Abstract. Hepatocellular carcinoma (HCC) is one of the most common and difficult to treat types of cancer worldwide. Antigen-targeted immunotherapy has the potential to be a novel and effective adjuvant for use in HCC. In the present study, recombinant adeno-associated virus carrying the α-fetoprotein gene (rAAV/AFP) and cancer cell lysates were used to pulse antigen-presenting dendritic cells (DCs) in order to stimulate a cytotoxic T lymphocyte (CTL) response against HCC. rAAV/AFP-pulsed and cancer cell lysate-pulsed DCs resulted in a mature DC phenotype with high expression of major histocompatibility complex (MHC) class I, MHC class II, CD80, CD83 and CD86 molecules. However, rAAV/AFP-pulsed DCs exhibited superiority over cancer cell lysate-pulsed DCs in terms of stimulating proliferation of T cells, activating T cells to secret interferon-γ (IFN-γ) and inducing an α-fetoprotein (AFP)-specific MHC class I-restricted CTL response. The current data suggest that pulsing of DCs using rAAV/AFP is more effective than the cancer cell lysate-pulsing technique, and that this technique may be used for the development of immunotherapy in AFP-positive HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the primary causes of cancer-related mortality, with a global incidence of >500,000 cases per year (1). The incidence and mortality of this disease have increased amongst all races and in the two genders over the past two decades. HCC has a poor prognosis, and there are few treatment options. Curative surgery and liver transplantation are only available to a small minority of patients with early-stage HCC. The majority of cases are detected at an advanced stage. Other commonly-used therapies are predominantly palliative (2,3).

α-fetoprotein (AFP) is an oncofetal antigen. Suppression of AFP synthesis occurs shortly subsequent to birth. However, 50-80% of adults with HCC exhibit re-expression of AFP during tumor progression (2,3). Human T cell repertoires are able to recognize AFP-derived peptide epitopes in the context of major histocompatibility complex (MHC) class I molecules and to induce AFP-specific protection (2,3). Therefore, AFP, as a tumor-associated antigen, may be a suitable target for dendritic cell-based cytotoxic T lymphocyte (CTL)-mediated antigen-targeted immunotherapy in HCC.

Pulsing dendritic cells (DCs) with tumor antigen is a versatile approach by which to generate cancer vaccines (4-8). DCs are the most effective type of antigen-presenting cells and are able to stimulate naive T lymphocytes to initiate a primary immune response (4). DCs are scarce, representing only 0.2% of total white blood cells. Therefore, a number of protocols in order to procure DCs from peripheral blood mononuclear cells (PBMCs) in vitro have been developed (9). These protocols allow the in vitro manipulation of DCs for clinical and laboratory-based studies (10). Techniques for generating antigen-loaded DCs include pulsing or incubating DCs with cancer cell lysate, utilizing cancer cells undergoing apoptosis, tumor antigen peptide and specific tumor protein, and the delivery of tumor antigen gene into DCs using viral vectors (5-7,11-15).

A phase II clinical trial demonstrated that vaccination with mature autologous DCs pulsed ex vivo with tumor cell line lysate in patients with advanced HCC was safe and well-tolerated, with evidence of antitumor efficacy assessed radiologically and serologically (8). The results from a separate study supported the superiority of lentivirus-AFP-engineered DC vaccine over AFP peptide-pulsed DC vaccine (16). Recombinant adeno-associated virus (rAAV) is one of the safest virus vectors used in gene therapy, as the wild-type virus has never been shown to cause human disease. A further advantage of rAAV is the absence of viral coding sequences, which may diminish the elimination of transduced DCs.
by virus-specific CTL (17-19). In addition, rAAV is able to transduce dividing and nondividing cells, which may allow the transduction of DCs in various states of activation and maturation, from a broad range of sources (19-21). For these reasons above, rAAV rather than a lentivirus was selected as the vector-carrying AFP gene for pulsing DCs in the present study, and was compared with cancer lysate-pulsed DCs.

The HPV-16 E7 antigen gene has been successfully transduced into DCs using rAAV, which resulted in the induction of a CTL response against cervical cancer cells (22). This result indicated that AFP gene delivery into DCs by rAAV may also be an effective approach for priming CTL response against HCC. The aim of the present study was to demonstrate whether rAAV was able to transduce the AFP gene into DCs effectively and prime an AFP-specific MHC-class I-restricted CTL response.

Materials and methods

Cell lines. The HepG2 and SK-HEP-1 HCC cell lines, and the K562 and HEK293 myelogenous leukemia cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The BEL7402 and SMMC7721 HCC cell lines were purchased from the Cancer Research Department of the China Medical Science Institute (Beijing, China). Cells were cultured in complete Dulbecco's modified Eagle's medium or RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 5 or 10% fetal bovine serum (Invitrogen Life Technologies). PBMCs obtained from healthy donors were separated using the Ficoll density-gradient centrifugation at 400 x g for 20 min and incubated in six-well culture plates at 37°C for 2 h in AIM-V medium. Following incubation, nonadherent cells were removed, and adherent PBMCs were cultured in AIM-V medium containing 800 units/ml granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN, USA). Adherent PBMCs were infected with 10^9 eg/ml rAAV/AFP. Following incubation for 8 h, the medium/virus solution was removed, the cells were washed and fresh AIM-V medium was added to the cultures. Throughout the culture period, 800 IU/ml GM-CSF was included in the medium. In order to induce the maturation of DCs, 1,000 IU/ml of human interleukin-4 (IL-4; R&D Systems) was added at 24 h. This permitted a brief period of monocyte proliferation, which promoted a higher level of rAAV transduction.

Cancer cell lysates were generated by four rapid freeze-thaw cycles of HepG2 cells. Cancer cell lysate-loading of DCs was performed by incubation of DCs with cancer cell lysate in the absence of transfection reagents. In brief, adherent PBMCs were cultured in AIM-V medium containing 800 units/ml GM-CSF and 1000 IU/ml IL-4. On day three, cancer cell lysates were added (100 µg lysate/5x10^3 DCs) and incubated with immature DCs. Every two days, the culture medium was replaced with fresh medium containing the cytokines. At day four, 50 IU/ml of human tumor necrosis factor-α (R&D Systems) was added into the culture. After six days of culturing, monocye-derived dendritic cells were harvested from nonadherent and loosely adherent cells. The survival of DCs upon viral infection was tested by trypsin blue stain (Sigma-Aldrich, St. Louis, MO, USA).

AFP expression of DCs and cell lines. DCs were harvested at six days post-transduction, and intracellular staining was performed in order to analyze AFP expression in rAAV/AFP-pulsed DCs. In brief, the cells were fixed, permeabilized and incubated with phycoerythrin (PE)-conjugated mouse anti-human AFP monoclonal antibody (1:100; 563002; BD Pharmingen, San Diego, CA, USA). A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) was used for data acquisitions. For each sample ≥10,000 cells were counted. Lysate-pulsed or untreated DCs were used as a control. AFP expression of the cell lines was tested using the same procedure.

**Construction of the rAAV vector.** The wild-type AAV type 2 genome, pSM620, was digested in order to delete the internal AAV sequences from map units 3-97, including the p5 promoter, and a specially designed polylinker was ligated in place, resulting in the rAAV vector plasmid, dl3-97 (23).

The cytomegalovirus (CMV) enhancer and the SV40 early mRNA polyadenylation signal DNA were derived from the p-enhanced green fluorescent protein (EGFP)-N1 plasmid (Clontech, Mountain View, CA, USA) and inserted into the dl3-97 vector. Subsequently, the CMV immediate early promoter was inserted into the dl3-97 vector (dl3-97/CMVp), which was derived from pEGFP-N1. Human AFP cDNA was amplified using reverse transcription-polymerase chain reaction (RT-PCR). Total mRNA was isolated from the HepG2 cells using an Oligotex mRNA isolation kit (Qiagen, Valencia, CA, USA). Once the first-strand cDNA was generated, PCR amplification of AFP sequence from nucleotides 12 to 1902 was conducted using the following primers: Forward: 5'-CTT CCACCCTGCAATAAC-3' and reverse: 5'-TTGTCTTCTTCTCCCCCCTG-3' (24). The AFP cDNA was then inserted into the dl3-97 vectors for 8 h at 4°C in order to generate the rAAV/AFP vector.

**Generation of rAAV virus.** The pSH3 plasmid is able to express the AAV type 2 rep and cap genes and the adenovirus type 5 E2A, VA1 and E4 genes, to allow rAAV DNA replication and packaging into viral particles without contaminating the wild-type AAV and adenovirus (25). The rAAV vector was co-lipofected into HEK293 cells with the pSH3 plasmid, and the rAAV was harvested after four days. A one-step column purification technique, which used gravity flow based on its affinity to heparin, without ultracentrifugation, was performed in order to generate the purified rAAV (26). The rAAV was titered by dot blot hybridization as described previously (22).

**Generation and pulsing of monocyte-derived DCs.** PBMCs were obtained from HLA-A2-positive healthy volunteers, separated by Ficoll density-gradient centrifugation at 400 x g for 20 min and incubated in six-well culture plates at 37°C for 2 h in AIM-V medium. Following incubation, nonadherent cells were removed, and adherent PBMCs were cultured in AIM-V medium containing 800 units/ml granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN, USA). Adherent PBMCs were infected with 10^9 eg/ml rAAV/AFP. Following incubation for 8 h, the medium/virus solution was removed, the cells were washed and fresh AIM-V medium was added to the cultures. Throughout the culture period, 800 IU/ml GM-CSF was included in the medium. In order to induce the maturation of DCs, 1,000 IU/ml of human interleukin-4 (IL-4; R&D Systems) was added at 24 h. This permitted a brief period of monocyte proliferation, which promoted a higher level of rAAV transduction.

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Cell surface marker analysis of DCs and cell lines. For analysis of the phenotype of DCs, the non-adherent and loosely adherent cells were collected by gentle pipetting following six days in culture. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse anti-human antibodies directed against MHC class I (HLA-ABC; 1:100; 555552), MHC class II (HLA-DR; 1:100; 555811), CD80 (1:100; 555683), CD83 (1:100; 555910) and CD86 (1:100; 555657), which were all purchased from BD Pharmingen. All samples were then analyzed using a FACSCalibur flow cytometer. HLA-A2 expression of the cell lines were tested using FITC-conjugated mouse anti-human monoclonal antibodies against HLA-A2 (1:100; 551285; BD Pharmingen).

T-cell proliferation assay. CD3+ T cells were isolated from PBMCs using a Pan T Cell Isolation kit II (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer’s instructions. The rAAV/AFP-pulsed or lysate-pulsed DCs were co-cultured with CD3+ T cells at a ratio of 1:20 in the presence of 20 units/ml recombinant human IL-2 and 20 ng/ml IL-7 (R&D Systems). The wild type AAV-pulsed and untreated DCs were used as controls. The DC-T cell culture medium was replaced by fresh medium plus the cytokines every two days. On day eight, T cell proliferation was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA) and 3H-thymidine uptake assay. For the Cell Titer 96 AQueous One Solution Cell Proliferation Assay, dye solution was added to each well and incubated for 4 h. A microplate imaging system was used to measure the absorbance of the samples at 490 nm. For 3H-thymidine uptake assay, the cultures were pulsed with 1 µCi 3H-thymidine (New England Nuclear, Boston, MA, USA) per well for 12 h. 3H-thymidine uptake was counted using an LS 6000SE liquid scintillation counter (Beckman Coulter, Brea, CA, USA).

Cytokine expression level analysis in primed T cells. T cells were harvested eight days after priming. The intracellular staining assay described above was performed to analyze interferon-γ (IFN-γ) and IL-4 expression using FITC-conjugated mouse anti-human IFN-γ (1:100; 552882) and PE-conjugated mouse anti-human IL-4 (1:100; 559333) monoclonal antibodies (BD Pharmingen).

Cytotoxicity assays. After eight days of the DC-T cell culture, 6 h chromium-51 (51Cr) release assays were used to analyze the killing activity of the CTL, which had been elicited by the rAAV/AFP-pulsed, lysate-pulsed or untreated DCs against the target cells. The targets included HepG2, BEL7402, SMMC7721, Sk-Hep-1 and natural killer cell (NK)-sensitive K562 tumor cells. The 51Cr-labeled target cells were mixed with the CTL (1:20) and incubated for 6 h at 37°C with 5% CO2. In order to determine the structures on the target cells, the mouse anti-human HLA-A2 monoclonal antibody (1:100; 551230; BD Pharmingen) was used to block cytotoxicity. The 51Cr-labeled targets were pre-incubated with mouse anti-human HLA-A2 antibody for 2 h prior to performance of the 51Cr-release assay. In order to demonstrate the AFP-specific killing activity of the CTL, a series of AFP-negative cells were also tested. K562 cells were used as targets to observe NK activity.

Statistical analysis. All data are expressed as the mean ± standard deviation and differences between groups were analyzed using the Student’s t-test with SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Construction and generation of rAAV. To construct the rAAV vectors for this study, the CMV immediate early promoters were successfully inserted into the dl3-97 vectors. AFP mRNA was isolated from HepG2 cells and amplified by RT-PCR (data not shown). cDNA was successfully cloned into the rAAV vectors, sequenced and determined to be identical to the published sequence (24,27). The viral stocks of rAAV were generated and titered (data not shown). The viral titer was 10^11 eg/ml.
Levels of antigen-positive DCs following rAAV delivery. In order to assess the efficiency of rAAV pulsing of DCs, the level of AFP protein in rAAV/AFP-pulsed DCs was analyzed using intracellular staining at six days post-transduction. The results, shown in Fig. 1A, demonstrated that rAAV/AFP infection of DCs results in a high percentage of cells containing intracellular AFP protein (82.8%).

Characterization of DCs. DCs were generated from healthy volunteers. The phenotypes of DCs were examined by flow cytometric analysis in order to determine whether significant differences in phenotype were discernible among untreated, lysate-pulsed and rAAV/AFP-pulsed DC populations. The results demonstrated that the DCs generated from all three techniques displayed a characteristic phenotype, with expression of MHC class I (HLA-ABC), MHC class II (HLA-DR), CD80, CD83 and CD86 (P>0.05; Fig. 1B). The survival of DCs following viral infection, was measured using trypan blue staining. The results showed that rAAV/AFP-pulsing or wild type AAV-pulsing has no significant effect on the viability of DCs (data not shown).

Proliferation of T cells stimulated by rAAV/AFP-pulsed DCs. In order to assess the stimulatory capacity of DCs, rAAV/AFP-pulsed, lysate-pulsed, wild type AAV-pulsed and untreated DCs were co-cultured for eight days with T cells from the donors from which the DCs had been obtained. The formation of large T cell clusters was observed in T cells co-cultured with rAAV/AFP-pulsed DCs. Smaller clusters were observed in T cells that had been co-cultured with lysate-pulsed and untreated DCs (Fig. 2A). T cell proliferation was induced by rAAV/AFP-pulsed, lysate-pulsed, wild type AAV-pulsed and untreated DCs. However, proliferation levels were lower for the lysate-pulsed, wild type AAV-pulsed and untreated groups that for the rAAV/AFP-pulsed groups (P<0.05; Fig. 2B and C).

Cytokine production of CD4+ and CD8+ T cells. The cytokine production of T cells was measured using intracellular staining in order to determine T cell activation. T cells co-cultured with DCs were collected on day eight and analyzed for expression of IFN-γ and IL-4. A relatively high level of IFN-γ production was detected in CD4+ (30.2%) and CD8+ (32.8%) T cells stimulated by rAAV/AFP-pulsed DCs. A smaller proportion of IFN-γ producing T cells were observed in the T cell populations co-cultured with either cancer cell lysate-pulsed DCs (10.5% of CD4-positive cells and 9.6% of CD8-positive cells) or untreated DCs (5.6 and 3.2% of CD4 and CD8-positive cells, respectively; P<0.05). Minimal production of IL-4 was detected in CD4+ and CD8+ T cells (Fig. 3).

rAAV/AFP-pulsed DCs primed and propagated AFP-specific and HLA class I-restricted CTLs. T cells were co-cultured...
with rAAV/AFP-pulsed, lysate-pulsed or untreated DCs at a ratio of 20:1 for eight days. A 6 h chromium-51 (51Cr) release assay was used to assess the induction of the CTL response. In order to determine antigen-specific and HLA class I-restricted tumor lysis by CTLs, multiple tumor targets were used (Fig. 4A). The T cells stimulated by rAAV/AFP-pulsed DCs exhibited 42.9% lysis of HepG2 (HLA-A2+, AFP+) cells and 48.5% lysis of BEL7402 (HLA-A2+, AFP+) cells. By contrast, there was reduced CTL activity against SMMC-7721 (HLA-A2+, AFP-), SK-Hep1 (HLA-A2+, AFP-) and K562 cells (HLA-A2-, AFP-; P<0.05). Furthermore, lysis of HepG2 and BEL7402 cells was significantly reduced by preincubation of the tumor cells with anti-HLA-A2 mAb (Fig. 4B). As shown in Fig. 4C, only 22.4% lysis of HepG2 cells was observed when using T cells stimulated by HepG2-lysate-pulsed DCs, which was significantly lower than that induced by T cells stimulated by rAAV/AFP-pulsed DCs (P<0.05). The killing activity of CTLs stimulated by lysate-pulsed DCs against BEL7402, SMMC-7721 and SK-Hep1 cells was observed. Cell death was not observed using the untreated DC-stimulated T cells (Fig. 4D).

Discussion

DCs are potent antigen-presenting cells that are involved in regulating immune responses (28-30). In human tumors, the presence of functional immunogenic DCs is rare. Defective DC recruitment, differentiation, maturation and survival may contribute to the low levels of functional DCs, observed in human cancer (31). An approach which may be effective in combatting these low levels is to generate functional DCs in vitro and transplant the genetically manipulated DCs into patients. Immunization of patients with ex vivo-generated DCs is feasible and enhances antigen-specific immune responses in humans.

In the present study, DCs transduced with antigen-expressing rAAV were compared with control lysate-pulsed DCs. The results demonstrated that direct transduction of DCs with antigen-expressing rAAV was more efficient than loading DCs with tumor lysate. There are a number of possible reasons that may explain this result. Due to protein degradation and MHC molecule cycling, protein pulsing of DCs may be an inefficient way to deliver an antigen to target cells. By contrast, gene transfer ensures long-lasting expression of the target antigen.
and may provide an opportunity for the repeated stimulation of CTLs. Viral entry into DCs is often more efficient than other approaches for the introduction of antigen. Viral delivery of antigens may result in the production of an entire array of epitopes presented by the autologous MHC class I and MHC class II molecules, which results in a more efficient activation of multiclonal T cells (32‑34). Viral gene delivery results in the production and endogenous expression of a number of epitopes in DCs, which are theoretically more representative and immunoreactive than that produced by alternative approaches.

AFP is an ideal antigen target for immunotherapy in HCC, due to the high level of expression in the majority of HCCs, and the low level of expression in healthy liver and other cell types. The present study demonstrated that the rAAV/AFP virus effectively loaded freshly adherent DC precursors with the AFP gene. In addition, the transduction of the AFP gene into DCs resulted in functionally active AFP‑epitope‑presenting DCs, without impacting upon the phenotype, viability and functions of these cells. One explanation for this result may be that rAAV is one of the safest virus vectors used in gene therapy. The wild-type virus has never been shown to cause human disease.

The rAAV/AFP-pulsed DCs were able to prime and propagate AFP antigen-specific CTLs in an efficient manner. A notable phenomenon observed during the priming process was the formation of large DC-T cell clusters two days after addition of T cells, which may suggest enhanced priming in this group (35-37). T cell proliferation were also induced by wild type AAV-pulsed DCs. However, the proliferation level was significantly lower in this group, which indicates that proliferation is specific for AFP, but not for the cytokines produced by DCs in response to a viral infection.

The T cells primed by rAAV/AFP-pulsed DCs exhibited low levels of IL-4, a characteristic cytokine in the Th2 response, and increased levels of IFN-γ, a typical Th1 cytokine. These data suggest that the use of rAAV/AFP-pulsed DCs is effective in generating a significant Th1 response.

Marked CTL activity, induced by rAAV/AFP-pulsed DCs against MHC class I-matched, AFP-positive HCC cell lines was observed after eight days of priming. The majority of DC loading/priming protocols require a far longer period in order to demonstrate CTL activity. The underlying reason for this is unknown. However, there are a number of possible explanations. Firstly, the high transduction efficiency of the rAAV vector. Secondly, the effective formation of the DC-T cell clusters and proliferation of T cells. Thirdly, the high levels of CD80, CD83 and CD86 expression. Finally, higher IFN-γ secretion with no detectable IL-4 secretion, which is associated with the desirable Th1 response. Blockage of HLA-A2 inhibited cytotoxicity, which was in accordance with HLA-A2-restriction. No significant cytotoxicity was observed against AFP-negative target cells or NK-sensitive K562 cells, indicating that the cytotoxicity is antigen-specific and MHC class I restricted.

In conclusion, rAAV/AFP pulsing of DCs represents a promising technique for effectively introducing the AFP antigen gene into DCs in order to stimulate an AFP-specific CTL response against HCC. The protocol described in the current study may
in future be used to develop an adjuvant immunotherapy for the treatment of HCC. In addition, the present study provides a foundation for future studies, involving transduction of alternative tumor antigen genes into DCs using rAAV in order to elicit a CTL response against the transduced antigen.

References


