Regulating Immunity and Inhibiting Tumor Growth by the Recombinant Peptide sPD-1-CH50

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Abstract. *Background: Soluble programmed death-1 (sPD-1) has been proven to bind PD-1 ligands (PD-Ls), block PD-L/PD-1 interactions, and enhance the cytolysis of cytotoxic T-lymphocytes (CTLs). CH50, a two-domain molecule of fibronectin, has been shown to suppress the development of tumors via enhancing the activity of macrophages. This study evaluated the influence of the recombinant peptide sPD-1-CH50 on antitumor immunity. Materials and Methods: A secretory recombinant peptide, sPD-1-CH50, containing three functional domains was constructed, and its antitumor effect and mechanism were studied. Results: The data demonstrated that sPD-1-CH50 was able to increase the cytolytic activity of both macrophages and CTLs, especially towards B7-H1-positive tumor cells. The effect correlated with the enhanced production of inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). In vivo studies showed that sPD-1-CH50 inhibited the invasiveness and growth of hepatoma. Conclusion: Regulation of immune cells by sPD-1-CH50 has potential therapeutic value for the inhibition of invasion and growth of hepatoma.*

Antitumor immune responses involve cytotoxic T-lymphocyte (CTLs)-mediated specific antitumor immunity, and nonspecific antitumor immunity mediated by natural killer cells and macrophages. At present, immunotherapy strategies, including modified cancer cells, tumor antigen vaccines, co-stimulation, and immune-activating antibodies, are generally aimed at triggering CTL responses to inhibit and kill tumor cells (1, 2). Although these approaches can elicit some T-cell responses, these often do not correlate with regression of tumors. This has been attributed to functional deficiencies or tolerance of T-cells, including suppression, anergy, ignorance, and programmed death in the microenvironment of tumors (3-5).

Programmed death-1 (PD-1) is a receptor expressed on T-cells, which can negatively regulate T-cell receptor signaling by interacting with its specific ligands, B7-H1 and B7-DC, also known as PD-L1 and PD-L2, respectively (6). Studies suggest that the differential binding properties of B7-H1 and B7-DC may be responsible for their differential contributions to immune responses (7). Tumor-associated B7-H1 is thought to contribute to immune escape of cancer cells by interacting with the PD-1 receptor, which promotes apoptosis of activated effector T-cells, and to decrease the susceptibility of tumor cells to lysis by activated T-cells. Blockade of B7-H1 by specific monoclonal antibodies can enhance the tumor-specific CTL response and cause tumor rejection (8-11). In previous studies, we constructed a eukaryotic expression plasmid (pPD-1A) that expresses the extracellular domain of murine PD-1 (sPD-1). This protein can bind PD-1 ligands, block PD-L/PD-1 interactions, and enhance the cytotoxicity of tumor-specific CTLs, up-regulating the expression of 4-1BB, B7-1, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) by lymphocytes and down-regulating that of OX40 (CD134) and interleukin-10 (IL-10) (12-14).

Activated macrophages are the most important effector cells in nonspecific antitumor immunity and can kill tumor cells prior to the effect of CTLs. Fibronectin (FN) is a multi-domain functional glycoprotein that is part of the extracellular matrix. The bifunctional domain recombinant peptide CH50 contains CellI and HepII domains and has been prepared in our laboratory (15, 16). In prior studies, we have found that recombinant FN polypeptide CH50 had a regulatory effect on macrophages, and inhibited tumor growth, invasion and metastasis by decreasing the

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expressions of cell division cycle 2(CDC2), αvβ3 integrin and matrix metalloproteinases MMP-2 and MMP-9 in the tumor milieu (17, 18).

Here we constructed a recombinant secretory eukaryotic expression plasmid psPD-1-CH50, containing the coding sequence of the extracellular domain of murine PD-1 (sPD-1) and the CellI-HepII domains of the CH50 polypeptide, to investigate further the antitumor activities of sPD-1-CH50.

Materials and Methods

Cell culture. The baby hamster kidney cell line (BHK) and hepatoma cell line H22 were obtained from the China Type Culture Collection (CCTC Wuhan, China) and cultured in DMEM (Invitrogen Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml streptomycin, and 100 mg/ml penicillin (complete medium). T-lymphocytes and macrophages were isolated from murine spleen and peritoneal cavity, respectively, as described elsewhere (12, 19). The cells were cultured at 37˚C in a humidified incubator with 5% CO_2.

Construction of expression plasmid vectors. Recombinant plasmids pPD-1A and pCH510 were constructed by our laboratory, and contained the coding sequences of the extracellular domain of murine PD-1 (sPD-1) and CellI-HepII domains of FN, respectively (12, 15). To construct the secretory expression plasmid psPD-1-CH50, carrying a cDNA encoding sPD-1 and CH50, specific primers were used as follows: *sPD-1* sense 5'-GGTTCATA AAGCTTTCTGAAGGCGAC ACTGCC-3', containing a *HindIII* restriction site, antisense 5'-TTTGCGGCCGCAGATC CTCCACCTCGTGATTGAAACCG-3', containing the linked peptide SRGGGS coding sequence and a *NotI* restriction site; *CH50* sense 5'-GATATAGCGGCCGCTCCC ACTGACCTG-3', containing a *NotI* restriction site, antisense 5'-TGCACGTCTAGACGCTTGCTTTGACT-3', containing a termination codon and an *XbaI* restriction site. The DNA was amplified by PCR with primers and templates, pPD-1A and pCH510. The amplified fragment was digested by restriction enzymes *HindIII*, *NotI* and *XbaI* (Promega, Madison, WI, USA), and inserted into compatible enzyme restriction sites of pSecTagA (Invitrogen Life Technologies, Carlsbad, CA, USA). The plasmids psPD-1 and pCH50 were constructed by inserting the DNA sequence of sPD-1 and that of CH50 into pSecTagA, respectively. All constructed plasmids were identified by sequencing and compared with the published sequences.

Cell transfection and expression analysis. For measurement of the biological activity of secretory recombinant proteins sPD-1-CH50, sPD-1 and CH50, BHK cells were transfected with the plasmids psPD-1-CH50, psPD-1, pCH50 and pSecTagA, using the Dosper liposomal transfection reagent (Boehringer Mannheim, Mannheim, Germany) following the manufacturer's instructions. After transfection for 48 h, cells were observed and analyzed using RT-PCR. For stable transfection, transfected BHK cells were selected on the basis of G418 resistance (800 μg/ml) for 3 weeks. The expression product in the supernatant was analyzed by both SDS-PAGE and Western blot (19). Anti-FN (Ab-1) mouse mAb (N-294) was purchased from Merck Ltd. (Darmstadt, Germany). Goat anti-mouse PD-1 polyclonal Ab was obtained from R&D Systems (Minneapolis, MN, USA). The culture supernatant of stably transfected BHK cells was collected and used for biological analysis.

ELISA for chemokines. Murine peritoneal macrophages were cultured with the supernatant of stably transfected BHK cells in 96 well culture plates for 24 h and 48 h. Production of TNF-α and IFN-γ in the supernatant was measured by ELISA, using murine TNF-α or IFN-γ ELISA Kit (eBioscience, San Diego, CA, USA), according to the manufacturer's protocol.

Conventional reverse transcription-PCR and real-time PCR. Total RNA was isolated, using TRIzol reagent, from cells or muscle tissues of normal mice or tumor marginal tissues of tumor-bearing mice. RT-PCR was used to determine relative quantities of mRNA (One-Step RT-PCR kit; Qiagen, Valencia, CA, USA). Twenty-eight PCR cycles were used for all of the analyses and the mRNA of *β-actin* was used as the internal control. The primers for detection of mRNA of *sPD-1*, *CH50* and *sPD-1-CH50* were described above. Quantitative real-time PCR for *iNOS, TNF-α, IFN-γ* and *β-actin* genes was performed as described previously (20). The results were expressed as the expression level of each gene relative to that of housekeeping gene *β-actin*.

Cytotoxicity assay. Standard ^{51}Cr-release assays were performed. Briefly, target H22 cells cultured with the supernatants of BHK cells transfected with psPD-1, pCH50 or psPD-1-CH50 for 1 h, were labeled with $Na^{51}CrO_4$ (0.1 μCi/10^6 cells; Amersham Pharmacia Biotech, Piscataway, NY, USA) at 37˚C for 1 h. After extensive washing, target cells were incubated with effectors, spleen T-lymphocytes or macrophages, at different effector:target (E:T) ratios in triplicate for 4 h at 37˚C, and ^{51}Cr release (cpm) into the supernatants was measured in a gamma counter to calculate the percentage specific release. The percentage specific lysis was calculated by the formula: percentage specific release=100× (experimental cpm – spontaneous release)/(maximum release – spontaneous release). Spontaneous release was ≤20% of maximum release in all experiments.

Gene therapy protocol. Six-week-old female BALB/c mice (Center of Medical Experimental Animals of Hubei Province, Wuhan, P.R. of China) were injected with 1×10^5 H22 ascitic hepatoma cells into the right hind thigh muscle. On day 3 after inoculation, mice from the treatment groups received 100 μg of plasmid DNA by *i.m*. injection (local naked DNA transfection). Equal dosage of psPD-1 and pCH50 (100 μg each) were used in the combination treatment. Plasmids were injected every 3 days for six times. Mice of the control group received an equal volume of saline or an equal amount of pSecTagA. Plasmid DNA used for gene therapy was prepared as described previously (21). Tumor size was measured using calipers fitted with a Vernier scale when the tumor could be palpated. The tumor diameter was calculated using the formula $(a\times b)/2$, where a is the larger diameter and b is the smaller diameter. The average inhibition rate percentage=[(average tumor weight of pSecTagA plasmid control group – average tumor weight of treated group)/(average tumor weight of pSecTagA plasmid control group)] ×100%.

Histological analysis. Mouse muscle tissues of injection sites from treated mice were surgically excised, fixed for 12-24 h in 4% formaldehyde, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (H&E) according to standard procedures.

Statistical analysis. Each experiment was performed 3 to 5 times, and results are expressed as the mean±SD. Student's *t*-test was used to assess significant differences, with $p<0.05$ being considered as significant. Western blot analysis data show representative data of at least three independent experiments.

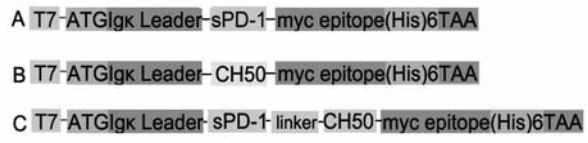

Figure 1. *Schematic of the three recombinant peptides used in this study. All constructs have an N-terminal Igκ leader and C-terminal polyhistidine tag (His tag). The linked peptide amino acid sequence inserted between sPD-1 and CH50 is SRGGGS. Plasmids: A, psPD-1; B, pCH50; C, psPD-1-CH50.*

Results

Plasmid construction, expression and characterization. Three new recombinant peptide constructs, psPD-1, pCH50 and psPD-1-CH50, were designed for this work (Figure 1). In all constructs, an Igκ leader and a polyhistidine tag were included on the *N*-terminus and *C*-terminus of the protein, respectively. A linked peptide sequence, SRGGGS, was inserted between the sPD-1 and CH50 domains in the construct sPD-1-CH50. DNA sequencing confirmed that the sequences were identical to those published. The transcription and expression of the recombinant protein sPD-1-CH50 were identified by RT-PCR and Western blotting respectively. The transcription of sPD-1 was detected in BHK cells transfected with psPD-1 and psPD-1-CH50. *CH50* transcription was observed in the pCH50 and psPD-1-CH50 groups. The expression of the sPD-1-CH50 transcript was only examined in BHK cells transfected with the plasmid psPD-1-CH50 (Figure 2A). Western blots showed similar results (Figure 2B). SDS-PAGE was performed to identify the molecular weight of the recombinant protein in cell culture supernatants. The result of RT-PCR, SDS-PAGE and Western blot demonstrated that the molecular weight of sPD-1, CH50 and sPD-1-CH50 was 20 kDa, 31 kDa and 51 kDa respectively.

Activating murine peritoneal macrophages by sPD-1-CH50. To examine the biological effect of sPD-1-CH50, the supernatant of stably transfected BHK cells was harvested, and then co-incubated with macrophages for 24 to 48 h. Levels of IFN-γ, TNF-α and iNOS were tested with ELISA and RT-PCR.

IFN-γ, which is often found as the regulatory cytokine in the nitrite production pathway, was found to be enhanced by psPD-1-CH50, at both the protein (Figure 3A) and mRNA levels (Figure 3C). The levels of IFN-γ in the psPD-1 and pCH50 groups were also higher than that in the pSecTagA group, but lower than that in the sPD-1-CH50 group. The level of TNF-α was also higher in both the sPD-1-CH50 and the CH50 groups, detected as the protein (Figure 3B) and mRNA (Figure 3C). In addition, in the psPD-1-CH50 group, there was a 1.8-fold increase of the mRNA level of iNOS compared with the pSecTagA group, which was higher than in the psPD-1 and pCH50 groups (Figure 3D). Thus, sPD-1-CH50 was able to activate the macrophages, and the effect was stronger than that of CH50 and not less than that of the sPD-1/CH50 combination.

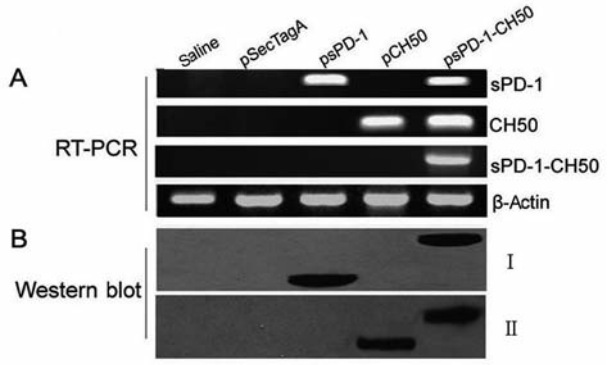

Figure 2. *Expression of secretory recombinant proteins sPD-1, CH50 and sPD-1-CH50 by BHK cells transfected with the corresponding plasmids. A, Relative quantitative real-time PCR analysis of sPD-1, CH50 and sPD-1-CH50 transcription. Each RNA sample was analyzed in triplicate at two RNA concentrations. B, Western blot analysis of sPD-1, CH50 and sPD-1-CH50 in the supernatants of BHK cells. I: analysis by using anti-PD-1 antibody; II: analysis by using anti-FN antibody. Each sample was analyzed in triplicate. Lane 1, saline was transfected into BHK cells; lane 2-5, the plasmids pSecTagA, psPD-1, pCH50 and psPD-1-CH50, respectively, were transfected into BHK cells.*

Blocking PD-1/B7-H1 interactions with sPD-1-CH50 and enhancing the cytotoxicity of spleen lymphocytes and macrophages. Our previous study showed that IFN-γ helps up-regulate the expression of B7-H1 in tumors (14), and that the latter mediates tumor resistance to immune attack. sPD-1 can bind specifically and efficiently to B7-H1 and block its function. To test whether the recombinant protein sPD-1-CH50 expressed by BHK cells was able to bind B7-H1 and block B7-H1-mediated immune resistance, we used 20 ng/ml IFN-γ to stimulate H22 cells for 24 h. FACS analysis data revealed that B7-H1 was hardly detectable on the untreated H22 cells. In contrast, IFN-γ treatment increased the percentage of B7-H1-positive H22 cells to 91.8%, consistent with previous results (13). Treated or untreated H22 cells were used as target cells, and the cytotoxic effect of activated lymphocytes (Figure 3E) and macrophages (Figure 3F) was examined (12, 13). In sPD-1, CH50 and sPD-1-CH50 groups, H22 cells stimulated with IFN-γ were significantly less susceptible to the cytotoxicity of the effector cells than those without stimulation. Treatment with psPD-1-CH50 was able to markedly increase the cytotoxicity of T lymphocytes and macrophages to tumor cells, especially to the B7-H1-positive cells. This effect was stronger than that of psPD-1 and pCH50 singly or in combination. Binding of sPD-1-CH50 to B7-H1 blocked B7-H1-mediated immune resistance of T lymphocytes, and also effectively stimulated macrophages to kill B7-H1-positive cells.

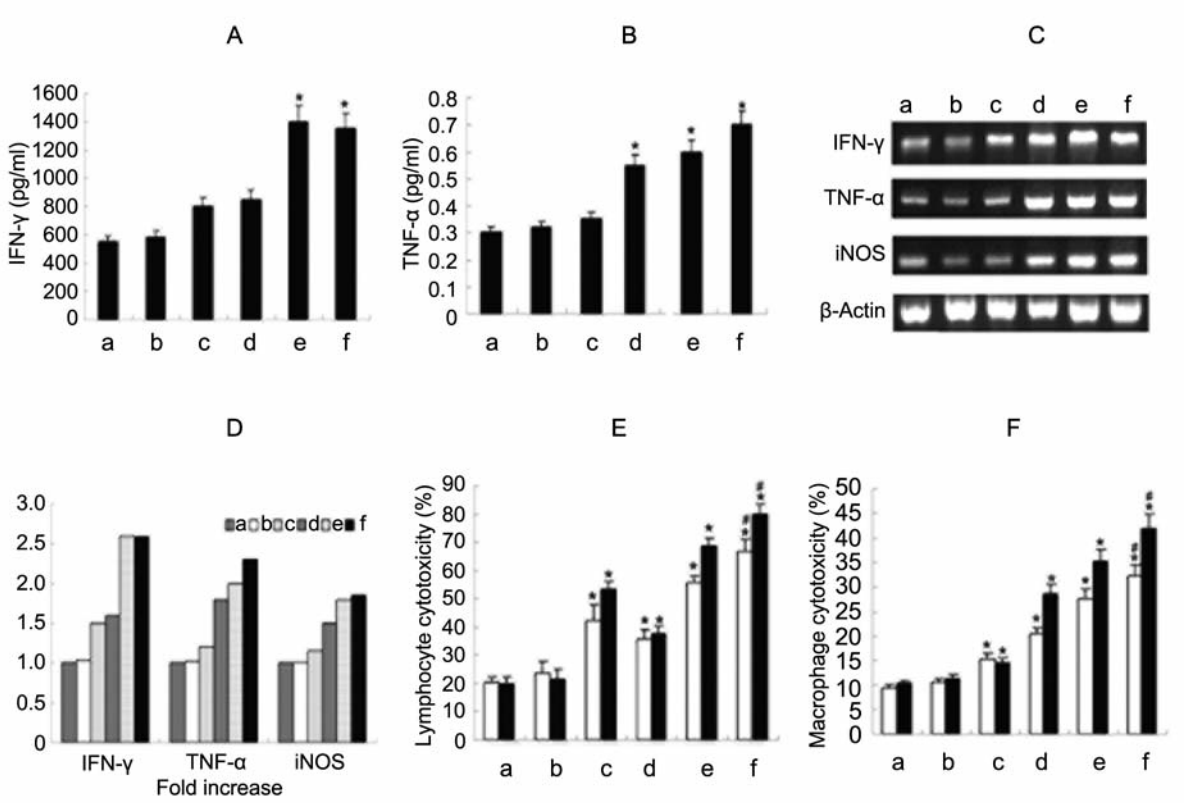

Figure 3. *Regulatory effect of the recombinant peptide sPD-1-CH50 on macrophages and T lymphocytes. A, B, ELISA detection of IFN-γ and TNF-α produced with peritoneal macrophages. C, D, Relative, quantitative real-time PCR analysis of IFN-γ, TNF-α and iNOS transcription in peritoneal macrophages. E, F, Cytotoxicity of spleen lymphocytes and macrophages to H22 cells (E:T=20:1). Target cells were stimulated (black) or unstimulated (white) by IFN-γ. In all cases: a: BHK cell culture supernatant; b: culture supernatant of BHK cells transfected with pSecTagA; c: culture supernatant of BHK cells transfected with psPD-1; d: culture supernatant of BHK cells transfected with pCH50; e: culture supernatant of BHK cells with psPD-1 and pCH50 (the dosage of each plasmid was the same in each group); f: culture supernatant of BHK cells transfected with psPD-1-CH50. Data are representative of three independent experiments. Each value represents the mean±SD; *p<0.05, compared with the control group (a); #p<0.05, compared with the psPD-1/pCH50 group (e).*

The immunotherapeutic effect of sPD-1-CH50 on tumor in vivo. The above results showed that sPD-1-CH50 effectively regulated the immune activities of macrophages and CTLs and exerted an antitumor effect *in vitro*. We next examined whether it could play an antitumor role in tumor-bearing mice *in vivo*. Relative quantitative real-time PCR showed that the transcription activity of treated genes was especially evident in the tumor periphery following the injection of the plasmids (data not shown). Tumors formed in all the animals, and the increase in tumor volume was inhibited by the expression of sPD-1-CH50. The volumes of subcutaneous primary tumors in mice treated with plasmids expressing sPD-1-CH50, sPD-1/CH50, sPD-1 and CH50 were inhibited by 80%, 83%, 50% and 42%, respectively, on day 25 compared to control mice given empty plasmid (n=8, $p<0.05$) (Figure 4). After 25 days' treatment, H&E staining of the tissues from the inoculation sites of these mice showed the existence of a few tumor cells and abundant immune

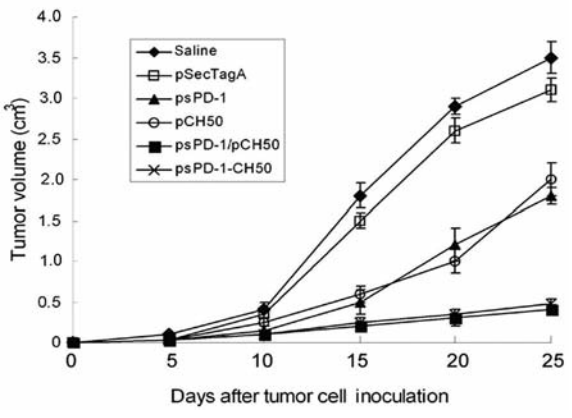

Figure 4. *Immunotherapeutic effect of psPD-1-CH50 on tumors in vivo. Suppression of primary tumor growth by psPD-1-CH50 gene delivery. Each value represents the mean±SD (n=8 in each group). Each condition was evaluated in at least four experiments.*

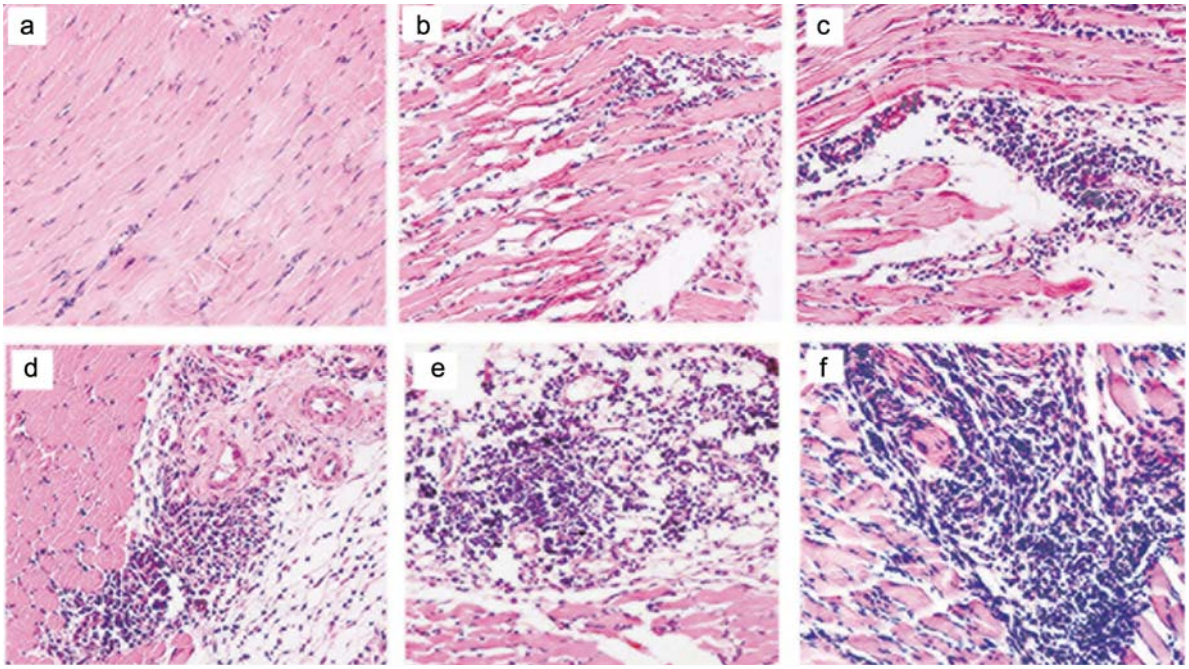

Figure 5. *Transfection with psPD-1-CH50 increased the chemotaxis of immune cells. After the treatment, the tissues from the inoculation site of the mice without palpable tumor were analyzed by H&E staining (×200). Treated with: a, saline; b, pSecTag A; c, psPD-1; d, pCH50; e, psPD-1 and pCH50; f, treated with psPD-1-CH50. Images are representative of multiple microscopic fields observed in at least eight mice per group.*

cells in the plasmid treated groups. Compared with psPD-1 and pCH50 treatment groups, there were more immune cells in the tumor milieu in the psPD-1/CH50 and psPD-1-CH50 groups (Figure 5).

Discussion

CTLs and macrophages are both important effector cells in antitumor immunity. In this study, we hypothesized that a recombinant protein exists that can regulate the activities of CTLs and macrophages simultaneously. We therefore constructed and expressed a recombinant, secretory protein, psPD-1-CH50, using gene cloning techniques, and examined its immune regulatory effect. We observed that, in addition to IFN-γ production, TNF-α and iNOS were also increased by psPD-1-CH50 treatment. Thus, both the oxygen-dependent and oxygen-independent pathways of macrophage killing mechanism were activated by sPD-1-CH50. This result indicates a total activation of macrophages by sPD-1-CH50 (22-25).

To investigate whether sPD-1-CH50 can boost the immune response of the host against tumor cells, we studied the effect of sPD-1-CH50 on the cytotoxic activity of murine peritoneal macrophages and spleen lymphocytes. We observed that sPD-1-CH50 not only enhanced the cytotoxicity of macrophages, but also enhanced the lysis of spleen lymphocytes. These results suggest that sPD-1-CH50 stimulates macrophages and lymphocytes. Blocking B7-H1/PD-1 interactions with sPD-1-CH50, not only removes the resistance of the highly B7-H1-positive tumor cells to the attack of CTLs, but also switches on the target cell killing activity of macrophages towards these cells.

On the basis of our *in vitro* experiments, we used naked plasmid to deliver sPD-1-CH50 into tumor inoculation sites. sPD-1-CH50 stimulates more effective antitumor activities than sPD-1, CH50 and sPD-1/CH50. The recombinant protein sPD-1-CH50 might mediate antitumor activity through at least two possibly related mechanisms: i) the recruitment of immune cells such as T lymphocytes and macrophages into local tissue, and ii) blockade of the interaction of PD-1 with PD-Ls in the tumor milieu. The inoculation sites of these mice had few tumor cells and abundant immune cells.

In summary, the therapeutic strategy with the recombinant protein sPD-1-CH50 boosts the possibility of the development of a successful antitumor immunotherapy, but also adds to the understanding of the synergism of specific and nonspecific immunity in the face of malignancies. It also suggests a method for the clearance of residual tumor cells after surgical removal of the tumor.

Conflict of Interest Statement

The authors declare that there are no financial or personal relationships with other people or organisations that could innapropriately influence (bias) their work.

Acknowledgements

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