Research Article

Multiple Antigenic Peptides of Human Heparanase Elicit a Much More Potent Immune Response against Tumors

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Abstract

Peptide vaccination for cancer immunotherapy requires an ideal immune response induced by epitope peptides derived from tumor-associated antigens (TAA). Heparanase is broadly expressed in various advanced tumors. Accumulating evidence suggests that heparanase can serve as a universal TAA for tumor immunotherapy. However, due to the low immunogenicity of peptide vaccines, an ideal immune response against tumors usually cannot be elicited in patients. To increase the immunogenicity of peptide vaccines, we designed three 4-branched multiple antigenic peptides (MAP) on the basis of the human leukocyte antigen (HLA)-A2–restricted cytotoxic T lymphocyte (CTL) epitopes of human heparanase that we identified previously as antigen carriers. Our results show that MAP vaccines based on the HLA-A2–restricted CTL epitopes of human heparanase were capable of inducing HLA-A2–restricted and heparanase-specific CTLs in vitro and in mice. Moreover, compared with their corresponding linear peptides, heparanase MAP vaccines elicited much stronger lysis of tumor cells by activating CD8⁺ T lymphocytes and increasing the releasing of IFN-γ. However, these heparanase-specific CTLs did not lyse heparanase-expressing autologous lymphocytes and dendritic cells, which confirm the safety of these MAP vaccines. Therefore, our findings indicate that MAP vaccines based on CTL epitopes of human heparanase can be used as potent immunogens for tumor immunotherapy because of advantages such as broad spectrum, high effectiveness, high specificity, and safety. Cancer Prev Res; 4(8); 1285–95. ©2011 AACR.

Introduction

Dendritic cell (DC)-based immunotherapy, which has the advantage of strong immunogenicity and minimal side effects, has become a hot topic in the study of malignant tumor therapy (1–3). Antigen presentation is critical for the initiation of an immune response, and DCs are important in this process because they are the most potent professional antigen-presenting cells (4, 5). To this end, DCs present tumor-associated antigens (TAA) to the immune system and initiate a TAA-specific immune response. TAA-loaded DC presentation is the major mechanism of tumor immunotherapy. Unfortunately, most TAAs that have been described possess tissue specificity, such as in the case of carcinoembryonic antigen (CEA) in colon and lung carcinoma, prostate-specific antigen (PSA) in prostatic carcinoma, and α-fetoprotein (AFP) in hepatocellular carcinoma. Immunotherapy targeting these TAAs has shown a narrow therapeutic window. Therefore, it is critical to identify universal TAAs for tumor immunotherapy.

Heparanase (Hpa) is the only endogenous endoglycosidase found thus far that can degrade the heparan sulphate proteoglycans in the extracellular matrix (ECM) and basement membrane (BM; ref. 6). Heparanase is found in almost all metastatic malignant tumor cells. Inhibition of heparanase can inhibit the proliferation and metastasis of tumor cells. Activation of heparanase allows tumor cells to break through the ECM and BM barrier, release cytokines, form new vessels, and cause the local permanent seeding of tumor cells, thus metastasizing (7–9). Our previous study showed that the DC-loaded full-length heparanase cDNA could induce a heparanase-specific cytotoxic T lymphocytes (CTL), which showed potent lysis of gastric carcinoma cells that were heparanase positive and human leukocyte antigen (HLA)-A2 matched (10). These results indicated that heparanase can serve as a TAA that can be used for tumor immunotherapy.

CTLs are chief mediators of tumor immunosurveillance through the recognition of TAAs as cognate peptides bound
to MHC molecules expressed on the surface of tumor cells. A major advance in tumor immunology in the last 20 years was the verification that CTL epitopes bind to MHC rather than to integral TAAs to induce CTL activation. These epitope peptides are often 8 to 10 amino acids long, with 2 to 3 primary anchor residues that interact with MHC class I molecules and 2 to 3 amino acid residues that bind to the T-cell receptor (11).

The first group of immunogenic epitopes in heparanase was discovered by Sommerfeldt and colleagues using the SYFPEITHI algorithm to identify nonapeptides of the heparanase amino acid sequence (12). By using HLA-A2–restricted epitope prediction algorithms based on supermotif and quantitative motif methods, we successfully predicted and identified another 3 HLA-A2–restricted CTL epitopes, Hpa277 (277–285, KMLKSFLKA), Hpa405 (405–413, WLSLLFKKL) and Hhtpa525 (525–553, PAFSYSFFV), in human heparanase and 2 H-2Kb–restricted CTL epitopes, mHpa398 (398–405, LSLLFKKL) and Hpa405 (405–413, WLSLLFKKL) and their corresponding 4-branched polypeptides, also called MAPs, were synthesized by Chinese Peptide Company (Fig. 1). An HLA-A2–restricted nonapeptide HIV/pol (476–484, ILLEPVHGV) derived from HIV was synthesized as a negative control. On the basis of the selected amino acid sequences, MAPs were produced by solid-phase peptide synthesis (SPPS) using Fmoc chemistry onto a branched lysine core resulting in four 9-mer peptides presented on 1 structure (21). The peptides were purified using reverse-phase high-performance liquid chromatography (HPLC) on a Vydac C18 column. The purity of the peptides was confirmed by analytic HPLC, and the identity was confirmed by mass spectrographic analysis. The purity of the peptides can reach 95% or more by purification with HPLC.

DC generation from human peripheral blood precursors and mouse bone marrow

DCs from peripheral blood mononuclear cells (PBMC) were isolated using the procedure previously described by Romani and colleagues (22). Briefly, PBMCs were isolated from healthy HLA-A2+ donors by Ficoll-Hypaque density gradient centrifugation and then seeded into culture flasks in RPMI-1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS. After monocytes adhered (incubation for 2 hours), the nonadherent cells were collected and frozen in freeze medium (60% RPMI-1640 and 30% FBS, 10% dimethyl sulfoxide) for further use in CTL assays. The adherent cells were cultured for 7 days in RPMI-1640 containing 1,000 U/mL of Interleukin-4 (IL-4) and GM-CSF.

Materials and Methods

Mice and cell lines

C57BL/6 transgenic mice, which express a chimeric heavy chain of the MHC-I molecule (HLA-A-2.1) and a2 as well as H-2Kb, transmembrane and intracytoplasmic domains), were kindly provided by Prof. Ni B from the Institute of Immunology, Third Military Medical University. Animal studies were conducted in agreement with the local ethics committee of the Third Military Medical University. The osteogenic sarcoma cell line U2OS (Hpa+, HLA-A2+), gastric cancer cell line KATO-III (Hpa+, HLA-A2+), colon cancer cell line SW480 (Hpa+, HLA-A2+), liver cancer cell line HepG2 (Hpa+, HLA-A2+), and breast cancer cell line MCF-7 (Hpa+, HLA-A2+) were maintained in our laboratory. U2OS, MCF-7, and HepG2 cells were cultured in Dulbecco’s modified Eagle’s media (DMEM; Hyclone) supplemented with 10% FBS, 10 U/mL penicillin, and 100 μg/mL streptomycin. KATO-III and SW480 cells were all cultured in RPMI-1640 medium containing 10% FBS, penicillin (200 U/mL), streptomycin (100 μg/mL). All cell lines mentioned previously were maintained at 37°C in a humidified atmosphere containing 5% CO2.

Polypeptide synthesis, purification, and identification

Three HLA-A2–restricted CTL epitopes peptide from human heparanase, Hpa (525–533, KMLKSFLKA), Hpa (277–285, WLSLLFKKL), and Hpa405 (405–413, PAFSYSFFV) and their corresponding 4-branched polypeptides, also called MAPs, were synthesized by Chinese Peptide Company (Fig. 1). To investigate the immune responses against various tumors, CTL activity induced by these heparanase MAP vaccines was determined by a 4-hour 51Cr release assay in vitro and ex vivo. The results showed that these MAP vaccines could elicit much more killing than their corresponding linear peptides. These results provide the theoretical foundation for heparanase MAP vaccine immunotherapy for patients with malignant tumors.
granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Inc.) and interleukin (IL-4; R&D Systems, Inc.) and were then cultured for an additional day in the presence of 1,000 U/mL of TNF-α (R&D Systems, Inc.) to induce final maturation. After 8 days of culture, the mature DCs were harvested. Phenotypic markers of DCs were analyzed by flow cytometry.

DCs from mouse bone marrow (mDC) were generated as described previously (14). Briefly, bone marrow was flushed from the tibias and femurs of C57BL/6-Tg mice and depleted of erythrocytes with commercial lysis buffer (Sigma). The cells were washed twice in serum-free RPMI-1640 medium and cultured in a 100-mm plastic Petri dish at 1 x 10⁵ cells/mL with RPMI-1640 medium containing 200 U/mL recombinant murine GM-CSF (mGM-CSF; R&D System, Inc.) and 400 U/mL recombinant murine IL-4 (mIL-4; R&D System, Inc.). On days 3, 5, and 7, half of the media was refreshed without discarding any cells, and fresh cytokine-containing (mGM-CSF and mIL-4) media was added. On day 8 of culture, mTNF-α (R&D System, Inc.) was added to the media. On day 9, nonadherent cells obtained from these cultures were considered as mature bone marrow–derived DCs. Flow cytometry confirmed the phenotypic markers of mDCs (BD FACSARia II).

Induction of peptide-specific CTLs with synthetic heparanase peptides

This assay was conducted as described previously (13–15). Briefly, DCs from PBMCs were loaded with different heparanase peptides and a negative control peptide at a final concentration of 30 μmol/L for 4 hours and then irradiated with 20 Gy, which prevented all outgrowths in the control cultures. Autologous T cells were restimulated every 7 days with the previously mentioned peptide-pulsed DCs to generate peptide-specific CTLs. Recombinant IL-2 at a concentration of 50 U/mL was added to the culture medium on day 3 after every stimulation. CTL activity was then assessed on day 23 by a 4-hour ⁵¹Cr release assay.

DCs generated from mouse bone marrow were cultured in 1 mL of RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin containing 30 μmol/L of each heparanase polypeptide. Cells were then cultured at 37°C for 3 hours with gentle shaking every 30 minutes. Next, cells were washed twice in PBS, and then, the above mentioned DCs (2 x 10⁶) with loaded peptides were used to immunize C57BL/6-Tg(HLA-A2)1ENGE/J mice 3 times (with a 7-day interval) by subcutaneous injection. Mice were sacrificed, and spleens were removed 7 days after the last peptide-pulsed DCs immunization. The T cells (4 x 10⁶/mL) were cultured with different heparanase peptides in 6-well plates in complete medium containing IL-2 at 50 units/mL. After 5 days of coculture, the in vivo restimulated splenocytes were assayed by 4-hour ⁵¹Cr release.

Cytotoxicity assay

To evaluate the levels of CTL activity, a 4-hour ⁵¹Cr release assay was used as previously described (13–15). Briefly, target cells were incubated with ⁵¹Cr (100 μCi per 1 x 10⁶ cells) for 2 hours in a 37°C water bath. After incubation with ⁵¹Cr, target cells were washed 3 times with PBS, resuspended in RPMI-1640 medium, and mixed with an effector cells at a 10:1, 20:1, 40:1, or 80:1 effector to target (E/T) ratio. Assays were conducted in triplicate for each sample at each ratio in a 96-well round-bottomed plate. After a 4-hour incubation, the supernatants were harvested, and the amount of released ⁵¹Cr was measured with a gamma counter. The percentage specific lysis was calculated according to the following formula:

Specific lysis (%) = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100%
Enzyme-linked immunospot assay for INF-γ

The generated CTLs from mice splenocytes were assayed in 24-hour enzyme-linked immunospot (ELISPOT) cultures (96-well coated microtiter plates; DAKEWE) for INF-γ production. Briefly, effectors were plated in triplicate at a final concentration of 1 × 10⁵ or 3 × 10⁵ cells per well in 96-well nitrocellulose plates. Effectors were stimulated by candidate peptides at a final concentration of 30 μmol/L. The plate was incubated in 37°C with 5% CO₂ for 24 hours. The plate was processed using a biotin-labeled anti-mouse INF-γ, enzyme-labeling marker, and anti-marker, then the freshly prepared developer was added and incubated in the dark at 37°C for 8 minutes (Quick Spot Mouse INF-γ Precoded ELISPOT kit; DAKEWE). Spots were quantified using the ELISPOT reader (BioReader 4000 Pro-X; BIOSYS).

To investigate whether heparanase-specific MAP vaccines trigger CD8⁺ T lymphocyte with the help of CD4⁺ T lymphocyte, we depleted the CD4⁺ T lymphocytes with a method reported previously (23). Briefly, the C57BL/6-Tg mice were injected with 100 μg of purified anti-mouse CD4 antibody (BioLegend Inc.) through the vena caudalis once a day for 3 days before the first immunization. Mice were sacrificed, and spleens were removed. The splenocytes were used as effectors for assaying INF-γ by ELISPOT.

Statistics

All experiments were run in triplicate, and the results are given as the mean ± SD of triplicate determinations. Statistical analysis was conducted using Student’s t test. Differences were considered statistically significant when the value of P was <0.05. All statistical analyses were conducted using SPSS 11.5 software.

Results

Polypeptide synthesis, purification, and identification

We synthesized 3 peptides, Hpa (525–533, KMLKSFLKA), Hpa (277–285, WLSLLFKKL), and Hpa (405–413, PAFSYSFFV) and their corresponding...
4-branched MAP peptides MAP4-Hpa525–533 (KMLKSF1KLA), MAP4-Hpa277–285(WL5LFFKKL), and MAP4-Hpa405–413(PAFS5SFEY) were generated from MAP4-Hpa525, MAP4-Hpa277, and MAP4-Hpa405 by SPPS. The purity of the peptides was confirmed by analytic reverse-phase HPLC. Human heparanase epitopes [Hpa(525–533), Hpa(277–285), and Hpa(405–413)] had retention times (RT) of 11.735, 8.307, and 9.794 minutes, respectively, and the peak area percentages were 98.1%, 97.6%, and 98.1%, respectively. The RTs for the MAP human heparanase epitopes [MAP4-Hpa525, MAP4-Hpa277, and MAP4-Hpa405] were 8.695, 9.175, and 10.934 minutes, respectively, and the peak area percentages were 96.7%, 98.2%, and 90.1%, respectively. The purity of the peptides reached 90% or more, which met the international reference standard for polypeptide experiments. The identification of the peptides was confirmed by mass spectrometric analysis. The molecular weight of the human heparanase epitopes [Hpa(525–533), Hpa(277–285), and Hpa(405–413)] were 1,065.40, 1,147.50, and 1,064.20 Da, respectively, and their theoretical value were 1,065.62, 1,147.46, and 1,066.66 Da, respectively. The molecular weight of MAPs of human heparanase epitopes [MAP4-Hpa525, MAP4-Hpa277, and MAP4-Hpa405] were 4,663.2, 4,991.5, and 4,658.4 Da, respectively, and their theoretical value were 4,663.0, 4,991.6, and 4,658.8 Da, respectively (Fig. 1). There was no significant discrepancy between the observed value and the theoretical value, which confirms the identity of the peptides.

Identification of DCs

The phenotype of DCs was assessed by flow cytometry. The results showed that the expression of CD86, CD83, CD11c, MHC-II, and HLA-A2 in DCs from PBMCs were 84.9%, 93.8%, 95.0%, and 99.8%, respectively (Fig. 2C), whereas the expression of CD86, CD11c, MHC-II, and HLA-A2 in DCs from mice were 96.3%, 73%, 95%, and 89.5%, respectively (Fig. 2D). These data indicate that these DCs are mature and have antigen-presenting capacity.

MAP vaccines of heparanase are more potent than linear peptide in inducing killing activity

To detect whether the heparanase MAP vaccines could elicit stronger heparanase-specific CTLs than their corresponding linear peptide, DCs were loaded with heparanase MAP vaccines and their corresponding linear peptides to induce heparanase-specific CTLs. Killing of various tumor cell lines was measured by a 4-hour 51Cr release assay. The results showed that the MAP vaccines from human heparanase (MAP4-Hpa525, MAP4-Hpa277, MAP4-Hpa405) and their corresponding linear peptides (Hpa525, Hpa277, Hpa405) could induce heparanase-specific killing of KATO-III gastric cancer cells, U2OS osteogenic sarcoma cells, and SW48 colon cancer cells, which express both heparanase and HLA-A2 (Fig. 2A). At the highest E/T ratio (80:1), the lysis rates generated from MAP4-Hpa525, MAP4-Hpa277, and MAP4-Hpa405 were 78.1%, 78.6%, and 70.2%, respectively, in vitro and 82.3%, 73.3%, and 77.5%, respectively, ex vivo. The lysis rates generated from Hpa525, Hpa277, and Hpa405 were 57.3%, 56.2%, and 53.3% respectively, in vitro and 59.1%, 51.4%, and 53.6%, respectively, ex vivo. The induced effectors generated from the negative peptide could not lyse the target cells (Fig. 3). These results indicate that MAP vaccines from human heparanase are capable of eliciting much more potent killing of tumor cells than their corresponding linear peptides in vitro and ex vivo.

MAP vaccines of heparanase induce killing activity to different tumor cell lines in a heparanase-specific and MHC-restricted manner

To further confirm the heparanase specificity of the CTLs in vitro and ex vivo, we used the HLA-A2–positive, heparanase-negative breast cancer cell line MCF-7 (Fig. 2A and B; ref. 24). MCF-7 cells were transfected with recombinant, replication-defective adenovirus (Ad-Hpa) encoding a full-length cDNA of human heparanase at a multiplicity of infection (MOI) of 200 and cultured for 2 days in fresh DMEM medium containing 10% FCS (10). Western blot analysis showed that heparanase protein could be detected in MCF-7/Hpa cells, whereas in MCF-7 cells, the expression of heparanase is very weak (Fig. 2A). Heparanase-specific CTLs were generated using peptide-pulsed DCs from HLA-A2–positive PBMCs and DCs from the HLA-A2 bone marrow of C57BL/6-Tg transgenic mice. After 3 stimulations, the killing of MCF-7 and MCF-7/Hpa cells by CTLs induced by MAP4-Hpa525, MAP4-Hpa277, MAP4-Hpa405, Hpa525, Hpa277, or Hpa405 were determined at various E/T ratios by a 4-hour 51Cr release assay. The results showed that these heparanase-specific CTLs could lyse MCF-7/Hpa, whereas no obvious lysis of MCF-7 cells was detected even at the highest E/T ratio. Furthermore, compared with the corresponding linear peptides of heparanase, heparanase MAP vaccines could elicit stronger specific lysis of MCF-7/Hpa cells. The lysis rates generated from MAP4-Hpa525, MAP4-Hpa277, and MAP4-Hpa405 were 78.9%, 79.6%, and 78.3%, respectively, in vitro and 78.2%, 80.9%, and 73.9%, respectively, ex vivo. The lysis rates generated from Hpa525, Hpa277, and Hpa405 were 53.6%, 51.9%, and 50.4%, respectively, in vitro and 54.7%, 57.1%, and 59.8%, respectively, ex vivo (Fig. 4). These results clearly indicate that the CTLs are specifically targeted against heparanase peptides that were presented in the context of HLA-A2, and the MAP vaccines of human heparanase could elicit much more potent killing effects compared with their corresponding linear peptides.

To further confirm that these CTL epitopes were restricted by HLA-A2, we took advantage of the heparanase-positive HLA-A2–negative liver cancer cell line HepG2 (Fig. 2A and B; refs. 13, 15). HepG2 cells were transduced with a eukaryotic vector containing the full-length cDNA of HLA-A2 bone marrow of C57BL/6-Tg transgenic mice. After 3 stimulations, the killing of HepG2 cells by CTLs induced by MAP4-Hpa525, MAP4-Hpa277, MAP4-Hpa405, Hpa525, Hpa277, or Hpa405 were determined at various E/T ratios by a 4-hour 51Cr release assay. The results showed that these heparanase-specific CTLs could lyse HepG2 cells, whereas no obvious lysis of HepG2 cells was detected even at the highest E/T ratio. Furthermore, compared with the corresponding linear peptides of heparanase, heparanase MAP vaccines could elicit stronger specific lysis of HepG2 cells. The lysis rates generated from MAP4-Hpa525, MAP4-Hpa277, and MAP4-Hpa405 were 78.9%, 79.6%, and 78.3%, respectively, in vitro and 78.2%, 80.9%, and 73.9%, respectively, ex vivo. The lysis rates generated from Hpa525, Hpa277, and Hpa405 were 53.6%, 51.9%, and 50.4%, respectively, in vitro and 54.7%, 57.1%, and 59.8%, respectively, ex vivo (Fig. 4). These results clearly indicate that the CTLs are specifically targeted against heparanase peptides that were presented in the context of HLA-A2, and the MAP vaccines of human heparanase could elicit much more potent killing effects compared with their corresponding linear peptides.
After 24 hours of transduction, 400 μg/mL G418 was added to the RPMI-1640 medium. After G418 selection for 4 weeks, drug-resistant individual clones were randomly collected from the transduced cultures. The selected clone was named HepG2/HLA-A2. HLA-A2 was expressed in 71.4% of HepG2/HLA-A2 compared with 1.3% in control HepG2 cells (Fig. 2B). The 51Cr release assay showed that CTLs generated from Hpa-MAP525, Hpa-MAP277, Hpa-MAP405, Hpa525, Hpa277, and Hpa405 peptide-pulsed DCs could lyse HepG2/HLA-A2 in vitro and ex vivo. However, the polypeptide-induced effectors could not lyse HepG2 cells, even at the highest E/T ratio. Moreover, compared with their corresponding heparanase linear peptides, heparanase MAP vaccines could elicit much more potent lysis of HepG2/HLA-A2 cells. At the highest E/T ratio (80:1), the lysis rate was 39% in vitro and 24% ex vivo (Fig. 4). These results clearly show that the polypeptide-induced CTLs are restricted by MHC I molecules.

Heparanase-specific CTLs do not lyse autologous lymphocytes or DCs

It was reported that heparanase is expressed in immunologically competent cells, natural killer cells and inflammatory cells such as neutrophils, granulocytes, and activated T and B cells (8). Theoretically, immunotherapy aimed at heparanase may have side effects on the immune system. To investigate the effect of heparanase-specific CTLs on immunologically activated lymphocytes, CTLs induced by heparanase-specific peptides

![Figure 3. Specific lysis of CTLs generated from different heparanase-derived peptides against various target cells in vitro and ex vivo. CTLs generated from the HLA-A2–restricted nonapeptide HIVpol(476–484, ILLEPVHGV) derived from HIV served as the negative peptide (NP). A, specific lysis of CTLs induced by MAP vaccines or their corresponding linear polypeptide-pulsed DCs from PBMCs against U2OS (a), SW480 (b), or KATO-III (c) in vitro. B, specific lysis of CTLs induced by MAP vaccines or their corresponding linear polypeptide-pulsed DCs from bone marrow against U2OS (a), SW480 (b), or KATO-III (c) ex vivo. Splenocytes served as effectors.](image-url)
were also used to lyse autologous lymphocytes and DCs. The results indicate that MAP vaccines and their corresponding linear peptides from heparanase did not lyse autologous lymphocytes or DCs in vitro or ex vivo (Fig. 5).

**ELISPOT assay for IFN-γ**

Because CTLs are known to produce the Th1 cytokine IFN-γ, peptide-specific T cells were quantitated by measuring IFN-γ-producing cells by ELISPOT assay. As shown in Figure 6, MAP4-Hpa525, MAP4-Hpa277,
MAP4-Hpa405, and their corresponding linear peptides were found to generate strong peptide-specific T-cell responses by virtue of their ability to induce increased frequencies of IFN-γ-producing T cells as compared with a control peptide (P < 0.05). We also found that mice immunized by MAP4-Hpa525, MAP4-Hpa277, or MAP4-Hpa405 produce many more IFN-γ spots than their corresponding linear peptides (P < 0.05). To study the role of CD4+ T cells in the development of IFN-γ-producing cells, we depleted the CD4+ T lymphocytes by treating C57BL/6-Tg mice with anti-CD4 monoclonal antibody (mAb) before the first immunization. The results showed that IFN-γ-producing cells were significantly reduced in CD4-depleted mice compared with their counterparts in nondepleted mice (P < 0.05; Fig. 6). The results allow us to hypothesize that the immune response of CD8+ T cell against tumors is CD4+ T-cell dependent.

Discussion

Active cancer immunotherapy relies on the fundamental concept that tumor antigens exist and are present in the context of MHC for recognition by specific effector T cells. However, heterogeneous expression of most of the characterized tumor antigens limits the broad applicability of cancer vaccines that target such antigens (25, 26). Therefore, it is necessary for clinicians to identify universal TAAs for immunotherapy of different types of tumors. It has been reported that heparanase could be detected in almost all metastatic malignant tumors. The occurrence of metastasis depends critically on the activation of heparanase, which allows tumor cells to break through the BM and ECM, release cytokines, form new vessels, and allow the local permanent seeding of tumor cells (27). Inhibition of heparanase thus can inhibit the proliferation and metastasis of tumor cells (28). Furthermore, if some tumors evade the immune system through downregulating expression of heparanase, then proliferation and metastasis of tumor cells may be inhibited. Therefore, heparanase is a potentially universal TAA for immunotherapy of advanced stage tumors.

Sommerfeldt and colleagues first proved that heparanase could serve as a universal TAA for tumor immunotherapy, and several CTL epitopes of human heparanase were identified in 2006 (12). Since then, several other epitopes have been reported for both murine and human heparanase and tested for their antitumor activity on various tumor cells in vitro and in vivo (13–15). Despite the fact that peptide vaccines are easy to produce, free from pathogen contamination, and have minimal oncogenic potential, peptide vaccines usually cannot elicit an ideal immune response in the body due to small molecular weight and single structure, which limit their clinical application. To circumvent this issue, the epitope peptide cross-linking carrier protein method

Figure 5. Heparanase-specific CTL killing of autologous lymphocytes and DCs in vitro and ex vivo. CTLs generated from the HLA-A2-restricted nonapeptide HIVpol(476–484, ILLEPVHGV) derived from HIV serves as an NP. Specific lysis of CTLs generated from heparanase MAP vaccines and their corresponding linear peptides against autologous lymphocytes and DCs in vitro (A and B) and ex vivo (C and D).
is traditionally used to improve immunogenicity (29). However, because the carrier proteins are foreign antigenic macromolecules, the induced antibodies are often against the carrier proteins instead of against the target polypeptide.

In the past several decades, much progress has been made toward the development and structural design of complex polypeptides to be used as constituents of a single immunogen. MAP conjugates provide a means to include different stage-specific peptides on one molecule, resulting in a multiepitope, multistage vaccine molecule that can potentially improve the immunogenicity of polypeptides. MAPs offer a very attractive alternative to the conventional linear peptide approach based on a small immunologically inert core molecule of radial branching lysine residues onto which a number of peptide antigens can be anchored (17). This results in a large macromolecule with a unique 3-dimensional configuration that has a high molar ratio of peptide antigen to core molecule and does not require a carrier protein for elicitation of the immune response. The MAP system can deliver not only multiple B-cell but also T-cell epitopes and has already been shown to be valuable in vaccine development for infectious diseases and tumors (18–20, 30).

Although MAP vaccines based on B-cell epitopes from human heparanase have been reported (20), there have not been studies on MAP vaccines based on T-cell epitopes of human heparanase. Thus, in the present study, we synthesized three 4-branched MAP vaccines on the basis of 3 HLA-A2–restricted CTL epitopes of human heparanase that we previously identified (13, 15). The CTL activity induced by these MAP vaccines against various tumor cells was studied in vitro and ex vivo. The results show that MAP vaccines can induce stronger specific lysis to target cells compared with their corresponding linear polypeptides in vitro and ex vivo, which suggests that these MAP vaccines have much more immunogenicity than their corresponding linear polypeptides. In addition, we also found that these MAP vaccines based on HLA-A2–restricted CTL epitopes from human heparanase were both heparanase specific and HLA-A2 restricted, as shown in a heparanase-negative MCF-7 breast cancer cell line and an HLA-A2–negative HepG2 liver cancer cell line.

Furthermore, in addition to heparanase-specific lysis induced by CD8+ T lymphocytes, these MAP vaccines may also promote the release of IFN-γ. Our study shows that after removing the CD4+ T lymphocytes from effectors, IFN-γ release is reduced. The above results indicate that MAP vaccines may not only activate the CD8+ T lymphocytes, which display a heparanase-specific killing effect for tumor cells but may also increase the releasing of IFN-γ, which display nonspecific killing of tumor cells. Recently, it was reported that CD4+ T lymphocytes in the context of CTL-based vaccination is important to amplify the CTL repertoire and to ensure the development of memory CD8+ T lymphocytes (31, 32), and the CD4+ T help was crucial at an early time point, during initial CD8+ T-cell priming, and that this could influence the later development of memory CD8+ T cells (33).

From a safety standpoint, the use of heparanase CTL epitopes as a TAA in vaccination poses the risk of autoimmune side effects because heparanase is expressed not only in malignant tumors but also in some immune cells.
such as T and B lymphocytes, DCs, macrophages, neutrophils, and mast cells (8). Although our previous study showed that CTLs induced by linear polypeptide vaccines of heparanase could not lyse autologous lymphocytes and DCs that expressed heparanase (13–15) due to their much greater immunogenicity over linear polypeptides, whether heparanase-specific CTLs induced by these MAP vaccines could lyse these cells remained unknown. To this end, MAP vaccine–induced CTLs were used to lyse autologous lymphocytes and DCs. Our results show that lysis of lymphocytes or DCs do not occur in vitro or ex vivo.

In summary, this study suggests that MAP vaccines based on HLA-A2–restricted CTL epitopes of human heparanase are capable of inducing HLA-A2–restricted and heparanase-specific CTL in vitro and ex vivo. Furthermore, compared with their corresponding linear peptides, heparanase MAP vaccines could elicit much stronger lysis of target cells by activating CD8+ T lymphocytes. However, these heparanase-specific CTLs do not lyse heparanase-expressing autologous lymphocytes and DCs, confirming the safety of these MAP vaccines. Therefore, this study provides theoretical evidence of heparanase MAP vaccines for clinical application.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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