Effective Melanoma Immunotherapy with Interleukin-2 Delivered by a Novel Polymeric Nanoparticle

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Running title: Melanoma immunotherapy with IL-2 delivered by a nanopolymer

Keywords: Non-viral gene delivery vector; Interleukin-2; Immunotherapy; Gene
therapy; Melanoma

1The work was supported by the funding from the Innovation and Technology Fund (ITS/243/09 to MCL and SSN) of the Government of the Hong Kong Special Administrative Region, the National Natural Science Foundation of China (PC: 81001023) and the Natural Science Foundation of ChongQing (CSPC: 2010BC5007).

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3No potential conflicts of interest were disclosed.
Abstract

Interleukin-2 (IL-2) has been demonstrated to possess antitumor activity in numerous preclinical and clinical studies. However, the short half-life of recombinant IL-2 protein in serum requires repeated high-dose injections, resulting in severe side effects. Although adenovirus-mediated IL-2 gene therapy has shown antitumor efficacy, the host antibody response to adenoviral particles and potential biosafety concerns still obstruct its clinical applications. Here we report a novel nanopolymer for IL-2 delivery, consisting of low molecular weight polyethylenimine (PEI; 600Da) linked by β-cyclodextrin and conjugated with folate (named H1). H1 was mixed with IL-2 plasmid to form H1/pIL-2 polyplexes of around 100 nm in diameter. Peritumoral injection of these polyplexes suppressed the tumor growth and prolonged the survival of C57/BL6 mice bearing B16-F1 melanoma grafts. Importantly, the antitumor effects of H1/pIL-2 (50μg DNA) were similar to those of recombinant adenoviruses expressing IL-2 (rAdv-IL-2; 2×10⁶pfu). Furthermore, we showed that H1/pIL-2 stimulated the activation and proliferation of CD8+, CD4+ T cell and natural killer cells in peripheral blood and increased the infiltration of CD8+, CD4+ T cells and NK cells into the tumor environment. In conclusion, these results demonstrate that H1/pIL-2 is an effective and safe melanoma therapeutic with an efficacy comparable to that of rAdv-IL-2. This treatment represents an alternative gene therapy strategy for melanoma.
Introduction

Melanoma is the most malignant skin cancer. With the frequency of cases rising rapidly, melanoma has become one of the most fatal cancers in the past few years worldwide, especially in the white population (1, 2). Surgical resection, chemotherapy and immunotherapy are the conventional therapeutic strategies for patients with malignant melanoma. Although the efficacy of these therapies have been improved over the past decade, the serious side effects induced by various cytotoxic drugs and the low response rates observed in melanoma patients have presented great challenges to physicians (3, 4).

As an immune-modulating glycoprotein, interleukin-2 (IL-2) possesses strong antitumor activity by stimulating the activity of T lymphocytes and inducing the growth of natural killer (NK) cells and B cells (5, 6). Compared with other immunotherapeutic approaches, IL-2 has the best anti-tumor activity in patients with malignant melanoma. Follow-up data through 2004 confirmed the durability of responses produced by high-dose IL-2 regimen in patients with metastasis melanoma. However, the short half-life of IL-2 protein in serum requires repeated high-dose injections, resulting in severe side effects (7).

In order to overcome these limitations, intralesional delivery of IL-2 gene by adenoviruses has been investigated in various preclinical animal models (8-11). This approach enhanced tumor immunogenicity and led to tumor regression both at the injection site and distant sites, followed by the development of protective immunity.
In particular, repeated intratumoral injections of recombinant adenoviruses expressing IL-2 (rAdv-IL-2) developed clinical responses in patients with metastatic melanoma (12). However, the host antibody response and potential biosafety issues associated with adenoviral vectors severely obstruct their clinical applications. To address these problems, non-viral vectors, including cationic lipids or polycationic polymers, are attractive candidates because of their low immunogenicity, relative safety and low cost. Direct intratumoral injection of IL-2 DNA/cationic lipid complex has been demonstrated to be safe and efficient in patients with metastatic melanoma or metastatic renal cell carcinoma in clinical trials (13, 14). However, the efficiency of these non-viral vectors for IL-2 gene delivery was lower than that of rAdv-IL-2.

Recently, we have synthesized a novel polycationic vector, H1, which consists of low molecular weight polyethylenimine (PEI; 600Da) linked by β-cyclodextrin and conjugated with folic acid. We have demonstrated that H1 is a biodegradable, tumor specific, and efficient gene delivery vector in vitro and in vivo (15). Here, we report that subcutaneous administration of H1-human interleukin 2 plasmid polyplexes (H1/pIL-2) could effectively reduce melanoma growth and prolong the survival of melanoma-bearing mice. Importantly, the antitumor effects of H1/pIL-2 (50μg DNA) were similar to those of rAdv-IL-2 (2 x 10⁸ pfu). Furthermore, we found that activation, proliferation and infiltration of CD4+, CD8+ T cells and natural killer (NK) cells were involved in the antitumor effects of H1/IL-2. Interestingly, the percentage of Foxp3+ in CD25+/CD4+ cells in mice treated with H1/pIL-2 was significantly lower than that of control mice, an observation which is different from the IL-2
treatment.

Materials and Methods

Production of H1 and Plasmids

H1 was synthesized using a method described by Yao et al. (15). Enhanced green fluorescent protein (EGFP), firefly luciferase and human interleukin-2 (IL-2) cDNAs were obtained by polymerase chain reaction (PCR) using pEGFP-N1 (BD Biosciences, San Jose, CA), pGL3 Luciferase reporter vector (Promega Corporation, USA) and pDC-IL2-WRPE (constructed by our laboratory) as templates, respectively. The cDNAs encoding EGFP, Luciferase and human IL-2 were subcloned into the pAM/CAG-WPRE-BGHpolyA plasmid and generate pAM/CAG-EGFP (pEGFP), pAM/CAG-Luciferase (pLuc) and pAM/CAG-IL-2 (pIL-2) plamids, respectively.

Production of a Recombinant Adenovirus Expressing Human Interleukin 2 (rAdv-IL-2) or Enhanced Green Fluorescent Protein (rAdv-EGFP)

The cDNAs encoding human IL-2 and EGFP were subcloned into pAd/CMV/DEST™ (Invitrogen) to generate pAd-IL-2 and pAd-EGFP, respectively. The pAd-IL-2 or pAd-EGFP plasmid was then transfected into 293A cells using Lipofectamine™ 2000 Reagent (Invitrogen) to produce recombinant adenoviruses expressing human IL-2 (rAdv-IL-2) or EGFP (rAdv-EGFP). The resulting viruses were purified by cesium chloride gradient centrifugation and tittered using tissue
culture infectious dose 50 (TCID_{50}) method (AdEasy™ Vector System Application manual, Qbiogene). The viruses were stored at -80°C prior to use.

**Characterization of H1/pIL-2 Polyplexes**

To prepare H1/pIL-2 polyplexes, the H1 polymer solution was mixed with the pIL-2 plasmid solution (at an N/P ratio of 20:1) with an equal volume of 5% Glucose. The H1/plasmid DNA polyplexes were characterized by transmission electron microscopy (TEM). Briefly, the H1/pIL-2 polyplexes (50μl) were dropped to glow-discharged, 400-mesh carbon-coated copper grids. After drying at room temperature, images were recorded by a Philips 201 electron microscope operated at 80 kV.

**Animals and Cells**

Female C57BL/6N mice (6-9 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and were housed under aseptic conditions and cared for according to the guidelines from the Laboratory Animal Unit of the University of Hong Kong. The mice were fed for three days before the experiment. All experimental protocols were approved by the Department of Health of the Government of Hong Kong Special Administrative Region and the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong.
Mouse melanoma B16-F1 cells were obtained from American Type Culture Collection (CRL-6323™, ATCC, Rockville, MD), which was banked after receipt, and passaged for less than 6 months before use in this study. ATCC characterize cell lines using short tandem repeat polymorphism analysis. The cells were cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) and incubated at 37°C in 5% CO₂. Before the start of this study, tumorigenicity of the B16-F1 cells were tested by subcutaneous injection of the cells (2×10⁵ cells/mice) into the flank of 10 C57/BLN mice (6-9 weeks old). The cells were tumorigenic in 100% of the injected mice.

**Transfection Efficiency of H1/pIL-2 in B16-F1 Cells In Vitro**

B16-F1 cells were seeded at a density of 1 ×10⁵ cells/well in a 24-well plate for 24 hours. The previously prepared H1/pIL-2 polyplexes solution was added in each well (1μg DNA, N/P ratio 20:1). At 48 hours after transfection, the total RNA of transfected cells was extracted. The following primer sequences were used to detect the cDNA of the human hIL-2 gene: Sense: 5’-ATGTACAGGATGCAACTCCTGTCT-3’; Anti-Sense: 5’-TCAAGTGTCGTGAGATGATGCTT-3’.

**Animal Model and Treatments**

B16-F1 cells were harvested at the exponential growth phase and one hundred microliters of the cell suspension (2×10⁵ cells) was subcutaneously injected in the
right flank of each C57BL/6 mouse. When the tumor grew to about 0.2-0.4cm, the mice were treated by peritumoral injection with H1/pIL-2 (50μg DNA), H1/pIL-2 (25μg DNA), H1/pEGFP (50μg DNA), 5% Glucose and IL-2 plasmid alone (50μg), respectively. Each mouse was treated once every two days till the desired endpoint. To compare the antitumor efficacy between rAdv-IL-2 and H1/pIL-2, the tumor-bearing mice were grouped as below: (i) rAdv-IL-2 (2×10³ pfu) injection once every 5 days; (ii) rAdv-IL-2 (2×10³ pfu) single injection; (iii) H1/pIL-2 (50μg DNA) once every 2 days; (iv) rAdv-EGFP (2×10³ pfu) and (v) PBS, respectively. Tumor growth was monitored by measuring the two maximum perpendicular tumor diameters with a caliper. Tumor volume ($V$) was calculated by the formula $V = \frac{1}{2} \times L \times S^2$, where $L$ and $S$ are the longest and shortest diameters of the tumor, respectively.

**In Vivo Imaging**

Mice were anesthetized and then D-luciferin (Xenogen) at a dose of 150μg per gram mouse body weight was injected intraperitoneally. After that, wait for 5 min and take picture. The images were obtained at exposure time from 1 to 10 min depending on the intensity of the emitted photons. The total photon flux (photons/second) in a region of interest (ROI) was quantitated by the Living Image® Software (Xenogen MA, USA).

**Histological Distribution of H1/pEGFP Polyplexes**

The mice were injected with H1/pEGFP (50μg DNA, N/P 20:1) by peritumoral
injection. At 24 hours post injection, the tumor tissues were excised, fixed and embedded in optimum cutting temperature (O.C.T.) medium. The embedded tissues were sectioned at 6 μm by a freezing microtome. The specimen was mounted with propidium iodide (PI) solution and then directly examined by fluorescence microscope.

**Immune Cell and Cytokine Profiles in Blood**

The peripheral blood was collected from mice and then mixed with anticoagulant immediately. Serum and plasma samples were separated by centrifugation at 300×g for 10 min at 4 °C. The serum cytokine profiles were analyzed by Th1/Th2 10plex kit and FlowCytomixPro 1.0 Software (Bender MedSysterms, USA) according to the manufacturer’s instructions. The immune cell profiles of the plasma samples were analyzed by flow cytometry. To detect the expression of CD69 in T cells and NK cells, plasma samples were labeled with PE-CD4, PE/Cy5-CD8, PE-conjugated rat anti-mouse pan-NK and FITC-CD69 cocktail of antibodies. To detect Tregs in the plasma samples, a mouse regulatory T cell staining kit (eBioscience) was used. Treg staining was performed by the following the protocol (eBioscience). To detect Ki67 antigen in CD4+ and CD8+ T cells, an intracellular staining protocol recommended by BD Pharmingen™ was used. Flow cytometry analysis was performed by Epics Altra (Beckman Coulter, Miami, FL) and WinMDI (version 2.9) was used for data analysis.
Immunohistochemical Staining of Tumor Tissue

The specimens of tumor tissues were incubated by the anti-mouse monoclonal antibody of CD4-PE/Foxp3-FITC; CD8-FITC and CD49-PE alone, CD8-FITC/CD25-PE and CD49b-PE, respectively. CD16/CD32 antibody was used to block nonspecific binding. DAPI solution was used for nuclear staining before mounting the slides.

Stereologic Quantification of Tumor-infiltrating T Cells

To quantify tumor-infiltrating T cells, the stereologic method depicted by Henrik et al. was used with minor modifications (16). Briefly, immunohistochemical fluorescence images taken from tumor sections were analyzed by the software of Image-Pro Plus 6.0 (Media Cybernetics, USA) at 20× magnification. The density of tumor-infiltrating lymphocytes was scored by counting all positive cells intratumorally and relating them to an area of interest, representing the total tumor area measured stereologically, excluding necrotic tumor tissue and nontumor tissue such as peritumoral connective tissue. One section of each sample was examined in the stereologic study.

Results

Characterization of H1/pIL-2 Polyplexes and Expression of IL-2 in B16-F1 Cells
Transfected with H1/pIL-2

The schematic diagrams of pEGFP, pIL-2 and pLuc plasmids are shown in Fig. 1A. As shown in Fig. 1B, H1 could condense and package the pIL-2 plasmids to a compact globular structure with a particle size of around 100 nm. The expression of IL-2 in H1/pIL-2-transfected B16-F1 cells was determined by RT-PCR at 48 hours post transfection. A high level of IL-2 mRNA expression was detected in B16-F1 cells transfected with H1/pIL-2, whereas IL-2 mRNA was absent in the control B16-F1 cells transfected with H1/pEGFP (Fig. 1C).

Expression and Histological Distribution of Transgene Induced by H1/pLuc or H1/pEGFP Polyplexes

To evaluate the dynamic change of transgene expression over time in mice injected with H1/pLuc polyplexes, C57/BL6 mice bearing B16-F1 tumors were injected with naked pLuc (50 μg), H1/pLuc (25 μg DNA, N/P ratio 20:1) or H1/pLuc (50 μg DNA, N/P ratio 20:1) by peritumoral injection. The expression of luciferase was then monitored by a cooled CCD camera from day 1 to day 5. As shown in Fig. 1D, the peak value of luciferase activity induced by H1/pLuc (50 μg, DNA) occurred at 24 hours post transfection. It was then reduced by 40% and 60% at 48 hours and 72 hours, respectively. The peak luciferase activity of H1/pLuc (25 μg DNA) also appeared at 24 hours post transfection, but the value was only one third of that of H1/pLuc (50 μg DNA) and the bioluminescent signals were observed only for 3 days. These indicated that efficiency and duration time of gene expression were dependent...
on the dosage of H1/pLuc injected. For the mice injected with naked pLuc (50μg DNA), the signals could be observed only when the exposure time was extended to 10 min, indicating that the luciferase activity produced by naked pLuc was very weak.

On day 3 after the first injection, the second injection was performed in the same mice. Twenty four hours after the second injection, the luciferase activity rebounded and was almost similar to the first injection in all 3 groups (Fig. 1E). We also examined the tissue distribution of transgene expression in the mice injected with H1/pEGFP polyplexes. Histological analysis showed that EGFP expression was localized at the tumor border, subcutaneous fibro connective tissue and muscle fibro tissue near the tumor mass (Fig. 1F).

H1/pIL-2 and rAdv-IL-2 Effectively Reduced B16-F1 Melanoma Growth

Next, we tested whether H1/pIL-2 is efficacious in treating melanoma in a mouse model. Compared with the control mice injected with H1/pEGFP, pIL-2 plasmid alone, or 5% Glucose, the growth of subcutaneously inoculated B16-F1 melanoma was effectively inhibited in the mice receiving H1/pIL-2 (25μg DNA) or H1/pIL-2 (50μg DNA). The tumor growth was much slower in the group treated with H1/pIL-2 (50μg DNA) than the group treated with H1/pIL-2 (25μg DNA) (Fig. 2A). In addition, the survival of the mice treated with H1/pIL-2 (25μg DNA) or H1/pIL-2 (50μg DNA) was markedly prolonged as compared to that of the control mice (Fig. 2B).

We also compared the efficacy of H1/pIL-2 and rAdv-IL-2 in suppressing the growth of melanoma in mice bearing the B16-F1 tumor grafts. In mice receiving
single or multiple intratumoral injections of rAdv-IL-2 \((2\times10^8\text{pfu})\) or peritumoral injection of H1/pIL-2 \((50\mu\text{g DNA, N/P ratio 20:1})\), tumor growth was greatly inhibited as compared to those treated with rAdv-EGFP \((2\times10^8\text{pfu})\) or PBS. In particular, the tumor growth of mice treated with H1/pIL-2 was slower than that of mice treated with a single injection of rAdv-IL-2. Furthermore, H1/pIL-2-injected mice had tumor sizes almost similar to those receiving multiple injections of rAdv-IL-2, which elicited the most potent antitumor effects among the five experimental groups (Fig. 3A and B).

To evaluate the antitumor function of peritumoral H1/pIL-2 treatment on the metastatic melanoma, we established an additional melanoma mouse model by inoculating melanoma cells subcutaneously into both left and right flanks of the mice. The tumor inoculated in the left-flank was treated with peritumoral injection of H1/pIL-2\((50\mu\text{g DNA})\) or H1/pEGFP \((50\mu\text{g DNA})\), whereas the right-flank tumor (distal tumor) was kept untreated. We found that H1/pIL-2, but not the control H1/pEGFP, significantly suppressed the growth of the untreated distal tumors. However, in the H1/pIL-2-treated mice, the growth of the untreated distal tumors was still faster than that of the treated tumors (Fig. 3C).

**Stimulation of Activation and Proliferation of CD4+ T Cells, CD8+ T Cells and NK Cells in Peripheral Blood After Administration of H1/pIL-2**

We used flow cytometry to analyze the immune responses elicited in the
H1/pIL-2-treated mice. CD69 is recognized as a cell surface antigen expressed by activated NK cells, T cells, B cells and other subsets of immune cells. It is a pleiotropic molecule for the mediation of T cells and NK cells, which lead to the secretion of Th1/Th2 cytokines and activation of immune cell proliferation and cytotoxicity (17). On day 3 after the first treatment, the percentage of CD49+/CD69+ cells in the peripheral blood lymphocytes (PBL) of the H1/pIL-2-treated mice was 5-fold and 30-fold of that in the control H1/pEGFP-treated and 5% Glucose-treated mice, respectively. Similarly, the percentage of CD4+/CD69+ and CD8+/CD49+ cells in the H1/pIL-2-treated mice was significantly higher than that in the control mice. (Fig. 4A and B, **" P<0.05, n=4, student’s t-test). These results suggested that CD4+, CD8+ T cells and NK cells were activated in peripheral blood of mice treated by H1/pIL-2. Meanwhile, we also detected Ki67 expression in the CD4+ and CD8+ T cells of PBL by intracellular staining. The percentage of Ki67+/CD4+ and Ki67+/CD8+ T cells of mice treated by H1/pIL-2 was 2.6-fold and 3.7-fold of that in the control H1/pEGFP-treated mice, respectively (Fig. 4C and D), suggesting that the CD4+ and CD8+ effectors T cells were proliferative after H1/pIL-2 treatment.

In addition to the mentioned CD8+, CD4+ and CD49+ effector cells, we also investigated the effect of H1/pIL-2 treatment on Tregs. The percentage of CD25+/CD4+ cells in the PBL of the mice treated with H1/pIL-2 was 3.1-fold and 3.7-fold higher than that of mice treated with the H1/pEGFP and 5% Glucose-treated mice, respectively (Fig. 4E). When we gated on CD4+ cells, the percentage of CD25+/Foxp3+ cells of the mice treated by H1/pIL-2 were 0.9-fold and 1.3-fold...
lower than that of H1/pEGFP-treated and 5% Glucose-treated mice, respectively (Fig. 4F). By contrast, CD25+/Foxp3- population was significantly increased and CD25-/Foxp3- population was not changed by the H1/pIL-2 treatment.

On day 7 after the first treatment, peripheral blood samples were collected respectively from mice receiving subcutaneous administration of H1/pIL-2 (25μg DNA), H1/pIL-2 (50μg DNA) or H1/pEGFP (50μg DNA). We found that the populations of CD4+ and CD8+ T cells from mice treated with H1/pIL-2 (25μg DNA) increased slightly, while the mice treated with H1/pIL-2 (50μg DNA) increased significantly compared with those treated with H1/pEGFP (Fig. 5A and B). The population of CD49+ NK cells in mice treated with H1/pIL-2 (50μg DNA) and H1/pIL-2 (25μg DNA) was 2-fold and 1 fold higher than those treated with H1/pEGFP, respectively (“*” P <0.05, “**” P <0.001, n = 4, student’s t-test). On day 14 post-treatment, higher levels of CD4+ and CD49+ cells were also detected in the mice treated with H1/pIL-2, whereas the population of CD8+ cells declined and there was no significant difference between the percentage of CD8+ cells in the PBL of H1/pIL-2-, H1/pEGFP- and 5% Glucose-treated mice (Fig. 5C).

Increase of Th1, Th2 and Th17 Cytokine Secretion Induced by H1/pIL-2 Administration

To investigate the changes in the blood cytokine levels of mice treated with H1/pIL-2 polyplexes, serum samples from mice treated with H1/pIL-2 (25μg DNA),
H1/pIL-2 (50μg DNA), or H1/pEGFP (50μg DNA) were collected and analyzed on day 7 after the first treatment. The serum Th1 cytokines (e.g. IL-2, IFN-γ, and TNF-beta), Th2 cytokines (IL-4, IL-6) and IL-17 in the mice treated with H1/pIL-2 (25 μg DNA) and H1/pIL-2 (50μg DNA) increased significantly compared with those mice treated with H1/pEGFP. IL-2, IFN-γ, TNF-beta, IL-4, IL-6, and IL-17 in the mice treated with H1/pIL-2 (50μg DNA) were 2.0-, 2.6-, 2.7-, 4.5-, 3.5- and 2.1-fold higher than the mice treated with H1/pEGFP (50μg DNA), respectively (Fig. 5D; “*” P <0.05, “**” P <0.001, n=4, student’s t-test). The increase of Th1 and Th2 cytokines in peripheral blood of mice suggested that H1/pIL-2 treatment activated the cell-mediated and humoral immune responses of tumor bearing mice.

**H1/pIL-2 Increased The Density of Tumor-Infiltrating T Cells and NK cells**

We also assessed the effects of H1/pIL-2 administration on the degree of CD4+/Foxp3+, CD8+ T cells and NK cells infiltration into the tumor. Immunofluorescence staining was used to stain B16 tumor specimens taken out on day 3 after the first H1/pIL-2 treatment. One section from each tumor sample was stained and the density of tumor-infiltrating lymphocytes (TIL) was scored by counting positively stained cells in a stereologically measured area of interest, which excluded peritumoral connective tissue and necrotic tissue. The density of CD8+ TIL in the mice treated with H1/pIL-2 was 4-fold higher than that of 5% Glucose-treated group (Fig. 6A and B). In all sections stained by Foxp3-FITC/CD4-PE cocktail antibodies, Foxp3 positive signals were absent in the tumor cells and only a low level
of Foxp3 could be detected in the peritumoral connective tissue, which did not merge with CD4 positive signals. We therefore defined those CD4 positive cells as CD4+/Foxp3- cells. As shown in Fig. 6C and D, the density of CD4+/Foxp3- TIL in the mice treated with H1/pIL-2 was 1.4-fold higher than that of 5% Glucose-treated group. We also used CD49-PE monoclonal antibody to detect NK cells in the tumor tissue. We found that the density of tumor-infiltrating NK cells in the mice treated with H1/pIL-2 was 4.1-fold higher than 5% Glucose treated mice. (Fig. 6E and F, n=4, “***” P<0.001, student’s t-test).

Discussion

Our in vivo imaging data indicated that transgene expression induced by H1/pLuc peaked at 24 h post-injection and subsequently decreased. When the secondary injection was performed, a high level transgene expression recurred. Based on these results, in order to maintain a high level of IL-2 expression, we injected the tumor-bearing mice once every two days with H1/pIL-2 by peritumoral injection. Anti-tumor activities were observed in mice treated with H1/pIL-2 (50μg DNA) or H1/pIL-2 (25μg DNA), but not in those treated with 5% Glucose, H1/pEGFP (50μg DNA) or IL-2 (50μg DNA) plasmid alone. We also found that the anti-tumor effects of H1/pIL-2 (50μg DNA) were better than H1/pIL-2 (25μg DNA). These results indicate that a high level of IL-2 induced by H1/pIL-2 injection plays a major role in inhibiting the growth of melanoma.

It has been documented that rAdv-IL-2 can induce regression and immunity in...
Hence, we used rAdv-IL-2 as a benchmark to compare the efficacy of H1/pIL-2 to inhibit melanoma growth. We found that the antitumor efficacy of H1/pIL-2 (50μg DNA) was better than that of rAdv-IL-2 (2×10⁸pfu) with single injection and comparable to that of rAdv-IL-2 (2×10⁸pfu) with multiple injections. Our previous study showed that the level of luciferase gene expression transduced by a single injection of rAdv-luc peaked at 48 hours, declined subsequently, and then maintained at a constant level for 7-10 days (15). Thus, the amount of IL-2 produced by a single injection of rAdv-IL-2 could induce tumor suppression only at an early stage. The decline of the luciferase gene expression after 48 hours is likely due to the presence of anti-adenoviral neutralizing antibodies in the mice. On the other hand, repeated peritumoral injection of H1/pIL-2 could maintain IL-2 at a constantly high level for tumor suppression, and the polyplexes would not be attenuated by specific host antibodies. Therefore, the anti-tumor efficacy of H1/pIL-2 was comparable to that of rAdv-IL-2 with multiple injections. Together, these results show that a continued expression of IL-2 is necessary for its antitumor activity in our melanoma model.

We also analyzed the local and systemic immune responses elicited by H1/pIL-2 treatment. We found that H1/pIL-2 significantly increased the density of CD8+, CD4+/Foxp3- and CD49+ tumor infiltrating lymphocytes, suggesting that the antitumor effects induced by H1/pIL-2 may be attributed to the activated and tumor infiltrating CD8+, CD4+/ Foxp3- T cells and NK cells. Furthermore, peritumoral injection of H1/pIL-2 could provoke a systemic antitumor response by increasing the
activation and proliferation of the circulating CD8+, CD4+/Foxp3- T cells and NK cells, which may be responsible for inhibiting the growth of the untreated distal tumor in our mouse melanoma model.

Besides inducing these antitumor immune effectors, IL-2 also regulates the Treg lymphocyte balance, which plays an important role in peripheral tolerance to self-antigen and suppress anti-tumor immune response (18, 19). Treg cells are generally defined as CD4+/CD25+ T cells with an expression of transcription factor Foxp3. Foxp3 acts as a key regulator for the development and immune repression function of Tregs (20, 21). In this study, we found that H1/pIL-2 significantly up-regulated the expression of CD25 in CD4+ T cells but the frequency of Foxp3 in CD4+/CD25+ cells was reduced. The increase of CD25 expression was consistent with the activation of IL-2 on T cells (22). However, reduction of Foxp3+ Tregs population by H1/pIL-2 treatment was different from the systemic increase in Tregs observed in IL-2 therapy. We speculate that more complex mechanisms are involved in regulating Foxp3 expression in Tregs present in the H1/pIL-2-treated mice. In this study, a high level of IL-6, IL-4 and IL-17 cytokines were detected in the peripheral blood of mice injection with H1/pIL-2. IL-6 and IL-4 are proinflammatory cytokines which have been documented to decrease Foxp3 expression and down-regulate the functions of Tregs in vivo and in vitro (23-26). IL-17 is also a proinflammatory cytokine which is secreted by Th17 cells. The high level of IL-17 and low level of Tregs existing in peripheral blood of H1/pIL-2 treated mice is in line with the previous findings that IL-6 can promote the differentiation of naive T cells into Th17
cells rather than Foxp3+ Tregs (27).

Our immunofluorescence staining showed that little CD4+/Foxp3+ Tregs were rarely found within the H1/pIL-2-treated tumors, indicating that mechanisms other than Tregs are responsible for the regulating the antitumor effects induced by H1/pIL-2. Based on our data showing the increased infiltration of CD8+ T cells and NK cells into the tumor, it is also likely that innate immune cells like plasmacytoid dendritic cells and NKT cells played important roles in this process. Liu et al. reported that CpG-activated plasmacytoid dendritic cells could activate NK cells, conventional dendritic cells and CD8+ T cells to initiate effective and systemic antitumor immunity (28). Zaini et al. showed that NKT cells were responsible for the antitumor immunity induced by activated DC cells (29). In their B16 melanoma transplant model, these innate immune cells, when activated by Toll-like receptor (TLR) ligands, could produce chemokines and cytokines which in turn activate CD8+ T cells. The plasmid used in our experiments is a bacterially derived plasmid, which contains unmethylated CpG dinucleotides (30, 31). We speculate that the CpG dinucleotides present in the plasmid could be the potential TLR ligands which stimulate dendritic cells and macrophages by the Toll pathway and eventually activate plasmacytoid dendritic cells and NKT cells. Meanwhile, these activated antigen presenting cells also produced pro-inflammatory cytokines (e.g. IL-6 and IL-4) (32-33), which could decrease Foxp3 expression and the population of Foxp3+ Tregs in circulating CD4+ T cells. Because the H1/pEGFP polyplexes did not induce any potent anti-tumor response, the plasmid CpG alone could not account for the observed anti-tumor activity in the
H1/pIL-2-treated mice. We hypothesize that the innate immune cells may act as an initiating factor to break the immune tolerance induced by tumor cells, and tip the immune balance regulated by IL-2 to anti-tumor immunity rather than immune tolerance.

In conclusion, we have demonstrated that peritumoral injection of H1/pIL-2 suppressed tumor growth and prolonged the survival in C57/BL6 mice bearing B16-F1 melanoma grafts. The anti-tumor effect of H1/pIL-2 can be attributed mainly to the activation, proliferation and infiltration of effector T cells and NK cells into tumor. The therapeutic efficacy of H1/pIL-2 was dose-dependent, of low toxicity, and similar to that of rAdv-IL-2 (2×10^8 pfu). Overall, our data show that H1/pIL-2 represents a promising gene therapeutic strategy for melanoma.

Acknowledgments

We would like to thank Mr. Hong-Ping Xia, Mr. Yan-Xin Ren and Mr. Su-Guang Zuo for their supports in the animal study.

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Figure legends

Fig. 1. Expression and histological distribution of IL-2 delivered by H1. (A) Schematic diagrams of pAM/CAG-EGFP (pEGFP), pAM/CAG-luciferase (pLuc) and pAM/CAG-interlukin2 (pIL-2) plasmids. (B) Transmission electron microscopy image showing the globular morphology of H1/pIL-2 polyplexes at an N/P ratio of 20. Scale bar = 200 nm. (C) RT-PCR showing the mRNA expression of IL-2 and GAPDH in B16-F1 cells at 48 h after transfection with H1/pIL-2 or H1/pEGFP. (D) Representative images of C57BL/6 mice with B16-F1 grafts displaying in vivo bioluminescence at 24, 48, and 72 h after peritumoral injection with H1/pLuc (50 μg DNA, N/P ratio 20:1). The total photon flux (photons/second) in a region of interest (ROI) is shown in red boxes. (E) The dynamic change of gene expression transduced by peritumoral injection with H1/pLuc (50 μg DNA), H1/pLuc (25 μg DNA) and pLuc (50 μg DNA) alone in melanoma grafts in mice. The total flux of ROI over time (ROI/min) was used as a parameter to compare their transfection efficiency. The red arrow indicates the time point when the second injection was performed (n = 4). (F) Representative histological sections of the tumor tissues collected from the mice peritumorally injected with H1/pEGFP (50 μg DNA) and the 5% Glucose, respectively.
The nuclei were stained by propidium iodide (PI). Abbreviations: TB, tumor border; SC, subcutaneous connective tissue.

Fig. 2. Effects of H1/pIL-2 polyplexes on tumor growth and survival of mice bearing B16-F1 grafts. (A) The volume of melanoma grafts in mice treated with H1/pIL-2 (50μg DNA), H1/pIL-2 (25μg DNA), 5% Glucose, H1/pEGFP (50μg DNA), and pIL-2 (50μg DNA), respectively. (B) Survival of melanoma grafts in mice treated with the same reagents mentioned above.

Fig. 3. The comparison of antitumor effects of H1/pIL-2 and rAdv-IL-2 treatments. (A) Survival of melanoma grafts in mice treated with PBS, rAdv-EGFP (2×10^8pfu), rAdv-IL-2 (2×10^8pfu) single injection, H1/pIL-2 (50μg DNA) and rAdv-IL-2 (2×10^8pfu) multiple injection, respectively. (B) The volume of melanoma grafts in mice treated with the same reagents mentioned above. (C) The volume of the melanoma grafts in mice where both flanks were inoculated with melanoma cells ("***" means P<0.05, n=4, student’s t-tests).

Fig. 4. Activation of CD8+ T cells, CD4+ T cells, NK cells, and CD4+/CD25+ T cells (but not Foxp3+/CD4+/CD25+ T cells) in the peripheral blood of tumor bearing mice treated with H1/pIL-2. (A) The percentage of CD49+/CD69+, CD4+/CD69+ and CD8+/CD69+ lymphocytes in H1/pIL-2-, H1/pEGFP- and 5% Glucose-treated mice, respectively. (B) Representative density plots showing the percentage of
CD49+/CD69+, CD4+/CD69+ and CD8+/CD69+ cells in the peripheral blood of mice treated with H1/pIL-2, H1/pEGFP and 5% Glucose, respectively. (C) and (D) Ki67 expression in CD4+ and CD8+ T cells of H1/pIL-2-, H1/pEGFP-, and 5% Glucose-treated mice, respectively. (E) The percentage of CD4+/CD25+ cells in H1/pIL-2-, H1/pEGFP-, and 5% Glucose-treated mice, respectively. The bottom panel shows the relative representative density plots. The square frames in the density plots represent the CD4 gated region for subsequent analysis of Foxp3+/CD25+ cells. (F) The percentage of Foxp3+/CD25+ cells when we gated on CD4+ lymphocytes. The bottom panel shows the representative density plots.

Fig. 5. Lymphocyte and cytokine profiles in the peripheral blood of tumor bearing mice treated with H1/pIL-2 and H1/pEGFP, respectively. (A) The percentage of CD4+ T cells, CD8+ T cells and CD49+ NK cells of H1/pIL-2(50 μg DNA)-, H1/pIL-2 (25 μg DNA)- and H1/pEGFP(50 μg DNA)-treated mice, respectively, on day 7 post-treatment. (B) Representative histograms of flow cytometric analyses showing the percentage of CD4+, CD8+ and CD49+ cells in PBL of the treated mice. (C) The percentage of CD4+, CD8+ and CD49+ cells of H1/pIL-2-, 5% Glucose- and H1/pEGFP-treated mice, respectively, on day 14 post-treatment. (D) Cytokines profiles of the peripheral blood in mice treated with H1/pIL-2 (25μg DNA), H1/pIL-2 (50μg DNA) and H1/pEGFP (50μg DNA), respectively.

Fig. 6. Immunohistochemical staining of tumor tissues. (A), (C) and (E)
Representative immunofluorescence photos showing CD8+, CD4+/Foxp3- T cells and NK cells in the tumor tissues excised from the mice on day 3 after first H1/pIL-2 treatment (Magnification of A and C = 200×; Magnification of E = 320×). Green signals are CD8+ T cells, red signals are CD4+ T cells or CD49+ cells and blue signals are DAPI-stained cell nuclei. (B), (D) and (F) Stereological analysis showed that the density of TIL such as CD8+, CD4+/Foxp3- T cells and NK cells in the mice treated by H1/pIL-2 and 5% Glucose, respectively (n=4, “***” means P<0.001, student’s t-test).
Fig. 1

A

ITR CAG EGFP cDNA WPRE BGHpolyA ITR

ITR CAG IL2 cDNA WPRE BGHpolyA ITR

ITR CAG Luciferase cDNA WPRE BGHpolyA ITR

B

![Image showing 200 nm scale]

C

H1/pEGFP H1/pIL-2

460 bp → IL-2

200 bp → GAPDH

D

24 hours 48 hours 72 hours

Exposure time 1 minute

ROI = 39662
ROI = 23468
ROI = 15736

E

![Graph showing ROI/min vs. days after I.S. Injection]

F

GFP PI Merge

5% Glucose

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Fig. 2

A

![Graph A: Tumor Volume (mm$^3$) vs. Days after tumor cell inoculation](image)

- 5% Glucose
- H1/pEGFP 50ug
- pIL-2 50ug
- H1/pIL-2 25ug
- H1/pIL-2 50ug

B

![Graph B: Percentage of Survival vs. Days post tumor cell inoculation](image)

- 5% Glucose
- H1/pEGFP 50ug
- pIL-2 50ug
- H1/pIL-2 25ug
- H1/pIL-2 50ug
Fig. 3

A

B

C

Percentage of Survival

Tumor Volume (mm³)

Days after tumor cell inoculation

Days after tumor cell inoculation

Days after tumor cell inoculation

H1/pIL-2 50ug

rAdv-IL-2 Multiple

H1/pIL-2 untreated

H1/pEGFP untreated

H1/pIL-2 treated

H1/EGFP treated
Fig. 5

A

B

C

D

Percentage of PBL

CD4+ CD8+ CD49+

H1/pEGFP 50ug
H1/pIL-2 25ug
H1/pIL-2 50ug

% Glucose

H1/pEGFP 50ug
H1/pIL-2 25ug
H1/pIL-2 50ug

Concentration(pg/ml)

IL1 IL2 IL5 IL6 IL10 IFN TNF GM IL4 IL17
Fig. 6

A

CD8-FITC  DAPI  Merge

H1/pIL-2

5% Glucose

B

CD8+ T cells/mm² AOI

H1/pIL-2  5% Glucose

C

CD4-PE  DAPI  Merge

H1/pIL-2

5% Glucose

D

CD4+ T cells/mm² AOI

H1/pIL-2  5% Glucose

E

CD49-PE  DAPI  Merge

H1/pIL-2

5% Glucose

F

NK cells/mm² AOI

H1/pIL-2  5% Glucose
Molecular Cancer Therapeutics

Effective Melanoma Immunotherapy with Interleukin-2 Delivered by a Novel Polymeric Nanoparticle

Hong Yao, Samuel S. Ng, Long-Fei Huo, et al.

Mol Cancer Ther Published OnlineFirst April 25, 2011.

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