Adenovirus-mediated interleukin 21 gene transfer enhances antitumor immunity and reduces tumorigenicity of Hepa1-6 in mice

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Abstract. In the present study, adenovirus-mediated interleukin 21 (Ad5-IL-21-EGFP) gene expression was induced in Hepa1-6 cells to investigate whether IL-21 was capable of enhancing antitumor immunity and reducing tumorigenicity of Hepa1-6 in a mouse model. Mice were inoculated indermally into the right flank with Hepa1-6 cells or Hepa1-6 cells infected with Ad5 or Ad5-IL-21. Four weeks later, the mice were sacrificed humanely, and the tumor volume, tumor weight and mouse spleen index were measured. The levels of IL-21, IL-4 and interferon (IFN)-γ levels in mouse serum and tumor tissues were detected by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry. Cell counting kit-8 (CCK-8) assay was used to detect the killing ability of spleen T cells and natural killer (NK) cells, and the proliferation ability of T cells. The expression of IL-21 was confirmed by reverse transcription-polymerase chain reaction, western blot analysis and ELISA assay in Ad5-IL-21-EGFP-infected Hepa1-6 cells. The overexpression of IL-21 significantly reduced the tumorigenicity of Hepa1-6 cells. The tumor volumes and tumor weights in Ad5-IL-21-Hepa1-6 mice were much smaller than those in the Ad5-Hepa1-6 group and Hepa1-6 wild-type group. The immunohistochemistry and ELISA assay demonstrated that IL-21 and IFN-γ levels were much higher while the IL-4 level was much lower in the Ad5-IL-21-Hepa1-6 group than in the other two groups. CCK-8 assay revealed that the killing ability of NK cells and T cells, and the proliferation ability of T cells in Ad5-IL-21-Hepa1-6 mice were higher than in the other two groups; the spleen index of Ad5-IL-21-Hepa1-6 mice was also higher than in the other groups. The data had a significant difference (P<0.01). In conclusion, IL-21 reduces tumorigenicity of Hepa1-6 by a mechanism involving enhanced activation of cell-mediated immunity in tumor-bearing mice.

Introduction

Interleukin 21 (IL-21), a cytokine identified in 2000 (1), is mainly secreted by activated CD4+ T cells and NK T cells (2,3). It effectively enhances T cell proliferation and the killing function of NK cells, resulting in a strong antitumor immune response (4,5). Its receptor (IL-21R) is widely expressed in various cell types within the immune system, including NK cells, B cells, T cells, macrophages and dendritic cells (6-8). The widespread lymphoid distribution of the IL-21R leads to pleiotropic action of IL-21 in the innate and adaptive immune responses (6-8). For this reason, researchers are becoming increasingly interested in IL-21. Studies have suggested that IL-21 is capable of suppressing growth in certain tumors, and it has already been used in phase I or II trials of patients with melanoma and renal cell carcinoma, with promising antitumor results obtained (9,10). However, IL-21 may also promote growth in certain other tumors, including colitis-associated colorectal cancer by impairing tumor immunosurveillance (11). Therefore, the function of IL-21 is extremely complex, as its use in different cancers has been shown to lead to different results (9-11). Further research is required to investigate this notable cytokine.

Primary hepatic carcinoma (PHC) is currently the fourth most common cause of cancer-related mortality worldwide (12). Its incidence is rapidly increasing (13). To date, there have been no in-depth studies into the use of IL-21 in PHC. In the present study, we used adenovirus-mediated IL-21 gene expression in the mouse hepatic cancer cell line Hepa1-6 to investigate the influence of IL-21 on antitumor immunity and tumorigenicity in mice, with the aim of identifying a novel biological treatment for PHC.

Materials and methods

Materials. Murine hepatocellular carcinoma Hepa1-6 cells and the murine T-lymphoma YAC-1 cell line sensitive to NK cells
were obtained from the Immunology Laboratory of Weifang Medical University (Weifang, China). Mouse hepatoma carcinoma cell line Hepa1-6 cells were maintained in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution. YAC-1 cells were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin solution. The cell counting kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (Haimen, China).

A replication-defective recombinant Ad5 vector encoding mouse IL-21 (named Ad5-IL-21-EGFP) and a replication-defective recombinant Ad5 vector (named Ad5-EGFP) were purchased from Vector Gene Technology Company Ltd. (Beijing, China). The physical titer of the recombinant virus (vp/ml) was $5.3 \times 10^9$, and the infectious titer (TCID50/ml) was $4 \times 10^9$.

SPF male C57BL/6 mice (6-8 weeks old) were purchased from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The present study was approved by the ethics committee of Weifang Medical University.

Preparation of spleen mononuclear cells. The mouse spleen was removed and weighed in a sterile manner. Single spleen cell suspension was prepared by passing through nylon mesh (200-µm pore size; BD Biosciences, Baltimore, MD, USA) with a sterile rubber spatula. Mononuclear cells were obtained by ficoll-hypaque density methods. Briefly, 3-4 ml spleen cell suspension was added slowly on the surface of the ficoll along the tube's wall (so as not to break the interface), then balanced and centrifuged at 2000 $x$ g for 20 min. Mononuclear cells were carefully transferred into another tube. Following further washes with phosphate-buffered saline (PBS), the cells were re-suspended in complete RPMI-1640 supplemented with 10% (v/v) heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin).

Establishment of cell lines. Hepa1-6 cells seeded 24 h earlier were infected with Ad5-EGFP or Ad5-IL-21-EGFP in serum-free DMEM for 2 h, then the infection medium was replaced with normal medium. The infection efficiency was assessed by fluorescence microscopy 24 h later. The Hepa-6 cell line infected with Ad5-EGFP was named Ad5-Hepa1-6, and the Hepa-6 cell line infected with Ad5-IL-21-EGFP was named Ad5-IL-21-Hepa1-6. After cells had been infected with Ad5-IL-21-EGFP or Ad5-EGFP for 24 h, cell culture supernatant was collected to measure the IL-21 expression level using the mouse IL-21 enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go kit (eBioscience, San Diego, CA, USA). Next, total cellular RNA of certain cells was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcription was carried out by converting 0.5 µg RNA into complementary DNA (cDNA) using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The target gene was amplified by polymerase chain reaction (PCR) using the GreenTag™ Ready Mix PCR reaction mix (Sigma-Aldrich). The following primers were used: forward, 5'-CCGCTAGCCTGGAGCTCAGTCTG-3'; and reverse, 5'-CCCAGGTCTTCTAGGAGATGCCTG-3'. Certain cells were washed with pre-cooled PBS twice, and lysed in RIPA buffer containing protease inhibitor cocktail on ice. The protein sample was quantified using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc.; catalog no. 500-0014). Total protein samples were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis prior to electrophoretic transfer to a nitrocellulose membrane. Western blot analysis was carried out in pre-cooled transferring buffer at 100 mA for 1 h. The nitrocellulose membrane was then blocked in 5% milk in Tris-buffered saline with 0.05% Tween-20 solution. The membrane was then incubated with rat anti-mouse IL-21 mAbs (Peprotech 500-0278) at 4°C overnight. After washing three times with PBS with 0.05% Tween-20 (PBST), the membrane was incubated with horseradish peroxidase-conjugated goat anti-rat antibody for 2 h at room temperature. The membrane was then washed five times with PBST and developed using a chemiluminescence detection kit (Sangon Biotech, Co., Ltd., Shanghai, China). The target protein bands were detected following exposure of the membranes to X-ray film and β-actin was used as an internal control.

Establishment of tumor model. For the subcutaneous Hepa1-6 tumor model, Ad5-Hepa1-6, Ad5-IL-21-Hepa1-6 and Hepa1-6 wild type (WT) were washed with sterilized PBS twice, then the cell density was adjusted to 1x10⁷/ml. Cell suspension (100 µl) was inoculated intradermally into the right flank of each mouse. Each group contained four mice, and the studies were repeated three times. Four weeks later, tumors were removed and weighed, and the tumor length and width were measured with calipers after the mice had been sacrificed in a humane manner. The tumor volume (in mm³) was estimated using the standard formula: tumor volume (mm³) = length x width² x 0.5. Mouse sera were separated and stored at -70°C for cytokine detection. Mouse tumor tissues were weighed following sacrifice and fixed in formaldehyde for immunohistochemistry. The mouse spleen and body were weighed in order to estimate the spleen index using the standard formula: spleen index = spleen weight (mg) / mouse weight (g).

Assessment of IL-21, IL-4 and interferon (IFN)-γ levels by immunohistochemistry. IL-21, IL-4 and IFN-γ levels in mouse serum were detected using the ELISA Ready-SET-Go kit as described previously. Proteins expressed in the mouse tumor tissues were detected by immunohistochemistry, which was performed on serial 4-µm-thick paraffin sections. The slides were deparaffinized in xylene and re-hydrated through four decreasing grades of ethanol (100, 95, 80 and 70%) for 2 min each. Endogenous peroxidase activity was blocked by immersing the slides in 3% hydrogen peroxide in methanol for 15 min at room temperature. Heat-induced antigen retrieval was performed for 5 min with 1X citrate buffer (pH 6.3) in a microwave and then cooled for 5 min. This process was performed three times. To reduce the nonspecific binding of antibodies to the tissues, the slides were pre-incubated with blocking serum in 1% bovine serum albumin for 30 min at room temperature. They were left to incubate with rabbit polyclonal anti-IL-21, IL-4 and IFN-γ (antibody dilution, 1:200),
IL-21 significantly reduces Hepa-1-6 tumorigenicity. The subcutaneous tumor mouse model revealed that IL-21 significantly reduced the tumorigenicity of Hepa-1-6. In the Ad5-IL-21-Hepa-1-6 group, only one of the four mice grew an extremely small tumor, while in the other two groups all four mice grew larger tumors. The tumor volumes of the Ad5-IL-21-Hepa-1-6, Ad5-Hepa-1-6 and Hepa-1-6 WT group mice were 0.008±0.015 cm\(^3\), 0.677±0.208 cm\(^3\) and 0.716±0.335 cm\(^3\), respectively, and the tumor weights were 0.011±0.022 g, 0.772±0.080 g and 0.828±0.486 g, respectively (Fig. 2A and B). The tumor volume and weight in the IL-21-Hepa-1-6 group were much smaller than those in the other two groups, and the difference was significant (P<0.01).

IL-21 enhances antitumor immunity in tumor-bearing mice. Immunohistochemistry confirmed that IL-21 and IFN-γ levels were much higher in Ad5-IL-21-Hepa-1-6 mouse tumor tissues than in the other two groups, but IL-4 levels were much lower in Ad5-IL-21-Hepa-1-6 mouse tumor tissues than the other two groups (Fig. 3A). Similar results were observed in the ELISA assay (Fig. 3B), which confirmed the cytokine IL-21 levels in Ad5-IL-21-Hepa-1-6 mice serum to be 80.09±5.560 ng/ml; much higher than those of the Ad5-Hepa-1-6 group and Hepa-1-6 WT groups. IFN-γ levels were 50.46±4.567 ng/ml, much higher than those of the Ad5-Hepa-1-6 and Hepa-1-6 WT groups, which were 15.49±2.290 ng/ml and 8.58±1.678 ng/ml, respectively. IL-4 levels in Ad5-IL-21-Hepa-1-6 mouse serum were 1.41 ng/ml±0.692, much lower than those in the Ad5-Hepa-1-6 and Hepa-1-6 WT groups, which were 10.84±1.398 ng/ml and 16.99±1.046 ng/ml, respectively.

The spleen cell killing assay revealed the killing ability of NK and T cells in the Ad5-IL-21-Hepa-1-6 mice to be 66.61±3.230 and 82.46±6.531%, respectively, which was much higher than in the Ad5-Hepa-1-6 and Hepa-1-6 WT groups (Fig. 3C). T cell proliferation assay revealed that the stimulation index of spleen T cells in Ad5-IL-21-Hepa-1-6 mice was 3.1±0.261, which was much higher than that of the Ad5-Hepa-1-6 and Hepa-1-6 WT groups, which were 1.45±0.266 and 1.37±0.072, respectively (Fig. 3D). The spleen index of Ad5-IL-21-Hepa-1-6 mice was 10.22±1.329, which was much higher than that of the Ad5-Hepa-1-6 and Hepa-1-6 WT groups, which were 5.5±0.519 and 4.8±0.271, respectively (Fig. 3E).

Discussion

As a promising cytokine for cancer immunotherapy, whilst IL-21 suppresses growth in certain tumors, including renal cell carcinoma and melanoma (9,14), it may promote growth in others, including colitis-associated colorectal cancer and follicular lymphoma (11,15). The function of IL-21 is complex, as it appears to have different functions in different tumors.

PHC is currently the fourth-leading cause of cancer-related mortality (12), and its incidence is rapidly increasing (13). In a previous study, Pan et al (16) used the naked plasmids pmIL-21 and/or pSpD-1 for local gene transfer by injection into experimental H22 murine hepatocarcinoma. The immunotherapy with IL-21 in combination with sPD-1 was observed to induce antitumor immune response. Cheng et al (17) injected...
recombinant plasmid capable of co-expressing GM-SCF, IL-21 and Rae-1 into a H22 cell-bearing mouse, and the recombinant expression plasmid inhibited liver cancer by a mechanism that involved activation of cell-mediated immunity in liver cancer. However, the efficiency of gene expression by naked plasmid injection was limited, and the therapeutic effect requires improvement. At present, no other study has investigated whether IL-21 is able to prevent liver cancer, and there is no available vaccine, so further study is required in this field. In the present study, we used adenovirus-mediated transfection of the IL-21 gene in the hepatic cancer cell line Hepa-1-6 and investigated the effect of IL-21 on the tumorigenicity of Hepa-1-6 and the influence of IL-21 on antitumor immunity in a mouse model.
Using the subcutaneous liver tumor model, we first revealed that IL-21 gene expression in the Hepa1-6 cell line significantly decreases the tumorigenicity of Hepa1-6. In the Ad5-IL-21-Hepa1-6 group, only one of the four mice grew a small tumor, and the other three mice did not grow any tumors, while in the Ad5-Hepa1-6 and Hepa1-6 groups, all the mice grew tumors, and the tumor weights and volumes were much larger than in the Ad5-IL-21-Hepa1-6 group. We speculated that this was due to the infection and expression efficiency of IL-21 mediated by Ad5 compared with naked plasmid injection, as well as the effective strategy we used in our study. This is positive news for vaccine development.

We also observed that the antitumor immune response was significantly enhanced in Ad5-IL-21-Hepa1-6 mice, which is consistent with the results of Pan et al (16) and Cheng et al (17), but the antitumor effect is much better, due to the infection and expression efficiency of IL-21 mediated by Ad5 compared with naked plasmid injection. The killing ability of NK and T cells...
in mouse spleen was significantly enhanced. We also detected high IFN-γ and low IL-4 levels in the serum and tumor tissue in the Ad5-IL-21-Hepa1-6 group. Since IFN-γ is a typical Th1 cytokine and IL-4 is a typical Th2 cytokine, the results of the present study indicate that IL-21 promoted the immune response shift to a Th1 response in mice, which is superior to the Th2 response for antitumor reactions. These data explain why IL-21 reduced the tumorigenicity of Hepa1-6. Our study is likely to lay a strong foundation for future biological treatments of liver cancer and vaccine development.

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