Basic Research Paper

Inhibitory effects of human AFP-derived peptide-pulsed dendritic cells on mouse hepatocellular carcinoma

Xiong-Hao Pang,1,2 Min-Shan Chen,1,2 Wei-Hua Jia1,3 and Xin-Xi Zhou1,3,*

1State Key Laboratory of Oncology in South China; Guangzhou, Guangdong P.R. China; 2Department of Hepatobiliary Tumor; and 3Research Department; Cancer Center, Sun Yat-sen University; Guangzhou, Guangdong P.R. China

Key words: dendritic cells, liver neoplasm, human AFP-derived peptide, Hepa1-6 cell, nude mouse

Background and Objective: Alpha-fetoprotein (AFP) is a good candidate antigen for the immunotherapy of hepatocellular carcinoma (HCC). Overcoming the immune tolerance induced by autologous antigen is one of the key points for inducing effective anti-tumor immune reaction. This research investigates the effect of human AFP-derived peptide-pulsed dendritic cells (hAFP-DCs) on immunity against mouse HCC.

Methods: Bone marrow-originated DCs were prepared routinely. The activity of hAFP-DC-stimulated cytotoxic T lymphocyte (CTL) against Hepa1-6 cells was examined by MTT assay. C57BL/6 mice were inoculated subcutaneously with $7 \times 10^6$ Hepa1-6 cells to develop hepatoma, and received an intratumor injection of hAFP-DCs, DCs and PBS, respectively, twice a week. Tumor volume was evaluated and the survival of mice after inoculation with Hepa1-6 cells was examined.

Results: We successfully prepared DCs from the bone marrow of mice. The cytotoxic activity of CTLs stimulated by hAFP-DCs and DCs showed a stronger tendency than the control, but without significance. The mean tumor volume at 31 days after inoculation with Hepa1-6 cells was $(195.04 \pm 155.22)$ mm$^3$ in hAFP-DCs group, $(360.65 \pm 209.02)$ mm$^3$ in DCs group and $(756.19 \pm 503.24)$ mm$^3$ in PBS group. The differences among these three groups were significant ($p < 0.001$). The survival rate of mice at 40 days after inoculation was $100\%$ in hAFP-DCs group, $90\%$ in DCs group and $50\%$ in PBS group ($p = 0.008$).

Conclusion: Human AFP-derived peptide-pulsed DCs can efficiently enhance immunity against HCC in mice.

Hepatocellular carcinoma (HCC), also called hepatoma, is one of the most common malignant tumor in China. Characterized by latent onset, fast and invasive growth, frequent relapse, poor results of conservative treatment, high mortality as well as low survival rate (five year survival rate less than 5%), hepatocellular carcinoma has been recognized as a kind of common refractory malignant tumor. Due to insensitivity to radiotherapy and chemotherapy, HCC is mainly treated by surgery-based combination therapy. However, because the majority (85%) of Chinese HCC patients are complicated with chronic hepatitis and liver cirrhosis, with a limited hepatic function reservation, the resectability rate of HCC ranges only from 10% to 37%. Moreover, relatively strict indications for liver transplantation and donor liver shortage prevent liver transplantation from being widely conducted in China.

Recent data show that immuno-gene therapy has become a hot topic in the research field of tumor treatment. One of effective ways to induce autologous tumor-killing activity is to prepare tumor vaccines with various forms of tumor antigens, which are able to stimulate tumor-specific immune reaction.

Materials and Methods

Materials. Reagents. RPMI-1640 was purchased from Gibco BRL. Fetal calf serum (FCS) was purchased from Hangzhou
Sijiqing Biological Engeneering Materials. Recombinant mouse GM-CSF (rmGM-CSF) and recombinant mouse IL-4 (rmIL-4) were purchased from R&D Systems. PE-labeled rat anti-mouse CD86, CD80, MHC II and 33D1 (a specific surface marker for DCs) monoclonal antibodies were purchased from eBioscience. Lipopolysaccharide (LPS) was purchased from Sigma. The mouse lymphocyte separation medium was purchased from Guangzhou Whiga Technology.

**Animals and cell lines.** Healthy female C57BL/6 mice between four and six weeks old, SPF grade, were purchased from the Laboratory Animal Center of the School of Pharmaceutical Sciences of the Sun Yat-sen University (Number of the License for Use of Laboratory Animals: SKXY (yue)2007-0081; Number of the Qualification Certificate for Animals: No.0036221). The AFP-expressing C57BL/6 mouse hepatocellular carcinoma Hepa1-6 cell line was generously provided by Professor Lu Mingde at the First Affiliated Hospital of Sun Yat-sen University.

**Experimental instruments.** Cell incubators (Thermo Forma, Electron Corporation) and a flow cytometer (BD, Immunocytometry Systems, San Jose CA) were used.

**Screening and synthesis of human AFP peptide.** Based on the full-length human AFP protein sequence (including 609 amino acid residues) retrieved from NCBI, the ProPred-I database and program (http://www.imtech.res.in/raghava/propred1/index.html) were used to predict the potential binding regions of human AFP to C57BL/6 mouse MHC I molecules (H-2Kb). The peptide sequence RVAKGYQEL, located at amino acids of 372 to 380 of human AFP, had the highest score (Real Score 31.68) in ProPred-I prediction and was chosen as the pulsing peptide. The peptide was synthesized by Shenzhen Hanyu Pharmaceutical Co., Ltd. High performance liquid chromatographic (HPLC) analysis showed that peptide purity was above 99%.

**Culture and purification of mouse bone marrow-derived DCs.** DCs were cultured according to the Inaba method with minor modifications. C57BL/6 mice of five to six weeks old were killed by cervical dislocation. The thigh and shin bones were stripped under aseptic conditions. After cutting to expose the red bone marrow, a 30-mL syringe was used to aspirate RPMI-1640 medium to rinse the bone marrow into a petri dish. The obtained bone marrow suspension was filtered using a 100-mesh sieve and centrifuged at 458g for six minutes to harvest bone marrow cells. Three milliliters of pre-warmed 0.83% Tris-NH4Cl were added into cell pellet to lyse red blood cells for five minutes. Subsequently, 40 mL of RPMI-1640 medium were added. After centrifugation at 458g for six min, the supernatant was discarded. A trypsin blue dye exclusion assay was performed to count the number of viable cells (cell viability determined was greater than 95%). RPMI-1640 complete medium (containing 10% FBS) was then added into cell pellet. Cells were seeded in 6-well plates. After adding 10 ng/mL of rmGM-CSF and 5 ng/mL of IL-4, the volume of each well was brought to 4 mL by adding the complete medium. On day three, the cell culture medium was replaced with fresh complete medium containing the same concentrations of cytokines. On day five, half the volume of culture medium was replaced with fresh complete medium containing sufficient amounts of cytokines. On day six, cells were suspended by gentle pipetting and harvested. The harvested cells represented ex vivo expanded mouse bone marrow-derived DCs. The changes in the growth and morphology of cells were observed under a phase contrast microscope each day. After co-culture with 200 ng/mL of LPS for 24 h, human AFP (250 ng/mL) were added and incubated for an additional four hours. DCs were then harvested and rinsed with PBS to remove LPS and unbound peptide. The DCs were then adjusted to a final concentration of 1 x 10^6 cells/mL.

**Preparation of mouse splenic lymphocytes.** Male C57BL/6 mice were killed by cervical dislocation and entirely soaked into 75% ethanol for disinfection. After cutting the skin under aseptic conditions, the spleen was removed and cut into small pieces, which were then squeezed into a 100-mesh sieve to prepare single cell suspension using an aseptic 30-mL syringe needle. After centrifugation at 458g for six min, the supernatant was discarded and 3 mL of lysis solution were added into cell pellet to lyse red blood cells. After repeating centrifugation at 458g for six min, the supernatant was discarded and cell pellet was resuspended with physiological saline solution to obtain mouse splenic cell suspension. Routine density gradient centrifugation was then conducted to separate splenic lymphocytes.

**Determination of the killing activity of CTL by MTT assay.** In vitro cultured Hepa1-6 cells were harvested and seeded in 96-well plates at a density of 1 x 10^4 per well, and used as target cells. Splenic lymphocytes co-cultured with human AFP-derived peptide-pulsed dendritic cells or LPS-stimulated DCs or treated PBS were used as effector cells, respectively. The proportions of effector cells to target cells were 1:1, 30:1 and 60:1, respectively. Additionally, control wells containing only target cells or effector cells were also set. After co-culture for 48 h, MTT assay was conducted to determine the absorbance values (A_490) of each group at a wavelength of 490 nm. The killing activity of CTL was calculated according to the following formula:

\[
\text{The killing rate} = \frac{1 - \text{absorbance value of the well containing both target and effector cells} - \text{absorbance value of the well containing only effector cells}}{\text{absorbance value of the well containing only target cells}} \times 100\%
\]

**Flow cytometry analysis of the phenotype of DCs.** After seven days of culture, DCs were harvested and adjusted to a density of 5 x 10^5/mL. PE-labeled rat anti-mouse CD86, CD80, MHC II and 33D1 (a specific surface marker for DCs) monoclonal antibodies were added according to the instructions provided with the products. Blank controls were also set. After incubation for 30 min at 4°C in a dark place, cells were fixed with 4% paraformaldehyde and subjected to flow cytometry analysis.

**Development of a mouse subcutaneous tumor model.** Logarithmic phase Hepa1-6 cells were used to prepare cell suspension. A subcutaneous injection of 7 x 10^6 cells into the root of the right foreleg of each mouse was then performed. Mice were then reared under SPF conditions for eight days to allow a marked growth of subcutaneous tumors. Thirty SPF-grade tumor-bearing mice were randomly divided into three groups, ten mice in each group. Mice in the first group were injected with human AFP-derived peptide pulsed DCs (hAFP-DCs) while those in the
was calculated according to the following formula: 

\[ V (\text{mm}^3) = (\text{transverse diameter}^2 \times \text{longitudinal diameter})/2. \]

second group were injected with unpulsed DCs (LPS-stimulated). From day eight after the inoculation, each mouse received an intratumor injection of \(1 \times 10^6\) (approximately \(0.1 \text{ ml}\)) DCs, twice a week. The third group was blank control group, in which each mouse received an intratumor injection of 0.1 mL of PBS buffer, twice a week. The maximum longitudinal and transverse diameters (mm) of tumors were measured twice a week. Tumor volume (\(V\)) was calculated according to the following formula:

\[ V (\text{mm}^3) = (\text{transverse diameter}^2 \times \text{longitudinal diameter})/2. \]

Additionally, the survival duration of mice was observed and recorded to conduct survival analysis.

**Statistical analysis.** Data were expressed as mean ± standard deviation (SD). The SPSS13.0 software package was used to conduct the t-test, Repeated Measures ANOVA, one-way ANOVA and Kaplan-Meier survival analysis. Statistical significance was concluded when the p-value was less than 0.05.

**Results**

**Isolation, culture and identification of DCs.** The growth of DCs. After induction with rmIL-4, rmGM-CSF and LPS, mature mouse bone marrow-derived DCs were successfully developed. After four hours of culture, cells were small-sized, round-shaped, adherent and evenly distributed. On day two, a small number of suspended cells were seen. After removing these suspended cells, a large number of dot-shaped cell colonies become apparent. On day five, there was a significant increase in the number and size of cell colonies. After further culture, cells showed significantly enlarged size and irregular shape, forming three to 10 burr-like protrusions of different size. Under a high-power microscope, these cells were characterized by the presence of stellar dendritic-shaped surfaces. Some cells grew as masses, and colonies were scattered. Due to the increased number of suspended cells in the culture medium while those slightly adherent cells could be easily suspended by gentle pipetting.

**DC-stimulated in vitro killing activity of CTL against Hepa1-6 cells.** In vitro experiments showed that T lymphocytes treated with either human AFP-derived peptide-pulsed dendritic cells, unpulsed dendritic cells or PBS could kill Hepa1-6 cells, and one-way ANOVA indicated that there was no statistically significant difference (\(p > 0.05\)) in the killing activity among the three groups. In the PBS-treated group, the killing activity against Hepa1-6 cells showed a relatively weak tendency.

**In vivo antitumor effects of vaccination with human AFP-derived peptide-pulsed dendritic cells.** On day 31 after inoculation with Hepa1-6 cells, the mean tumor volumes in hAFP-DCs group, DCs group and PBS group were (195.04 ± 155.22), (360.65 ± 209.02) and (756.19 ± 503.24) mm\(^3\), respectively (Fig. 3). Repeated Measures ANOVA showed that there were significant differences in the size of tumors in tumor-bearing mice (\(p < 0.001\)) among each group. Dunnnett’s t-test showed that there were significant differences in the size of tumors in mice between the hAFP-DCs and PBS groups (\(p < 0.001\)) as well as between the DCs and PBS groups (\(p = 0.002\)), suggesting that both hAFP-DCs and unpulsed DCs have some inhibitory effects on tumor growth. Moreover, a significant difference was also noted between the hAFP-DCs and DCs groups (\(p = 0.043\)), suggesting that hAFP-DCs have more strong inhibitory effects on tumor growth than unpulsed DCs.

**Analysis of the survival duration of tumor-bearing mice in each group.** When the maximum diameter of subcutaneous tumors in mice exceeded 1,500 mm\(^3\), they were killed. Observations continued for a total of 40 days. The body weight of tumor-bearing mice showed no significant difference before and after treatment and no obvious hair loss was noted, suggesting that treatment with DCs is safe. During the period of 40-day observations, no mice died in the hAFP-DCs group while one and five mice died in the DCs and PBS groups, respectively. Kaplan-Meier survival analysis indicated that the cumulative survival rates in the three groups were 100%, 90% and 50%, respectively, showing statistically significant differences among each group (\(p = 0.008\)) (Fig. 4). These results suggest that vaccination with DCs has some in vivo anti-tumor effects.

**Discussion**

Tumor-associated antigen AFP is a specific marker for hepatoma. It is a glycoprotein produced by fetal liver cells and expressed at low levels during postnatal stages due to repressed
Inhibitory effects of human AFP-derived peptide-pulsed dendritic cells on mouse hepatocellular carcinoma

In contrast, AFP regains high-level expression in 60–80% of HCC. However, the majority of HCC patients with elevated AFP levels generate no effective and specific immune response. Um et al.\textsuperscript{11} found that human serum AFP could impair the function of antigen-presenting cells and induce the apoptosis of these DCs. Therefore, in previous studies, it was generally believed that the body’s immune system could generate immune tolerance or ignorance to AFP produced by HCC. In 1999, Vollmer et al.\textsuperscript{12} first reported that DCs transfected with mouse AFP were able to induce immune response and killing activity of T cells against spontaneous mouse lymphoma, suggesting that the immune tolerance is incomplete and can be overcome through different immunization strategies. Butterfield et al.\textsuperscript{13,14} found that, after DCs derived from AFP-positive hepatoma patients were co-cultured with four AFP peptides chosen with proteomic approaches, subcutaneous inoculation of these treated autologous DCs could activate the body’s specific T-cell response and upregu-
Inhibitory effects of human AFP-derived peptide-pulsed dendritic cells on mouse hepatocellular carcinoma

This investigation proved that vaccination with human AFP-derived peptide-pulsed dendritic cells could induce in vivo and in vitro immune response against hepatocellular carcinoma in mice. In a future study, we will investigate cell subpopulations involved in this process and analyze the mechanism behind the action of the DC vaccine, thereby providing more comprehensive preliminary data for clinical immunotherapy of hepatocellular carcinoma.

Acknowledgements

Grant: Sci-Tech Project of Guangdong Province (No. 2004B31201006).

References


late the expression of IFNγ, thereby promoting the killing activity against tumors. This result further suggests that, under certain conditions, AFP is involved in the activation of specific immune response. However, the results of Phase I/II clinical trials were not so satisfactory. Though the strategy of AFP-induced immunization showed some biological effects, its clinical benefit in patients was not evident.15

The idea that immunization with xenogenic homologous proteins generated during biological evolution could overcome immune tolerance and induce autologous anti-tumor immune response has been verified in recent years. In the present study, we used the bioinformatic tool ProPred-I to screen xenogenic human AFP peptide to search the epitope with the strongest binding activity to C57BL/6 mouse MHC I molecules. The peptide sequence RVAKGYQE had the highest score (Real Score 31.68) in ProPred-I prediction and was chosen as the peptide to pulse DCs to treat tumor-bearing mice. Through observing and comparing the in vitro killing activity and in vivo anti-tumor effects of this vaccine against mouse hepatoma, we investigated whether the antigen epitope of xenogenic homologous AFP peptide could effectively induce immune response against hepatoma.

Mature mouse bone marrow-derived DCs were successfully developed in this study. Under a high-power microscope, these cells formed suspended colonies and were characterized by the presence of burr-like protrusions on cell surface. The analysis of the phenotype of cultured DCs showed that the positive expression rates of mouse-specific marker 33D1 as well as mature DC-specific markers CD80, CD86 and MHC II molecules were all above 70%, meeting the requirements of the treatment.16 Due to the limitations of the available number of T lymphocytes isolated from the spleen of mice, the experiments on the in vitro killing activity of DC-induced mouse CTL against murine hepatocellular carcinoma Hepa1-6 cells were performed only in three groups. Statistical analysis indicated that there was no statistical significance. However, compared to the PBS group, cells in the hAFP-DCs and DCs groups showed stronger in vitro killing activity against Hepa1-6 cells. To further validate whether DCs in different groups have inhibitory effects on Hepa1-6 cell-derived tumors, in vivo antitumor experiments were conducted in mice. Compared to the PBS and DCs groups, from days 23, 27 and 31 after the inoculation, the tumors in mice in the hAFP-DCs group showed minimum growth size (p < 0.05). Repeated Measures ANOVA also suggested that hAFP-DCs had stronger inhibitory effects on tumor growth than DCs and PBS. The result for the survival duration of tumor-bearing mice was consistent with that of in vivo antitumor experiments. Mice in the hAFP-DCs group had longer survival duration than those in the DCs and PBS group, suggesting that injection of human AFP-derived peptide-pulsed dendritic cells into mice is able to generate significant inhibitory effects on tumor growth. This result is consistent with that reported by Tan Xiaohua et al.18 Additionally, we also used bioinformatic tools to screen effective AFP peptides. The peptide sequence RVAKGYQEL was identified as an antigen epitope of human AFP (xenogenic) which could induce autologous antitumor effects against mouse hepatoma, providing a new material and basis for the development of hepatocellular carcinoma vaccine.