation. Samples (n=3) were analyzed on a FACSCalibur flow cytometer, and data were analyzed with FlowJo software.

Ad-IFN β induces transient lymphocyte depletion and leads to Ag-specific T cell proliferation. To further understand the effects of Ad-IFN β on tumor regression, we analyzed the intratumoral CD8⁺ T cell population after intratumoral treatment with Ad-lacZ or Ad-IFN β . The CD8⁺ T cell population was decreased, rather than increased, within the tumor mass by Day 3, and was recovered by Day 7, after Ad-IFN β treatment. The decrease was not observed in the draining lymph nodes or spleen, but only within the tumor mass (Fig. 4A). CD4⁺ T-cell population showed the same tendency with the CD8⁺ T-cell population (data not shown). To investigate the mechanism underlying tumor regression, in spite of transient CD8⁺ T-cell depletion, we transferred CFSE-labeled 2C CD8⁺ T cells, having a specific T-cell receptor (TCR) for the L^d antigen, into Ag104L^d bearing mice. The 2C CD8⁺ T cells showed higher proliferation in the draining lymph nodes and in the tumor mass of the Ad-IFN β than in the Ad-lacZ treatment group, suggesting activation of antigen specific T cells through transient T-cell depletion.

Discussion

Although type I IFN, especially IFN- β , is a candidate for cancer treatment, the stability of IFN- β *in vivo* has been an obstacle to clinical application. However, it has been proposed that, should a suitable liposomal or viral delivery system be developed, IFN- β might be a potent anticancer drug. In this study, we confirmed that adenovirus-mediated IFN- β gene therapy was effective in greatly reducing primary solid and metastatic tumors. In addition, we showed for the first time

that the mechanism of antitumor action by IFN- β involved IFN- β -induced transient T cell depletion and concomitant activation of antigen-specific T cells.

Type I IFN is produced from plasmacytoid dendritic cells following virus infection, and contributes to the immune response against viral infection and the clearing of the virus-infected cells. Uncontrolled type I IFN expression has also been correlated with autoimmune disease (25,26). Furthermore, IFNs are negative regulators that inhibit cancer cell proliferation and induce apoptosis of cancerous cells (27). Indeed, we showed that application of adenovirally-delivered IFN- β also induces apoptosis of Ag104L^d and 4T1 cells *in vitro*. Despite this direct cytotoxic effect of Ad-IFN β on tumor cells, we demonstrated that CD8⁺ T cell activation by Ad-IFN β was more important than direct cytotoxicity in tumor regression by Ad-IFN β *in vivo*. Although NK cell activation partially contributed to the antitumor immune response, immunological activation of CD8⁺ T cells was a major cause of the therapeutic effect of IFN- β , as this therapeutic effect was negated by CD8⁺ T-cell depletion *in vivo*. Although Ad-IFN β affected the host immune response, such as cytokine production and antigen-specific T cell proliferation *in vivo*, this effect was too weak to cause tumor regression. This minor immune response is more likely due to reactivity of the innate immune system that is induced by adenovirus through Toll-like receptor-dependent and -independent pathways (28).

In this study, we found that transient T cell depletion occurred in the tumor mass after intratumoral administration of Ad-IFN β . This is probably due to IFN-induced apoptosis of CD8⁺ naïve T cells, which has been reported to occur during

the early stages of viral infection. In these previous reports, the loss of CD8⁺ T cells was preceded by the development of an antiviral T-cell response (29,30), while apoptosis of the CD8⁺ T cells was dependent on antigen specificity and interferon receptor-mediated signaling since the loss of CD8⁺ T cells disappeared in IFN- β KO. We speculate that Ad-IFN β treatment might induce tumor cell death, which would result in the release of tumor-associated antigens, and lead antigen presenting cells (APC) to produce inflammatory cytokines, induce communication between APC and NK cells, and would accelerate presentation of these antigens which induce the activation of Ag-specific T cells. This would result in the expansion of the antigen-specific T cell population, which would be activated to kill the tumor cells. By contrast, antigen non-specific T cells would only be lost in the tumor mass during Ad-IFN β treatment because IFN- β expression would be restricted to the tumor mass.

In summary, in this study, we propose a novel mechanism for the antitumor effects of an adenovirally-delivered IFN β involving induction of a relatively high antitumor immune response through loss of antigen-nonspecific T cells, similar to that which occurs during the early phase of viral infection.

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