Prevention of hepatocellular carcinoma in mice by IL-2 and B7-1 genes co-transfected liver cancer cell vaccines

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METHODS: The murine liver cancer cell line Hepal-6 was transfected with IL-2 and/or B7-1 gene using recombinant adenoviral vectors and the liver cancer vaccines were prepared. C57BL/6 mice were immunized with these vaccines and challenged with the parental Hepal-6 cells afterwards. The immunoprotection was investigated and the reactive T cell line was assayed.

RESULTS: The immunoprotection of the tumor vaccine was demonstrated. The effect of IL-2 and B7-1 genes co-transfected Hepal-6 liver cancer vaccine (Hep6-IL2/B7 vaccine) on the onset of tumor formation was the strongest. When attacked with wild Hepal-6 cells, the median survival period of the mice immunized with Hep6-IL2/B7 vaccine was the longest (68 days, $\chi^2=7.70-11.69$, $P<0.05$) and the implanted tumor was the smallest ($z=3.20-44.10$, $P<0.05$). The effect of single IL-2 or B7-1 gene-transfected vaccine was next to the IL2/B7 gene co-transfected group, and the mean survival periods were 59 and 54 days, respectively. The mean survival periods of wild or enhanced green fluorescence protein gene modified vaccine immunized group was 51 and 48 days, respectively. The mice in control group all died within 38 days and the implanted tumor was the largest ($z=3.20-40.21$, $P<0.05$). The cellular immunofunction test and cytotoxicity study showed that the natural killer (NK) cell, lymphokine activated killer (LAK) cell and cytotoxic T lymphocyte (CTL) activities were significantly increased in mice immunized with the Hep6-IL2/B7 vaccine, (29.5±2.5 %, 65.0±2.9 %, 83.3±1.5 % respectively, compared with other groups, $P<0.05$).

CONCLUSION: The Hep6-IL2/B7 liver cancer vaccines can induce the mice to produce activated and specific CTL against the parental tumor cells, and demonstrate stronger effect on the hepatocarcinogenesis than single gene modified or the regular tumor vaccine. Therefore, the vaccines may become a novel potential therapy for recurrence and metastasis of HCC.

Ge NL, Ye SL, Zheng N, Sun RX, Liu YK, Tang ZY. Prevention of hepatocellular carcinoma in mice by IL-2 and B7-1 genes co-transfected liver cancer cell vaccines. World J Gastroenterol 2003; 9(10): 2182-2185 http://www.wjgnet.com/1007-9327/9/2182.asp

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver in humans and the rate of incidence and mortality is very high in East Asia and China[1-6]. Though many approaches, such as surgical resection, transarterial chemoembolization (TACE), percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), radiotherapy and liver transplantation were developed to treat it, and the effective and survival rates were increased, a large number of patients died from recurrence and metastasis[7-10].

Some studies showed immunogene modified tumor vaccines could induce effective specific active T cell immune response to prevent tumor recurrence and metastasis[11-14]. Also some reports demonstrated that specific active immunotherapy based on specific antitumor T cell immunity could be a potential modality for further improving the survival of HCC patients on preventing tumor recurrence and metastasis. But HCC is poorly immunogenic. Poor or no immunogenicity and lack of costimulating molecules on the surface of tumor cells are some of the causes of most tumors including HCC[15].

The successful induction of an anti-tumor immune response has been reported in a number of tumor models including HCC animal models by using B7-1 (CD80) transfected tumor cells as a vaccine[16]. The rationale of B7-1 based immunotherapy is that T cells require both an antigen-specific signal delivered through the T-cell receptor and a co-stimulatory signal to be fully activated and proliferated, and secrete cytokines or generate cytotoxic T lymphocyte (CTL) to cytolyse tumor cells[17-19].

Interleukin 2 (IL-2) is a growth factor that stimulates the proliferation of cytotoxic T cells, helper T cells, natural killer (NK) cells, and lymphokine activated killer (LAK) cells, all of which can participate in the anti-tumor response[20-22]. Many HCC animal models demonstrated that local secretion of IL-2 abrogated the tumorigenicity of cytokine-producing tumor cells and inducing a long-lasting protective immune response against a subsequent tumor graft[23,24]. In this study, we compared the effect and immunological mechanism of mouse Hepal-6 liver cancer cell vaccine modified by IL-2 and/or B7-1 genes on preventing the C57BL/6 mice from challenge of wild parental Hepal-6 cells. The results provide some experimental evidences that gene modified tumor vaccines can prevent recurrence and metastasis of liver cancer.
Preparation of lymphocytes in spleens from mice immunized with gene modified Hepal-6 tumor cell vaccines

The mice in each group were killed by dislocating cervical vertebra on the 7th day after the final immunization, and the lymphocytes were harvested and maintained in 1 640 medium (GIBCO BRL, USA) containing 10 % FCS (HYCLONE, USA).

Immunostaining and FACS analysis

The freshly prepared spleocytes were analyzed by direct immunofluorescence staining with mAbs CD3-FITC, CD4-PE, CD8-PE and CD25-PE. Flow cytometry analysis was then performed with a FACSscan (Becton Dickson Co.) in the Department of FACS, the Chinese Academy of Sciences.

Induction of the cytolytic activity of LAK cells and CTL from the lymphocytes

The harvested lymphocytes (2×10^6 cell/ml) were cultured in 1 640 containing 10 % FCS with rhIL-2 at 1 000 U/ml at 37 °C in a 5 % CO_2 incubator for 5 days and then cocultured with mitomycin Created Hepal-6 cells (20:1 of responder to tumor cell ratio) in 1 640 containing 10 % FCS with rhIL-2 at 50 U/ml at 37 °C in a 5 % CO_2 incubator for 5-7 days.

In vitro cytolytic assay of NK, LAK cells, and CTL

MTS assay was employed to test the cytotoxic activity of NK cell, LAK cell and CTL from stimulated lymphocytes. Target cells were Yac-1, P815 and Hepal-6 respectively and incubated with various lymphocytes at 37 °C in a 5 % CO_2 incubator for 2-4 hours at 100:1 of effector/target ratio. Assays were performed in triplicate wells. Cytolytic activity was calculated according to the formula provided by the MTS kits: cytolytic activity (%) =[(OD_t-OD_b)-(OD_e-OD_b)-(OD_a-OD_b)]×100 %/(OD_t-OD_b).

Statistical analysis

The data were analyzed with SAS 6.12 and STATA 6.0 software, and expressed as ±s. The cytotoxic activity of NK cell, LAK cell and CTL was determined by \( t \) test. The growth curves of tumor volume were analyzed with a generalized regression model and the mice survival period was analyzed with Log-Rank test.

RESULTS

In vivo immuno-protection by various tumor cell vaccines

After the immunized mice were challenged with wild Hepal-6 cells, the change of the tumor volume was assessed as is shown in Figure 1. The tumors grew progressively in Hep6-EGFP and Hep6 tumor vaccine immunized mice. The tumors in Hep6-IL2 and Hep6-B7 tumor vaccine immunized mice grew more slowly than those in Hep6-EGFP. Hep6 tumor vaccine immunized mice and the control. Compared with the above 5 groups of mice, the tumors in Hep6-IL2B7 tumor vaccine immunized mice grew most slowly and the tumor volume was the smallest (\( P < 0.05 \)) and about 50 % of the tumor disappeared 68 days after attacked by the wild Hepal-6 cell.

The survival periods of each group of mice were compared. The median survival time of Hep6, Hep6-EGFP, Hep6-B7 and Hep6-IL2 tumor vaccine immunized mice was 51, 48, 54 and 59 days, respectively and significantly longer than that of the control group (\( \chi^2 = 10.61-23.81, P < 0.05 \)) and about 50 % of the mice died within 41 days. The median survival period of the control mice was the shortest and all the mice died within 41 days. The median survival period of the Hep6-IL2B7 tumor vaccine immunized mice was significantly longer than that of other groups (\( \chi^2 = 7.70-11.69, P < 0.05 \)) and all the mice in this group were alive for more than 68 days after attacked by the wild Hepal-6 cell.

Expression of B7-1 and IL-2 genes transduced Hepal-6 tumor vaccine

Flow cytometry analysis confirmed that the transfected tumor
vaccines Hep6-IL2B7 and Hep6-B7 expressed a higher level of CD80 (55.47 % and 59.11 %, respectively) than Hep6-IL2. Hep6 and Hep6-EGFP tumor vaccines (12.48 %, 11.87 %, and 7.93 %, respectively).

In the supernant of Hep6-B7, Hep6 and Hep6-EGFP tumor vaccines, no IL-2 was detected, while a high level IL-2 was detected in the supernant of Hep6-IL2 and Hep6-IL2B7 tumor vaccines (1 885 pg and 460 pg per 3×10⁶ cells, respectively).

**Figure 1** Change of tumor volumes after cancer vaccine immunization.

**Flow cytometry analysis**

The CD4/CD8 ratio in lymphocytes of the Hep6-IL2B7 tumor vaccine immunized mice was the lowest (0.91) among all the study groups and the CD25 positive rate was the highest (32.85%). The CD4/CD8 ratios in lymphocytes of the Hep6-B7, Hep6-IL2 and Hep6 tumor vaccine immunized mice and normal mice were 1.02, 1.17, 1.24 and 1.31, respectively, while the CD25 positive rates were 28.62 %, 26.13 %, 19.32 % and 3.59 %, respectively.

**Cytolytic assay of NK cell, LAK cell, CTL of spleen lymphocytes**

The results are shown in Table 1. The cytolytic activities of the NK cells in each group of tumor vaccine immunized mice were all significantly higher than those in the normal mice (t=11.3-15.5, P<0.05) and it was the highest in the Hep6-IL2B7 group (29.5±2.5 %, t=10.4-15.5, P<0.05). The cytolytic activities of the LAK cells in the Hep6-IL2B7 tumor vaccine immunized mice were significantly higher than those in the normal mice (60.9±1.7 %, 65.0±2.9 %, t=10.6-40.2, P<0.05). The cytolytic activities of the CTL induced by Hep6-IL2B7 tumor vaccine were significantly higher than those in other groups (83.1±1.5 %, t=13.7-53.3, P<0.05).

**Table 1** The NK cell, LAK cell and CTL activities of each group mice against different target cells (x±s %)

| Effector cell | Target cell | Hepal-6 |
|--------------|-------------|---------|
| Normal group | Yac-1       | 19.0±1.9| 29.9±1.3 |
| Hep6-IL2 cancer vaccine | 32.5±2.7 | 60.9±1.7 | 64.4±12.3 |
| immunized group | | | |
| Hep6-B7 cancer vaccine | 17.0±1.7 | 47.7±1.2 | 71.2±1.5 |
| immunized group | | | |
| Hep6-IL2B7 cancer vaccine | 29.5±2.5 | 65.0±2.9 | 83.1±5.5 |
| immunized group | | | |
| Hep6 cancer vaccine | 18.5±1.4 | 42.5±1.3 | 52.8±1.2 |
| immunized group | | | |

NK cell activities: P<0.05 vs normal, Hep6-B7 and Hep6 cancer vaccine immunized group; LAK cell activities: P<0.05 vs Hep6-IL2 and Hep6-IL2B7 cancer vaccine immunized group; CTL activities: P<0.05 vs Hep6-IL2B7 cancer vaccine immunized group.

**DISCUSSION**

Hepatocellular carcinoma was poor immunogenicity partly because of poor antigen expression and lack of co-stimulatory molecules[26]. Though successful induction of anti-tumor immunity by means of B7-1 gene[11,14,16] or cytokine (IL-2, IL-4, IL-12, GM-CSF, IFN, and so on) gene[29,12,14,27] transfection has been reported in some HCC animal models, prevention of tumor recurrence and metastasis still needs to be improved.

Some animal experiments showed that IL-2 gene-modified tumor vaccine constantly secreted a relatively high level of IL-2 in local areas, induced local and systematic specific antitumor immuno-reactions and immuno-memory T cells in the body of the animals, prevented the challenge of the next parental tumor and decreased the recurrence rate[28-30]. Reports showed the locally secreted high level of IL-2 generated much less side effects than a systemically administrated high dose of IL-2[31].

Double signal systems were needed in the process of inducing effective specific active antitumor T cell immune response[31]. B7-1 was an important co-stimulating molecule for tumor antigen presentation, and could provide the second signal system for T cell immune[11,32,34]. Tumors could escape from the immune surveillance of host when they were lack of it[31]. B7-1 gene-modified tumor vaccine could break the immuno-tolerance of the tumor to the host and induce effective specific antitumor immunology[32,34].

Some reports showed a single immunogene modified tumor vaccine could not induce very effective immune response because double signal systems were needed in the process[31,39] and multi-gene modified tumor vaccine could improve the immunogenicity of tumor vaccines in different ways and promote T cell immunity[31,32,34,39]. So in our experiment, we investigated the immunoprotection of single IL-2 or B7-1 gene or both IL-2 and B7-1 genes immunized mice HCC cell line Hepal-6 tumor vaccines.

In our experiment, the results showed Hepal-6 liver cancer cell line expressed a low level of B7-1 molecule and no IL-2 was secreted, and the tumorigenicity was 100 %. The vaccine prepared from wild Hepal-6 cells showed very weak protection against wild tumor cell attacking. The tumor vaccine modified with IL-2 or B7-1 gene induced a stronger immunoprotective effect than the wild vaccines, and could significantly improve the survival rate and median survival time. More excitingly, the immunoprotection induced by IL-2 and B7-1 genes co-transfected tumor cell vaccine was even better than that of either of the genes. These two genes improved the immunogenicity of the liver cancer cell vaccine synergically and induced the mice to generate stronger anti-tumor immunoreactions. The immunological analysis of spleno-lymphocytes from the mice showed that IL-2 and B7-1 genes co-transfected liver cancer cell vaccine induced lymphocytes to express a significantly higher CD25 positive level and a lower CD4/CD8 ratio. This result suggested the T cells were activated. The cytotoxic analysis showed after immunization with this vaccine, specific CTLs were induced in the mice.

The results in this mouse liver cancer model demonstrated that after immunization with IL-2 and B7-1 genes co-transfected liver cancer cell vaccine, more effective systemic anti-tumor cell immunoreactions could be induce in mice than that with either of the gene modified vaccines, and the induced immuno-memory reaction could protect against the challenge of the wild parental Hepal-6 cell. The results suggest that the co-transfected gene tumor vaccine can prevent recurrence and metastasis of liver cancer.

**ACKNOWLEDGMENTS**

We are grateful to Drs. Yan Zhao and Wei-Hua Bao for their advice and help in the study.
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Edited by Zhang JZ and Wang XL