RESEARCH ARTICLE

Reconstructed Adeno-Associated Virus with the Extracellular Domain of Murine PD-1 Induces Antitumor Immunity

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Abstract

Background: The negative signaling provided by interactions of the co-inhibitory molecule, programmed death-1 (PD-1), and its ligands, B7-H1 (PD-L1) and B7-DC (PD-L2), is a critical mechanism contributing to tumor evasion; blockade of this pathway has been proven to enhance cytotoxic activity and mediate antitumor therapy. Here we evaluated the anti-tumor efficacy of AAV-mediated delivery of the extracellular domain of murine PD-1 (sPD-1) to a tumor site. Material and Methods: An rAAV vector was constructed in which the expression of sPD-1, a known negative regulator of TCR signals, is driven by human cytomegalovirus immediate early promoter (CMV-P), using a triple plasmid transfection system. Tumor-bearing mice were then treated with the AAV/sPD1 construct and expression of sPD-1 in tumor tissues was determined by semi quantitative RT-PCR, and tumor weights and cytotoxic activity of splenocytes were measured. Results: Analysis of tumor homogenates revealed sPD-1 mRNA to be significantly overexpressed in rAAV/sPD-1 treated mice as compared with control levels. Its use for local gene therapy at the inoculation site of H22 hepatoma cells could inhibit tumor growth, also enhancing lysis of tumor cells by lymphocytes stimulated specifically with an antigen. In addition, PD-1 was also found expressed on the surfaces of activated CD8+ T cells. Conclusion: This study confirmed that expression of the soluble extracellular domain of PD-1 molecule could reduce tumor microenvironment inhibitory effects on T cells and enhance cytotoxicity. This suggests that it might be a potential target for development of therapies to augment T-cell responses in patients with malignancies.

Keywords: PD-1 - sPD-1 - B7H1 - gene therapy - tumor immunotherapy

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Introduction

Currently cancer gene therapy using recombinant DNA technology provides a new and promising treatment models aim to destruction the malignant growth (Qian et al., 2004; Xing et al., 2011). A variety of gene therapy-based anticancer strategies have been tested in animal tumor models, including selective activation of prodrugs, genetic immunotherapy, antiangiogenic actions and replacement of tumor suppressor genes (Sangro et al., 2002; Borghouts et al., 2005; Cao et al., 2010), successful of these approaches was depend on efficient gene transfer process, numerous gene transfer vectors have been developed, most of these approaches have used retroviral vector producer, naked DNA, oligodendromer DNA coatings, electroporation or adenoviral vectors (Qian et al., 2000; Cross et al., 2006). However, retroviral vector producers may be impractical for human use due to low titer of the vector, and the transfer of naked DNA is typically an inefficient, transient process; adenoviral-mediated gene transfer is also complicated by transient transgene expression as well as induce immune responses against adenoviral antigens expressed on transduced cells (Streck et al., 2006). Adeno-associated virus (AAV) is a recently identified, nonpathogenic, helper-dependent member of the parvovirus family and becoming a promising alternative in cancer gene therapy which offer some potential advantages for gene transfer as compared to other gene delivery systems just mentioned. One advantage is the ability to transduce differentiated cells; another advantage, unlike other vectors of viral origin, it does not encode any wt viral genes and, hence, is less immunogenic. A third potential advantage is the capacity for persistent transgene expression (Aalbers et al., 2011). During the last few decades, many efforts have been made to create safe and effective tumor Immunotherapy strategies with the objective of tumor eradication (Rosenberg., 2001; Aguilar et al., 2011; Danylesko et al., 2012), including immune-activating antibodies, co-stimulation, tumor antigen vaccines and modified cancer cells are generally aimed to inducing CTL responses against tumor cells (Qiu et al., 2009). T lymphocytes represent a crucial component

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of the antitumor immunity; Ag-induced antitumor T cells activation and proliferation are regulated by both positive and negative co-stimulatory molecules (Phan et al., 2003; Capece et al., 2012). Although there are several potential mechanisms that could contribute to the resistance of solid tumors to host immunity surveillance, a major consideration is the engagement of negative regulatory receptors on activated T cells by ligands expressed in the tumor microenvironment (Blank et al., 2004; Topfer et al., 2011). Among these negative regulatory receptors, PD-1 is the most important one (Rozali et al., 2012), It has two known cognate ligands, B7-H1(PD-L1) and B7-DC (PD-L2), PD-Ls mRNA is not restricted to immune cells and can be found in other non lymphoid tissues such as heart, lung, liver and skeletal muscle cells (Hofmeyer et al., 2011). Furthermore, PD-L1 is expressed on tumors and subsequently contributes to the tumor immune evasion (Dong et al., 2002; Zeng et al., 2011). Recent evidence indicates that, the injection of specific anti-B7-H1Abs was inhibited the tumor growth in mice (Files et al., 2011). In the present study, we constructed a rAAV vector mediated-extracellular domain of PD-1 (sPD-1) expression, to counteract the negative immunoregulatory signals provides by PD-Ls/PD-1 pathway, and the improvement of cytotoxicity of T cells on tumor cells. We report the finding, the expressed sPD-1 could block the PD-Ls/PD-1 interactions, and local gene transfer of sPD-1 in tumor inoculation sites potently inhibited tumor growth and results in prolonged survival of mice. These results gain further insight into the function of sPD-1 expressed on tumor tissues in regulating antitumor immune response.

Materials and Methods

Mice and cell lines

Female BALB/c (H-2d) mice were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China) and housed in specific pathogen-free conditions. All studies involving mice were approved by Hubei province Animal Care and Use Committee. Murine H22 hepatoma cell line and 293T human embryonic kidney cell lines were purchased from China Center for Type Culture Collection (Wuhan, China). The cells were cultured in complete medium DMEM supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 100 μg/ml penicillin, and 100U/ml streptomycin. All cell culture reagents were obtained from GIBCOL, USA.

rAAV vector production and purification

All vectors used in this study are kindly provided by Feng Zuo Hua (Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science & Technology), while the enzymes used for construction of recombinant AAV were products of Promega (Madison, WI). The rAAV vector used in this study is based on the pAAV-MCS plasmid, which expresses the GFP gene under the control of human CMV immediate early promoter, for construction of the rAAV plasmid encoding sPD-1; first the DNA fragment containing the sPD-1 amino acids, about 450 coding sequence, was amplified by PCR using the plasmid pcDNA3.1/sPD-1 as a template. The forward primer 5-GGTACATAGAATTCCTGAAGGCGACACTGCC-3, which contained a BamHI site. Second, the sPD-1, PCR product as described above was inserted in the vector to generate pAAV/sPD-1. Cloning and propagation of AAV plasmids was carried out in the DH5α Escherichia coli strain. For Infectious virions particles production, packaging of the recombinant AAV plasmids was done in an adenovirus-free system, purification of virions was done by discontinuous iodixanol gradient centrifugation followed by affinity purification on a heparin-agarose column, as described (Zacchigna et al., 2004). Particle titers of the purified virions were determined by quantitative slot blot analysis as described previously (Ponnazhagan et al., 2004).

Intratumoral injection of rAAV/sPD-1 and tumor surveillance in vivo

Tumors were established by injection of 1×10^5 cells in 100 μl of PBS into right hind thigh muscle. Two days after inoculation, 2.5×10^{11} viral particles in a maximum volume of 400 μl of sterile saline was injected every other day for 6 times. Mice of control group received an equal volume of saline or equal amount of AAV. Tumor growth was inspected by Vernier caliper measurement every other day from day 6 after inoculation. Tumor volume was calculated according to the formula V= (a × b2)/2, with a as the larger diameter and b as the smaller diameter. The mouse survival rate was also recorded.

Semi-quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies) from from hind thigh muscle tissues of normal mice or tissues of BALB/c mice inoculated with H22 cells (3 mice/group). After being treated with DNase I (Promega), equal amounts of RNAs were reverse-transcribed and the cDNAs were amplified by the PCR with gene-specific primers at 94°C for denaturing, 54°C for annealing, and 72°C for extension. β-actin mRNA was used as an internal control and coamplified. Specific primers used were: extracellular domain of PD-1 (440bp), Sense 5'-GGTTCATAAAGCTTTCTGAAGGCGACACTGCC -3', antisense 5'- CCTGGTGAATTCATTGAAACCGGC CTTCTGG-3'; β-actin (542 bp) , sense 5'-ATGGGTCAGA AGGACTCCTATG-3', antisense 5'-ATCTCCTGCTCGA AGTCTAGAG-3'. The amplified products were analyzed by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Immunohistochemistry (IHC) analysis sPD-1 expression on H22 tumor cells

Tumor tissues were surgically excised and fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Immunohistochemistry was performed with the method of SP, as described (Wiendl et al., 2002; He et al., 2004). Goat anti-mouse PD-1 antibody (R&D systems Minneapolis, CA) was used to detect PD-1 expression at a dilution of 1: 200. A biotinylated secondary antibody was then added and visualized with streptavidin- labeled horseradish

peroxidase and chromogen solutions.

Histological analysis of Tumor infiltrating lymphocytes

Tumor tissues were surgically excised. Hematoxylin and eosin (H&E) staining was performed on sections 10 μm in thickness of 4% paraformaldehyde -fixed tissue. Sections were prepared on multiple levels, and 20-30 randomly chosen slice per group were evaluated for lymphocytic infiltration by a third-party pathologist.

Stimulation of spleen cells by HSP70- H22 peptide complex

Spleen cells were prepared and stimulated by HSP70-H22 peptides complex in vitro as previously described (Feng et al., 2002; Geng et al., 2006). The splenocytes were cultured at the concentration of 1 × 10^7/ml in RPMI 1640 supplemented with 20 U/ml IL-2 (PeproTech, London, UK) in a 96-well culture plate in the presence of 0.75 mg/ml of HSP70- H22 peptide complex. The cells were passaged and re-stimulated with HSP70-peptide complex every 2 days for 3 times. At each time of passages, half of spleen cells were harvested for analysis PD-1 expression on activated splenocytes.

Cytotoxicity assay

Splenocytes from tumor-bearing mice were cultured at 1×10^7 cells/ml and Re-stimulated with 0.75 μg/ml of HSP70-H22 peptide complex for 2 days. H22 target cells were labelled with Na51CrO4 (0.1 μCi/106 cells; Amersham Pharmacia Biotech) at 37°C for 1 h. After extensive washing, target cells were incubated with effectors cells at different E: T ratios in triplicate at 37°C for 4 h, and 51Cr released (cpm) into the supernatants was measured in a gamma-counter to calculate percentage specific release. Specific lysis was determined as follows: percent specific release = 100 × (experimental release - spontaneous release) / (maximum release – spontaneous release). Spontaneous release was ≤ 20% of maximum release in all experiments.

Statistics analysis

Results were expressed as mean values ± SD and the difference were determined by ANOVA test, except for survival rate determined by Wilcoxon's rank-test. A value less than 0.05 ($P<0.05$) was used for statistical significance.

Results

Construction and purification of rAAV/sPD-1

To achieve a constitutive, high-level expression of sPD-1, its PCR product about 450bp (Figure 1a), was cloned under the control of the (CMV) promoter to obtain the pAAV/sPD-1 plasmid, in which the expression cassette is flanked by the AAV inverted terminal repeats (Figure1b). This same construct was packaged in a helper virus-free system and purified using discontinuous gradient centrifugation and affinity chromatography. The vector titer was 2.38 × 10^{11} viral particle/ml. The GFP expression, which used as control was detected, as showed in (Figure 1c).

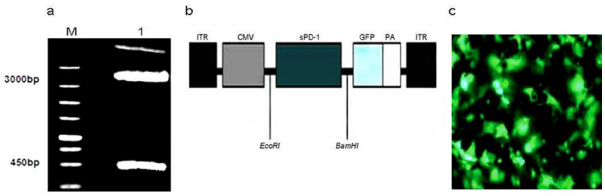

Figure 1. (a) sPD-1 Amplification. M:DL 5000 DNA Markers; Lane 1 sPD-1 PCR Product about 450 bp; **(b) Schematic Representation of the AAV/sPD-1 Expression Vector Used in this Study.** ITR, AAV terminal repeat sequences; CMV, cytomegalovirus promoter; GFP, green fluorescent protein gene and pA, polyadenylation site. Cloning restriction sites are indicated. **(c) Fluorescence Microscopy of 293t Cells**, transfected with pAAV/sPD-1 recombinant plasmid using triple plasmid transfection system, 72 hour post-transfection GFP-positive 293t cells

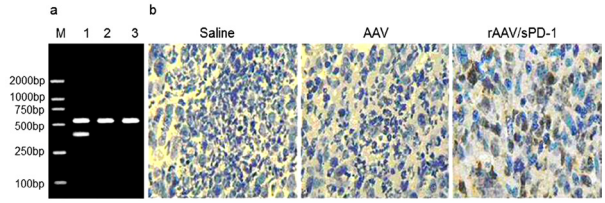

Figure 2. Analysis of Engineered Expression of sPD-1 in Vivo. (a) RT-PCR analysis of mouse sPD-1 gene expression in tumor. Primers annealing to sequences of sPD-1 cDNA generated a PCR product about 440 bp. Mouse â-actin was PCR amplified as a positive control. M: DL 2000 DNA Markers; Lane 1, AAV/sPD1 treated tumor; Lane 2, vector treated and lane 3, saline treated tumor. (b) Tumor tissue immunostaining (brown) revealed the presence of sPD-1 protein in rAAV/sPD-1 treated mice but not in saline and vector treated mice

Intratumoral gene transfer results in sPD-1 expression in situ

To determine whether rAAV/sPD-1 was efficiently taken up and direct sPD-1 expression in vivo. The mice tumor model was established by implantation of H22 cells into immune competent mice. The tumors were then treated by an intratumoral injection of saline, AAV, or rAAV/sPD-1. The expression of sPD-1 mRNA in tumor tissue was examined by RT-PCR. RT-PCR analysis of tumor homogenates revealed that sPD-1 mRNA was expressed in situ in rAAV/sPD-1 treated mice (Figure 2a), whereas, control homogenates from saline treated and vector treated tumors did not expression sPD-1 mRNA. Tumors were also sectioned 10 days following gene transfer, and immunohistochemical staining confirmed that considerable numbers of H22 tumor were stained with the anti-PD-1 Abs, which indicated express of sPD-1 on tumor cells. As expected, control sections from saline treated and vector treated tumors were not stained with the anti-PD-1 antibody (Figure 2b).

Regression of Established Tumors Following Intraumoral Injections of rAAV/sPD-1

We next tested whether intratumor injection of rAAV/sPD-1 would affect antitumor immunity. All mice inoculation with H22 tumor cells developed progressively growing tumors, and the mice treated with rAAV/sPD-1 also developed tumors. However, intratumoral injection of 2.5 × 10^{11} vector particles of rAAV/sPD-1 resulted in a

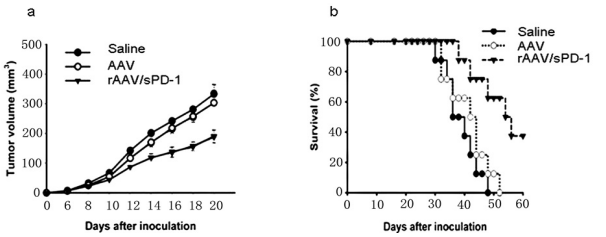

Figure 3. Intratumoral Injection of rAAV/sPD-1 Inhibits Tumor Growth. BALB/C Mice were Inoculated with 1×10^5/H22 Cells and Received Different Treatment as Described in Materials and Methods. Tumor sizes (a) were measured (mean diameter ±SD) every other day, starting on day 6, and survival time (b) was monitored. Each experiment group had 8 mice and the data were obtained from two independent experiments

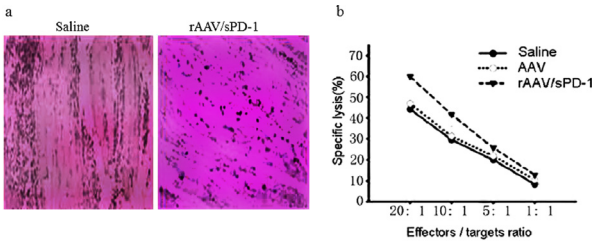

Figure 4. Expression of sPD-1 Enhances Antitumor Response. (a) Comparison of immune cell infiltration in rAAV/sPD-1 treated mice and control saline treated mice. Histological examination revealed severe immune cells infiltrate in rAAV/sPD-1 treated mice. (b) Expression of sPD-1 induced H22 specific CTL in vivo. Spleen cells were harvested and restimulated with HSP70-H22 peptide complex, and their cytolytic activity against H22 was determined in 4h – 51Cr release assay. Results are expressed as the mean ± SD of triplicate wells

significant inhibition of tumor growth. By day 20, at the time of sacrifice, the mean tumor volume in the saline treated animals was about 333.20 mm^3. And the mean tumor volume in the vector treated animals was about 303.33 mm^3, whereas in animals treated with rAAV/sPD-1, the tumor volume on average was 189.66 mm^3 (Figure 3a). In a separate experiment using the same protocol, the animal survival time also was monitored between rAAV/sPD-1 treated mice, vector treated mice and saline control mice. The mean survival time of the mice treated with saline and AAV was 39.00 ± 4.52 days and 41 ± 5.48, respectively, whereas, the mean survival time of mice receiving rAAV/sPD-1 was significantly prolonged ($P<0.01$) with 37.5% of mice surviving to day 60 (Figure 3b).

Induction of specific CTLs in mice treated with rAAV/sPD-1

Given the above results, we wished to determine whether the slower growth of rAAV/sPD-1 treated mice was due, at least in part, to the augmented immune cell response. Immune cells infiltration was evaluated by histological examination. On day 20 in the rAAV/sPD-1 treated mice showed a prominent infiltration of lymphocytes when compared with control treated tumors, which was possessed a larger capsule, displayed dense infiltration of mononuclear cells, neutrophils and lymphocytes. Also did not show muscle fiber degeneration, necrosis, as well as atrophy (Figure 4a). Tumor infiltration

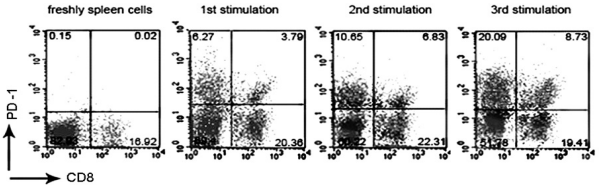

Figure 5. Induction of PD-1 Expression on CD8+ T Cells. The percentage of cells present in (top right) each quadrant was indicated

of both mononuclear cells and neutrophils may participate in the killing of residual tumor cells .To demonstrate PD-1 specific CTL, spleens were harvested from two mice/experimental group at the 20th day and re-stimulated with HSP70-H22 peptide complex. The splenocytes were then used in a standard 51Cr release assay for CTL activity against H22 targets. In mice injected with rAAV/sPD-1, significant increase in CTL activity against H22 cells was detected compared to CTL from vector treated mice (Figure 4b). The results indicated that expression of sPD-1 enhances the immunogencity of H22 tumor cells.

Expression of PD-1 on tumor-peptides activated CD8+ T cells

The existence of endogenous PD-1 on lymphocyte in antitumor immunity might help to understand the effect of expression of PD-1. To this end, we stimulate spleen cells with tumor-peptide complex in vitro and then detect PD-1 expression. Although freshly isolated spleen cells did not express any detectable amount of PD-1, considerable number of stimulated CD8+ T cell expressed PD-1. Furthermore, along with the rounds of stimulation, the percentage of PD-1 positive CD8+ T cells were increased (Figure 5).

Discussion

Expanding tumor resistance to immune system and continue to grow could become a major problem facing researchers to establish effective cancer immunotherapy. In fact, tumor growth was seldom controlled, despite of tumor infiltration of immune effectors cells, it have been clear that the tumor microenvironment can protect tumor cells from immune destruction. Cytotoxic T. lymphocyte cells represented one of the most important effectors cells in anti tumor immunity mechanisms. T cells activation and expansion are regulated by both positive and negative co-stimulatory receptors (Dulgerian et al., 2011). Several studies documented the usage of co-stimulatory molecule as an antitumor immunotherapy to trigger and enhance strong T-cell response against tumor by different combination approaches (Xiao et al., 2007). Programmed death-1(PD-1) is the type I transmembrane immunoinhibitory receptor expressed on activated T and B cells, consist of single extracellular Ig-like variable (IgV) domain and an intercellular part contains an immunoreceptor tyrosine-based inhibitory motif (Xu et al., 2006), which delivers negative signals upon ligation to its two ligands B7-H1 and B7-DC (Youngnak et al., 2003; Yokosuka et al., 2012). PD-L1 a recently identified co-inhibitory molecule belonging to the B7 family molecules (Lee et al., 2005), it is expressed in cells of lymphocyte

lineage and is also found in the tissues of various organs, including non-lymphoid organs, such as the heart, lung, placenta, kidney, and liver and several tumor cell lines (Freeman et al., 2000; Paterson et al., 2011), and it can promote the apoptosis of tumor specific CTL and leads to immune evasion of tumor (Dong et al., 2003; Wong et al., 2007). Studies carried out recently have revealed B7-H1 considered as effective molecular target for tumor immunotherapy. Generally they agreed, the blockade of B7-H1/PD-1 interactions pathway efficiently reduces tumor growth and improves survival (Qiu et al., 2009; Cao et al., 2011). Previously a eukaryotic expression plasmid (pPD1) encoded sPD-1 was constructed and its role to enhancement the anti tumor immunity was proven (He et al., 2005). In spite of efficient uptake and expression of gene delivered by naked plasmids, some limitations are restricting the usefulness of this method such as the gene transfer is limited to a small percentage of the cells near the injection site, in addition to the plasmid DNA remains episomal that present only transiently and the immediate precludeing long-term correction. In current study we used the rAAV to deliver sPD-1 into tumor sites, which can transduce a wide variety of cells, including liver, cardiac muscle, eyes, brain, lung and muscle cells, with the efficient cellular uptake, stable integration, low immunogenicity and have potential for long-term expression (Li et al., 2005; Keswani et al., 2012). Here we investigate the function activity of sPD-1 expression on nonlymphoid tissues in tumor inoculation model. Our results reveal that expression of sPD-1 on tumors tissues can promote tumor-specific immunity, a striking effect on local tumor infiltration was observed in immunocompetent mice, which might be correlated with retarded tumor growth and prolonged the survival of tumor-bearing mice. Our findings thus indicate that enforced expression of sPD-1 on tumor cells could stimulation of an effective antitumor response. The stimulation of an effective antitumor response following sPD-1 expression could be attributed to co-stimulatory molecules regulation. While this effect may be a consequence of the activation of a larger number T lymphocytes. It is equally likely to result from the removal of an inhibitory signal involved in T cell.

Acknowledgements

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