

Antitumor effect of membrane-type Tim-3 on hepatocellular carcinoma Hepa1-6 cells of ICR mice

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Abstract. In the present study, the inhibitory effect of trans-membrane Tim-3 on hepatocellular carcinoma Hepa1-6 cells and the potential application of Tim-3 on immune system of Institute of Cancer Research (ICR) mice loaded with Hepa1-6 hepatocellular carcinoma was investigated. The animal model was established via inoculation of Hepa1-6 hepatocarcinoma cells at the hind thigh of ICR mice. Recombinant vector plasmids were transfected at the same site for gene therapy by injection to observe the inhibitory effect of Tim-3 on tumor growth. A panel of genes from tumor tissues at various time intervals was analyzed by reverse transcription-polymerase chain reaction. Flow cytometry was used to evaluate the proliferation and cytotoxicity of splenocytes after Tim-3 transfection. Synergistic effects of Tim-3 with tumor abnormal protein-1 (TAP1) was also studied. It was revealed that the growth of tumor was significantly suppressed after the transfection of Tim-3. In the presence of Tim-3, the proliferation of splenocytes and cytotoxicity in the early phase of tumor development was significantly enhanced, and this antitumor effect was further improved by the synergistic effect of Tim-3 with TAP1. Therefore, the membrane-type Tim-3 may behave as an effective immunoregulator to enhance antitumor immune responses. Furthermore the present findings suggest that antitumor immunity was improved by the combined effect of Tim-3 and TAP1.

Introduction

Costimulatory signal is necessary to stimulate the response of effective cellular immune (1-3). The B7 family and other costimulatory molecules play an important role in the process

of costimulatory signal transmission (4,5). The binding of CD28 and B7 molecules on the surface of T cells seems to provide the primary costimulatory signal for T cell activation (6-8). Various membrane surface molecules are located onto T cell surface. These molecules guarantee the antigen recognition of T cell, interactions with other immune cells and receiving signal stimulation (8-12). Also, they provide an important fundamental for the identification and isolation of T cells and T cell subsets (7,11,13). Due to the generation and transmission of costimulatory signals, the apoptosis of target cells can regulate the immune response artificially (14-16). Tim-3 might act as a ligand for reverse transmission of signals to affect tumor immunity correspondingly (17,18). In order to investigate this new mechanism of Tim-3 molecules, hepatoma Hepa1-6 cell strain of ICR mouse was used to build a solid tumor model in thigh muscle to study the inhibitory effect of Tim-3 molecule on Hepa1-6 solid tumor and the influence on immune system of tumor bearing ICR mice.

Materials and methods

Materials. The cell strain of Hepa1-6 hepatocarcinoma was purchased from BeNa Culture Collection (Guangzhou, China). Male ICR mice (age: 4-6 weeks, weight: 18-22 g) were purchased from Animal Center of Jilin Medical University. The study was approved by the Academic Committee on the Ethics of Animal Experiments of Jilin Medical University [Jilin, China; permit no. SCXK (Jilin) 2007-0003]. All animals were treated in accordance with the Guidelines and Regulations for the Use and Care of Laboratory Animals of Jilin Laboratory Animal Monitoring Institute under the National Laboratory Animal Monitoring Institute of China. All animals were at the Animal Center of Jilin Medical University (Jilin, China) and acclimatized for two weeks at 24-28°C and 50-60% humidity. Trizol was purchased from Thermo Fisher Scientific. The reverse transcriptase and RNasin were purchased from Tiangen Biochemical Tech Co Ltd (Beijing). Taq DNA polymerase was purchased from Shanghai Haoran Company. 5-Carboxylfluorescein diacetate succinimidyl ester (CFSE) and propidium bromide were purchased from Sigma-Aldrich (China). When the tumor was measurable and the treatment was begun.

Tim-3 expression and detection. Twelve ICR mice were taken to conduct the measurements. The plasmid Tim-3 was

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dissolved in saline solution and injected into the muscle of left thigh of the mice using *in situ* injection method (0.1 mg per ICR mouse). After injection of Tim-3 solution, each three mice were killed after 12, 24, 36, and 48 h, respectively. RT-PCR method was applied to detect the expression of Tim-3 in the muscle tissue of ICR mice.

Tumor cell inoculation. Hepa1-6 hepatoma cells (1×10^6 /ml) were inoculated into the muscle of left thigh of mice (0.1 ml per mouse). The mice were randomly divided into normal saline group (the blank control group), pcDNA group (the plasmid control group) and Tim-3 group (the treatment group) with 20 mice in each group. Plasmid was injected on alternate day after the second day of inoculation with each injection of 0.1 ml (1 mg/ml), the injection site was the position of inoculated tumor cells. All mice inoculated with cells developed the tumor, when the mice were sacrificed.

Based on above experimental groups, TAP1 group (the treatment group) and Tim-3/TAP1 group (the combined treatment group, injection was half for each plasmid) were added with 20 mice in each group, Hepa1-6 hepatoma cells were inoculated with the same method and the same treatment was conduct for examination of synergistic effect of Tim-3 and TAP1.

Gene expression in tumor micro-environment. After 3rd and 6th day of inoculation, five mice that were randomly selected from normal saline group, pcDNA group and Tim-3 group, respectively were killed for tumor tissue sampling. The total RNA was extracted with Trizol reagent for reverse transcriptase reaction. Then 5 μ l samples were taken as template from 60 μ l reverse transcriptase product to amplify IL-13, fup-1 and TAP1 by PCR, respectively. There were 40 cycles at the conditions of 85°C for 40 sec, 60°C for 60 sec, 40°C for 90 sec, and 25°C for 120 sec. Gel imaging analysis system was used to analyze the expression level of mRNA and relative expression value was calculated by the following equation: relative expression value (%) = (gray value of amplified bands of the gene to be test / gray value of β -actin gene amplification band) \times 100%.

In vitro proliferation activity of spleen cells. After 4th day of tumor cells inoculation, six mice from normal saline group, pcDNA group and Tim-3 group, respectively were randomly selected for spleen sampling. Single spleen cell was prepared under sterile conditions for the following two experiments: The first experiment is proliferation of spleen cells *in vitro*. The concentration of mice spleen cell was adjusted to 1×10^6 /ml, suspended in culture medium containing 15% fetal calf serum followed by stained with CFSE. The solution was divided into two samples, one added Hepa1-6 antigen peptide and TAP1 protein complex (the final concentration was 0.58 μ g/ml). Both samples were transferred to a hole plate (100 μ l per hole) at 37°C in an incubator for 7 days. Flow cytometry was used to detect spleen cell proliferation index. The second experiment is spleen cell killing *in vitro*. Three samples of prepared single spleen cell suspension were taken (without CFSE staining), along with the spleen cells taken from a normal mouse which was used to determine the non-specific killing rate. All samples were cultured in culture medium containing 15%

fetal calf serum. Hepa1-6 antigen peptide and TAP1 protein complex (the final concentration was 0.58 μ g/ml) was added and cultured *in vitro* for 7 days as effector cells. Hepa1-6 cells were taken from mouse ascites and cultured in the same culture medium for overnight followed by stained with CFSE as target cells. The effector cells were mixed with the target cells according to a ratio of 30:1 at 37°C, the mixture was cultured in an incubator for 6 h, followed by propidium bromide second staining. Flow cytometry was used to detect the death rate of target cells (Hepa1-6 cells).

Statistical method. The *in vitro* experiments were repeated for 3 times. Single-factor ANOVA was used to compare the difference between the control group and the experimental group. $P < 0.05$ indicates statistical significance. The experimental data were analyzed by statistical software package SPSS13.

Results

Inhibitory effect of Tim-3 expression on tumor growth in vivo. Tim-3 plasmid was injected into the muscle of mice, and the expression of Tim-3 mRNA was detected in muscle tissue after 12 h, and the peak value reached to peak at 24–48 h, which could not be detected after 60 h (Fig. 1). After inoculation for 12 days, the average tumor weight (0.84 g) of Tim-3 group was less than that of pcDNA group (1.57 g) and normal saline group (1.61 g), which has significant difference ($P < 0.05$). However, there was no significant difference between pcDNA group and normal saline group, suggesting that the expression of Tim-3 could be inhibited by gene transfection in T cells, which could probably inhibit the growth of tumor cells.

Expression of immune related genes. After the 5th day of inoculation, IL-13, fup-1 and TAP1 of saline group and pcDNA group only exhibited trace expression, while the Tim-3 group displayed the expression of all three genes (Fig. 2). The expression of fup-1 was higher than IL-13, while the expression of TAP1 was markedly lower than the other two genes.

After the 10th day of inoculation, the expression of IL-13 and fup-1 in Tim-3 group did not show further increase compared with the results obtained above. However, the TAP1 expression in normal saline group, pcDNA group and Tim-3 group was all enhanced (Fig. 3). Therefore, it can be concluded that Tim-3 can promote the expression of positive immune related genes in the early stage of tumor growth.

Effects of Tim-3 on enhancement of splenocytes proliferation and cytotoxicity. After inoculation for one week, the spleen cells were taken for proliferation experiment. Under the conditions of adding specific antigenic peptide stimulation and without specific antigenic peptide stimulation, flow cytometry was used to detect the mice splenocyte proliferation index of each experimental group. The results (Fig. 4) showed that the Tim-3 group had a significant difference ($P < 0.05$) compared with the saline control group and the pcDNA group with specific antigenic peptide stimulation. Also, the mice splenocyte proliferation index of the Tim-3 group without specific antigenic peptide stimulation did not have significant difference comparing the other two groups.

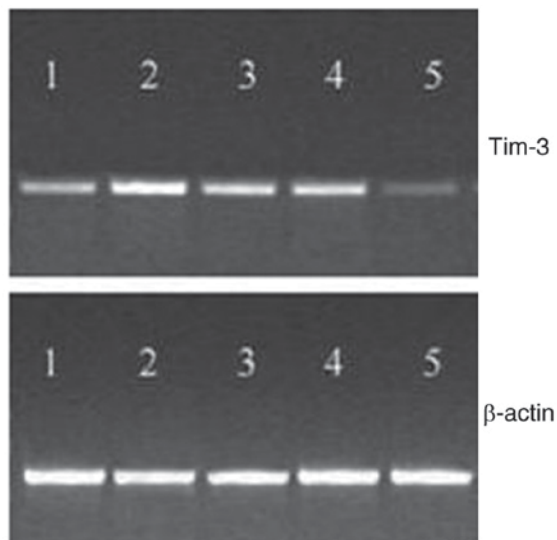


Figure 1. *In vivo* expression of Tim-3 in mice muscle tissues. Lane 1, after 12 h; lane 2, after 24 h; lane 3, after 36 h; lane 4, after 48 h; lane 5, after 60 h.

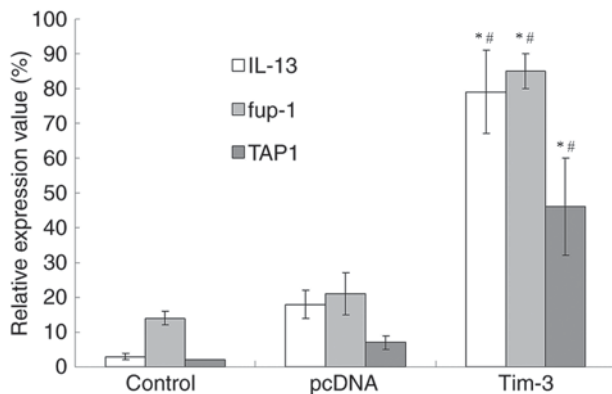


Figure 2. Relative expression values of various genes after five days of inoculation. * $P<0.05$ vs. Control; # $P<0.05$ vs. pcDNA.

The ratios of proliferation index obtained by adding specific antigenic peptide stimulation and without specific antigenic peptide stimulation for the three groups were 0.97, 0.99, and 1.62, respectively. This result indicates that the larger the ratio is, the higher the activity of spleen cell proliferation is under antigen stimulation.

The killing activity experiment of spleen cells further confirmed the specific killing activity of T cells. The specific killing function of Tim-3 group was stronger than pcDNA group ($P<0.05$) and saline control group ($P<0.01$). The killing rate was 32, 23, and 14%, respectively.

Synergistic effect of Tim-3 with TAP1. So far, we have demonstrated that transfection of Tim-3 into tumor-bearing mice significantly inhibited the growth of tumor. Furthermore, the growth was further inhibited when Tim-3 and TAP1 were simultaneously transfected. After inoculation for two weeks, of the average weight of tumor was only 0.42 g, significantly lower than that of Tim-3 transfected mice (0.84 g) or TAP1 transfected mice (1.57 g) and normal saline group (1.61 g). The experimental group treated with combined Tim-3 and TAP1

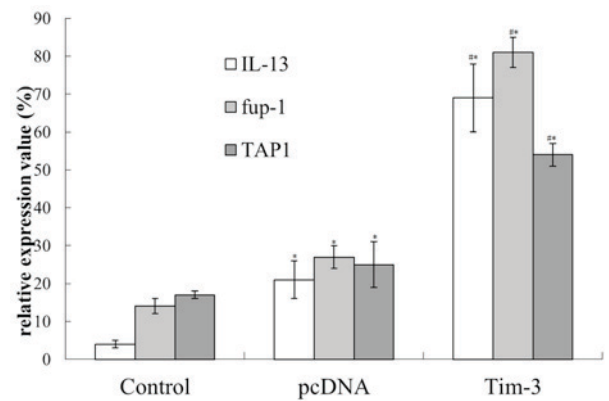


Figure 3. Relative expression values of various genes after ten days of inoculation. * $P<0.05$ vs. Control; # $P<0.05$ vs. pcDNA.

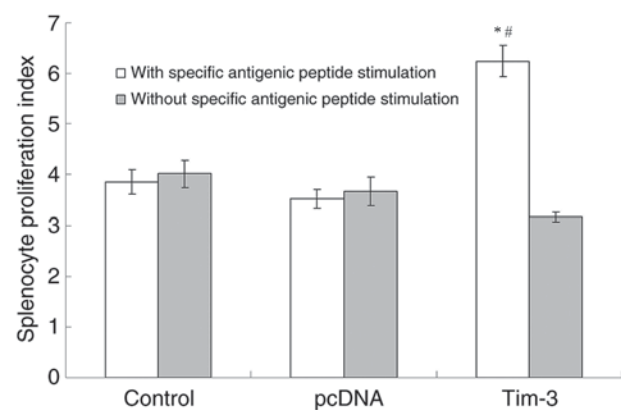


Figure 4. Splenocyte proliferation index of three groups detected by flow cytometry. * $P<0.05$ vs. Control; # $P<0.05$ vs. pcDNA.

has significant difference ($P<0.05$), suggesting that Tim-3 and TAP1 have stronger synergistic antitumor effect.

Discussion

So far, Tim-3 has been used as the receptor molecule on the surface of T3 cells in the studies of membrane-type Tim-3 (5-7). Generally, TAP1 is considered as the ligand of Tim-3 (8,9). The signal produced by synergistic effect of TAP1 and membrane-type Tim-3 can prevent the activation of the cell and negatively regulate the cell immunity. However, if membrane-type Tim-3 inhibits receptor only, soluble Tim-3 should enhance the immune response by blocking effect. Previous studies have found that soluble Tim-3 does not produce an immune enhancing effect, but generates an immunosuppressive effect (10-12).

In this study, it was found that T cells can enhance the immune function by transfecting the recombinant membrane-type Tim-3 eukaryotic expression plasmid into Hepal-6 hepatoma cells of vaccinated mice, which had significant antitumor effect. This result is not consistent with a previous publication which reported that membrane-type Tim-3 can induce T cell immune tolerance. The membrane-type Tim-3 is a receptor of the T cell surface, after binding with ligand for signal transduction. Tim-3 produced a series of changes within the cell. In current work, intramuscular injection of recombinant carrier

of membrane-type Tim-3 was directly conducted. Which carrier effectively expressed in muscle cells but was difficult to transfect T cells. The possibility that membrane-type Tim-3 as cell surface receptor expression in mature muscle cell surface to induce the produce of positive immune effect by muscle cells is very small. Another possibility is that the membrane-type Tim-3 has ligand properties, which produced positive immune regulatory effect through the interaction with receptor of immune cells surface. Because only TAP1 molecules are known to bind with membrane-type Tim-3, the existence of other receptor molecules remains unclear. In this research, the method of local transfection was taken to allow the expression of membrane-type Tim-3 only in specific position - the thigh muscle cells of ICR mice. We speculate that in this case the membrane-type Tim-3 may not have a direct effect on T cells, but indirectly activate T cells via activation of macrophages or antigen cells. Because TAP1 that combined with Tim-3 can express in a variety of tissue cells including macrophages, the Tim-3 molecule will react with these cells. The RT-PCR results obtained with pure Hepa1-6 cell samples showed very weak expression of TAP1 only, the possibility that Tim-3 molecules reacted with the Hepa1-6 cells locally inoculated can be excluded.

In this study, we have found that the membrane-type Tim-3 and TAP1 could have synergistic effect. TAP1 as a molecule having clear physiological functions can interact with its receptor (expressed on the surface of activated T cells) to maintain the activity of T cells or further enhance the activity and turn it into memory T cells. The transformation is independent and not restricted by other molecules, suggesting that the mechanisms of membrane-type Tim-3 signal is activation of T cells eventually, and produce synergistic effect with TAP1 based on the activation. After 2 weeks of tumor cells inoculation, TAP1 molecule started to function. Although T cells were activated at this moment, the effect of Tim-3 molecules is not further strengthened, suggesting that Tim-3 molecules do not influence the functions of T cells after activation.

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