Active Immunization Study of Colon Cancer Derived 1-8D Peptide in HHD Mice

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ABSTRACT

Background: 1-8D gene is a member of human 1-8 interferon inducible gene family and was shown to be overexpressed in fresh colon cancer tissues. Three peptides 1-6, 3-5 and 3-7 derived from human 1-8D gene were shown to have immunogenicity against colon cancer. **Methods:** To study tumor immunotherapy of three peptides we established an active immunization model using HHD mice. $D^{b-/-} \times \beta 2$ microglobulin ($\beta 2$ m) null mice transgenic for a chimeric HLA-A2.1/D^{b-} $\beta 2$ m single chain (HHD mice) were challenged with B16/HHD/1-8D tumor cells and were immunized with irradiated peptide-loaded RMA- S/HHD/B7.1 transfectants. In therapy model tumor growth was retarded in HHD mice that were injected with 3-5 peptide-loaded RMA-S/HHD/B7.1. In survival test vaccination with 1-8D-derived peptide protects HHD mice from tumor progression after tumor challenge. **Results:** These studies show that peptide 3-5 derived from 1-8D gene can be the most effective candidate for the vaccine of immunotherapy against colon cancer and highlight 1-8D gene as putative colon carcinoma associated antigens. **Conclusion:** We demonstrated that RMA-S/HHD/ B7.1 loaded with 1-8D peptides, especially 3-5, immunization generates potent antitumor immunity against tumor cells in HHD mice and designed active immunization as proper immunotherapeutic protocols. **(Immune Network 2005;5(3):157-162)**

Key Words: 1-8D gene, TAA (Tumor-associated antigen), Active immunization, HHD mice, Colon cancer, Peptide vaccine

Introduction

Colorectal cancer is the second most common type of malignancy in western nations. The incidence rate of colorectal cancer among Koreans is increasing rapidly. Given the relatively low success rate of current treatments, several approaches are being taken to define new therapeutic avenues for colorectal cancer, in common with many other tumor types, including signal-transduction inhibitor, vaccine, antivascular drug and gene therapy. Especially gene therapy deserves attention as it is the most logical extension of the information gained recently from the human genome project. It includes gene replacement, virus-directed enzyme-prodrug therapy, immune manipulation and virotherapy (1). Immunogenetic strategies for colorectal cancer gene therapy include the delivery of lymphoproliferative cytokines and tumor-associated antigens (TAAs), and attempt to enhance the immunogenicity of autologous tumors. Reasons proposed for why colorectal cancer cells are incapable of eliciting an immune response include an inability to process epitope, absent adhesion, or costimulatory molecules, the presence of inhibitory cytokines, or the fact that these tumors have low expression of MHC molecules (2).

Carcinoembryonic antigen (CEA) and Ep-CAM etc. are become known as the tumor-associated antigen of colorectal cancer until now. CEA and Ep-CAM are cell surface adhesion molecules expressed by more than 90% of colorectal cancers. Her-2/neu belongs to the family of epithelial growth factor receptors, is over-expressed in a subset of colorectal cancers and

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appears to be immunogenic in ovarian and breast cancers.

Utilizing the data of Zhang L. et al (3) comparing transcripts of colon tumor tissue samples and normal tissues excised from the same patients, a set of 26 overexpressed genes, expressed in tumors at least 5 folds higher than in normal tissue, was obtained. Using this data, 500 putative TAA peptides derived from overexpressed genes in colon carcinoma were synthesized recently (unpublished data).

Facing the problem of developing a method of discriminating between immunologically relevant and irrelevant MHC class I restricted peptides the $D^b \times \beta 2$ microglobulin ($\beta 2$ m) null mice transgenic for a recombinant HLA-A2.1/$D^b - \beta 2$ m single chain (HHD mice) were used. These mice combine classical HLA transgenesis with selective destruction of murine H-2. Therefore, unlike the classical HLA transgenics, these mice mount only HLA-A2.1-restricted Cytotoxicity T lymphocyte (CTL) responses and were demonstrated as a useful biological tool for identifying potential tumor associated antigen (TAA) HLA-A2.1 restricted epitopes and to establish hierarchy in their anti-tumor efficacy among these peptides (4,5).

Seven peptides from 500 putative TAA peptides were shown to be antigenic and immunogenic in HHD mice. Notably three (1-6, 3-5, 3-7) of the seven, derived from "Human 1-8D from interferon inducible gene (1-8D gene)", were found to react both *in vitro* and *in vivo* against a colon carcinoma cell line. Previously we showed anti-tumor CTL response against three peptides *in vitro* and *in vivo* using an adoptive transfer therapy (6). Peptide 3-5 was found to be highly effective in CTL activity. Adoptively transferred anti-peptide 3-5 CTL caused significant retardation in tumor growth.

The 1-8 gene family is inducible by both type I (α, β) and type II (γ) interferons. Three members of the family, 1-8D, 1-8U and 9-27 are linked on an 18 Kb fragment of chromosome 11 and are highly homologous (7). All three sequences are identical for 120 bp and show high similarity throughout much of the coding region. The single intron occurs at the same position in each gene. Also the sequence of amino acids and the structure of the peptides show high similarity each other, but 1-8 gene family presumably reflects differences in the interaction of the interferon-stimulable response elements (ISREs) with the various interferon-inducible and constitutive factors that govern the interferon response. The functional role of 1-8 genes has not become well known until now.

Active immunotherapy employs full-length or known epitopes of TAA administered systemically either alone or combined with other immunogens, with the aim of stimulating the patient's own CTL immune response. Active specific immunotherapy attempts to stimulate the immune system to target a particular antigen administered in various forms and combinations as a vaccine. The simplest strategy to stimulate the host's immune response against tumors is the subcutaneously injection of peptides derived from relevant TAA, often accompanied by an immunoadjuvant. Peptide immunization offers the possibility of specifically directing the immune response against tumor-expressed epitopes, avoiding the potential induction of autoreactivity towards sequences present in the remainder of the protein.

In this study, B16/HHD/1-8D that forms fast growing tumors in HHD mice was prepared by transfection of B16-F10.9 melanoma with the HHD and human 1-8D constructs. In the protection model, vaccination with 1-8D peptides prolonged survival in HHD mice and in the therapy model, tumor growth was retarded in HHD mice that were injected with peptide 3-5-loaded RMA-S/HHD/B7.1. These studies show that peptide 3-5 can be the most effective candidate vaccine against colon cancer.

Materials and Methods

Mice. The derivation of HLA-A2.1/$D^b - \beta 2$ monochain, transgenic, H-$2D^b \times \beta 2$m double-knockout mice (named HHD mice) has been described by Pascolo et al (1997). HHD mice were kindly provided by Dr. F.A. Lemonnier, Pasteur Institute, Paris. Animals were maintained and treated according to NIH guidelines.

Tumor cells. RMA-S is a TAP-2 deficient lymphoma clone of C57BL/6 origin. The RMA-S/HHD/B7.1 clone is a HHD transfectant expressing the murine B7.1 costimulatory molecule. Transfectants were grown in RPMI (Sigma, St Louis, USA) 1640 containing 10 % Fetal bovine serum, combined antibiotics, 500 μg/ml G418 (neomycin) and 0.5 μg/ml Puromycin.

F10.9 is highly metastatic subclone of the mouse B16 melanoma. The B16-F10.9/HHD/1-8D clone is a HHD and human 1-8D transfectant. Transfectants were grown in DMEM containing 10% Fetal bovine serum, combined antibiotics, 1,000 μg/ml G418 and 250 μg/ml Hygromycin B.

Peptide sequences. Peptide 1-6, 3-5 are derived from "Human 1-8D of interferon inducible gene (1-8D gene)". Peptide PAP is a prostate cancer associated antigen. These peptides have the following sequences: Peptide 1-6 EMLKEEQEV, Peptide 3-5 LILGIFMTI and Peptide PAP ILLWQPIPV. Peptides were purchased from Takara (Kyoto, Japan).

Stable Transfection of B16-F10.9 melanoma cells. For stable transfection B16-F10.9 melanoma cells were seeded in 100-mm-diameter plates at a density of 5×10^5

cells per plate. After 24 hours, using the Lipofectamine protocol the cells were transfected with HHD and 1-8D construct, which encoded the gene conferring resistance to G418 and Hygromycin B respectively. After 2 days, transfectants were selected in G418 (1,500 μg/ml; Calbiochem, La jolla, CA, USA) and Hygromycin B (600 μg/ml; Sigma, St Louis, USA). In 10 days cells were replated in the selective medium (200 cells/plate). After selection cells were grown in DMEM medium supplemented with 1,000 μg/ml G418 and 250 μg/ml Hygromycin B to allow colonies of resistant cells to grow.

The HHD construct encoding chimerical HLA-A2.1/$D^b - \beta 2$ microglobulin single chain molecule was inserted into pBR328 plasmid (4). 1-8D gene was inserted into pcDNA3.1 plasmid. These plasmids were kindly provided by Dr. Lea Eisenbach, Weizmann Institute, Israel.

FACS analysis. 5×10^6 B16/HHD/B7.1 were washed in FACS buffer (PBS with 0.5% BSA and 0.1% sodium azide), stained with fluorescent dye-conjugated monoclonal antibodies (mAb) and incubated with FACS buffer for 30 min at 4°C. Cells were washed with 3ml of FACS buffer. Then cells were added with 0.5 ml of PBS containing 0.1% sodium azide and analyzed by FACS. FITC (fluorescin isothiocyanate)-labeled anti-HLA Ab and FITC-labeled mouse IgG2b were used as monoclonal antibodies.

Real-time RT PCR analysis. 1-8D gene expression on B16/HHD/1-8D was quantified using Real-time RT-PCR analysis. Briefly, Total RNA from was extracted using standard Trizol RNA isolation protocol (Life Technologies, Grand island NY) and was reverse transcribed with Powerscript reverse transcriptase (Clontech, BD Biosciences, Oxford, UK) and oligo-dT primers.

PCR primers spanning exon-intron boundaries for analysis of 1-8D gene were designed using the web based Primer3 software (http://www-genome.wi. mit. edu/cgi-bin/primer/primer3_www.cgi) and were purchased from Bioneer (Daejeon, Korea).

The primers used for 1-8D were forward 5'-ATG TCG TCT GGT CCC TGT TC-3' and reverse 5'-TGA TGA CGA GCA GAA TGG TC-3'. The primer sequences of GAPDH were forward 5'-GAG TCA ACG GAT TTG GTC GT-3' and reverse 5'-TGA CAA GCT TCC CGT TCT CAG-3'. The combination of 1 μl of DNA sample with a reaction mixture containing 5 μl of SYBR Green PCR Master Mix (Bioneer, Daejeon, Korea) with optimized concentrations of specific primers, 1 μl forward primer, 1 μl reverse primer, 1 μl MgCl and 11 μl RNase-free water were used in a final volume of 20 μl. Real-time PCR amplification was carried out in Bioneer Exicycler (Bioneer, Daejeon, Korea). After a preincubation step at 94°C for 10 min in order to activate the HotStart Taq DNA polymerase, amplification was performed during 40 cycles including denaturation (94°C, 40 sec), annealing (52°C, 30 sec) and extension (72°C, 30 sec). After final extension at 72°C for 5 min, a melting curve analysis was performed. Relative expression quantification was performed using "2 *(-Delta Delta C(T)) Method*" (8).

Peptide loading of RMA-S/HHD/B7.1 cells. Peptide loading of RMA-S/HHD/B7.1 was performed as follows: the cells were washed three times in PBS, then cell surface expression of HHD monochain was stabilized by a 4-hour culture at 26°C. Synthetic peptides (1-6, 3-5, PAP 30 μg/ml) were added to 10×10^6 cells in 1ml of Opti-MEM medium (Gibco BRL, Gaithersburg, MD, USA). The cells were incubated overnight at 26°C and for an additional 3 hours at 37°C. RMA-S/HHD/B7.1 cells were loaded separately with each peptide and pooled before vaccination.

Active immunization in HHD mice. For the protection model, tumor challenge (2×10^6 cells/mouse) was performed 10 days after 3 times weekly intraperitoneally immunization with irradiated RMA-S/HHD/B7.1 loaded with different peptides (1×106 cells/mouse) in HHD mice (10~12 weeks female). Animal survival was monitored for 60 days.

For the therapy model, all HHD mice (10~12 weeks female) were injected subcutaneously with B16/HHD/1-8D (1×10^6 cells/mouse). After 10 days, HHD mice were injected intraperitoneally 3 times at 7-days intervals with irradiated RMA-S/HHD/B7.1 loaded with various peptides (1×10^6 cells/mouse).

Statistical Analysis. The results are expressed as means ±SE. The significance of differences between groups was determined by carrying out Student's t-test.

Results

B16-F10.9 melanoma cell growth in HHD mice. For active immunization study it is important to find aggressive human tumor lines that can grow in HHD mice. Because we couldn't find human colon carcinoma cell lines that grow fast in HHD mice, we have tested some murine tumor lines for tumorigenicity in HHD mice. HHD mice were injected subcutaneously with 1×10^6, 5×10^5 and 1×10^5 B16-F10.9 melanoma. After 10 days, we measured tumor size and found that B16-F10.9 melanoma form fast growing tumors in HHD mice (Fig. 1, solid line).

Stable transfection of B16-F10.9 melanoma cells with HHD and 1-8D. In order to design a model for active immunization against tumors in HHD mice B16-F10, 9 melanoma cells were used for stable transfection. The HHD and human 1-8D constructs were stably transfected into B16-F10.9 cell lines using Lipofectamine Reagents. After transfection, the cells were grown in

selection medium containing G418 and Hygromycin B. We investigated the expression of HHD by FACS analysis and 1-8D by Real-time RT-PCR on B16-F10.9 melanoma (Fig. 2). And then we injected 1×10^6 B16/HHD/1-8D transfectants subcutaneously in HHD mice. It also formed fast growing tumors in HHD mice (Fig. 1, dashed line).

Active immunization with 1-8D peptides in HHD mice. We tested whether irradiated RMA-S/HHD/B7.1 loaded with 1-8D peptides could induce protective antitumor immunity. All HHD mice were immunized intraperitoneally 3 times at 7-days intervals with irradiated RMA-S/HHD/B7.1 loaded with various peptides. Ten days after last immunization, the immunized HHD mice were challenged intraperitoneally with B16/HHD/1-8D and animal survival was monitored (Fig. 3).

Immunization with irradiated peptides 1-6 or 3-5-loaded RMA-S/HHD/B7.1 partially prolonged animal survival following B16/HHD/1-8D tumor challenge. Thus, this result indicated that vaccination with 1-8D derived peptides elicited potent protective immunity and prolonged animal survival after tumor challenge.

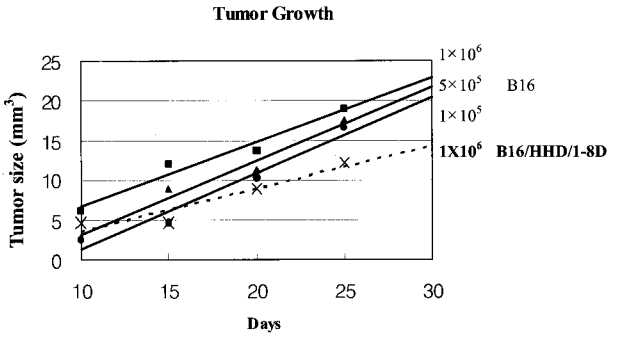

Figure 1. Tumor growth of B16-F10.9 and B16/HHD/1-8D in HHD mice. B16-F10.9 (solid line) and B16/HHD/1-8D transfectants (dashed line) were injected in HHD mice with different cell numbers (n=5). After 10 days, the growth of the tumor was monitored.

We next tested whether immunization with RMA-S/HHD/B7.1 loaded with 1-8D peptides could generate immune responses strong enough to inhibit the pre-existing B16/HHD/1-8D tumor. All HHD mice were injected subcutaneously with B16/HHD/1-8D. Ten days later, HHD mice were immunized intraperitoneally 3 times at 7-days intervals with irradiated RMA-S/HHD/B7.1 loaded with various peptides. We monitored rejection of pre- established tumors (Fig. 4). In contrast to the lack of any therapeutic effect in HHD mice immunized with irradiated peptide 1-6 or PAP-loaded RMA-S/HHD/B7.1 or with PBS, tumor growth was notably retarded in HHD mice that were immunized with irradiated peptide 3-5-loaded RMA-S/HHD/B7.1.

Discussion

We have examined the potential anti-tumoral use of novel 1-8D derived peptides as colon cancer-asso-

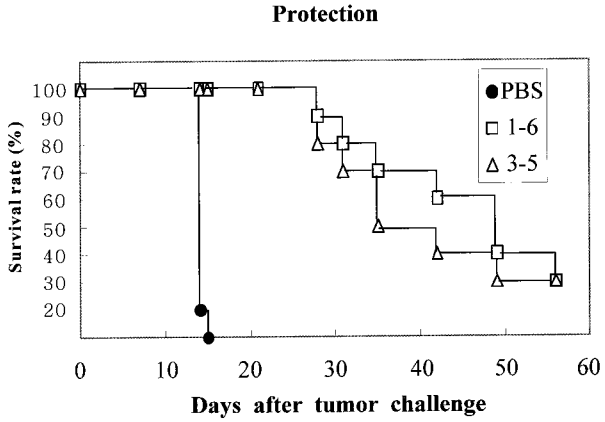

Figure 3. Survival rate of HHD mice after immunization with 1-8D peptides. HHD mice were vaccinated intraperitoneally 3 times, weekly, with RMA-S/HHD/B7.1 loaded with peptide 1-6 and 3-5, or injected with PBS (n=9). Ten days after the last immunization, the mice were challenged intraperitoneally with B16/HHD/B7.1 tumor cells.

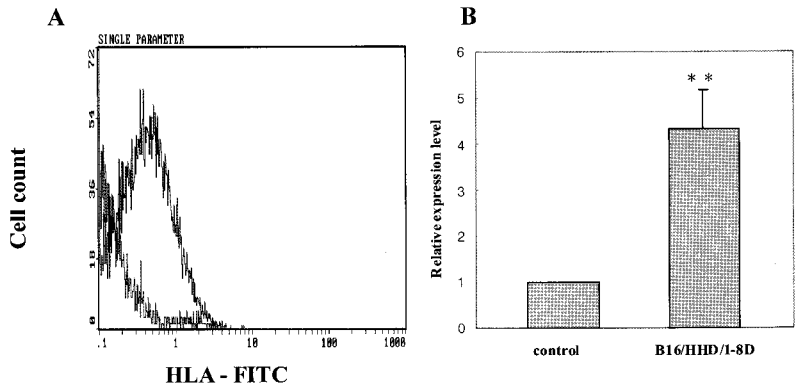

Figure 2. B16/HHD/1-8D cells highly express HHD and 1-8D. (A) Expression of HHD on B16/HHD/1-8D cells. Cells were stained with FITC conjugated anti-HLA Ab and analyzed by flow cytometry. Red histograms, control; Blue histograms, B16/HHD/1-8D cells. (B) Analysis of relative 1-8D gene expression on B16/HHD/1-8D cells using real-time quantitative PCR.

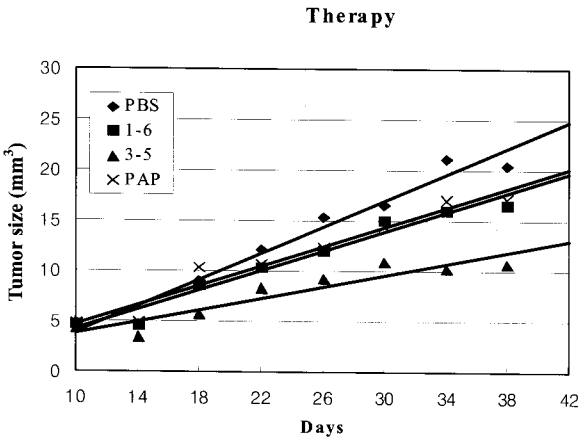

Figure 4. Immunization with 1-8D peptides induces therapeutic antitumor immunity in HHD mice. All HHD mice were challenged subcutaneously with B16/HHD/1-8D. After 10 days, HHD mice were immunized intraperitoneally 3 times at 7-days intervals with irradiated peptide-loaded RMA-S/HHD/B7.1 or injected with PBS (n=10).

ciated antigen. In our previous adoptive transfer therapy study, peptide 3-5 was found to be the most effective vaccine in HHD mice. Peptide 3-7 was nonimmunogenic *in vitro* and *in vivo* (6), so peptide 3-7 was not used in the current study. For the active immunization study it is important to find human colon cancer cell lines or other murine cell lines that grow fast in HHD mice. It was found that B16-F10.9 melanoma form fast growing tumor in HHD mice. B16/HHD/1-8D transfectants were prepared by stable transfection of B16-F10.9 with the HHD and human 1-8D constructs (Fig. 2). It formed fast growing tumors in HHD mice. Unexpectedly the growth of B16-F10.9 melanoma (Fig. 1, solid line) was faster than B16/HHD/1-8D transfectants (Fig. 1, dashed line) in HHD mice. But to design more effective and sophisticated experiment it is necessary to find human colon cancer cell line or more aggressive cell line. Then we monitored protection against a subsequent tumor challenge and rejection of pre-established tumors. Immunization with 1-8D derived peptides protects HHD mice from tumor progression (Fig. 3) and induces therapeutic antitumor immunity in HHD mice (Fig. 4). These results show that vaccination with peptide 3-5 can delay tumor growth and prolong survival in HHD mice. It is consistent with adoptive transfer therapy showing that peptide 3-5 was the most effective vaccine in HHD mice. However, in the protection study peptide 3-5 was not the most effective candidate for the vaccine even if 1-8D derived peptides could be effective vaccine. Vaccination with peptide 1-6 and 3-5 elicited similar protective effects, thus it seemed that our protection model was designed incompletely. Therefore if we find human colon cancer cell lines or set the each control group and cell number etc. peptide 3-5 will elicit stronger and more effective protective immunity against tumor challenge.

To this end, we used HHD mice, which can be used as a notable tool for identifying potential CTL epitopes. HHD mice have recently proven to be effective for identifying CTL epitopes from viral and tumor antigen (9-15).

And TAP-2 deficient RMA-S/HHD/B7.1 was used as an antigen delivery system. But over the past decade, Increasing attention has been focused on dendritic cells (DCs) as vehicle for antigen delivery in active immunotherapy trials. Mature DCs pulsed with peptides have proven effective in enhancing antitumor immunity, although most studies used foreign antigens or peptides as immune targets (16-18). Recently, both human and animal studies using mature DCs pulsed with tumor antigen-derived peptides showed some effect on increased T cell response and inhibited tumor growth (19-22). So we expect immune response against 1-8D derived peptides will be stronger and more effective against tumor growth by using autologous DCs of HHD mice and the statistical significance will be increased.

In conclusion, we demonstrated that RMA-S/HHD/B7.1 loaded with 1-8D peptides, especially 3-5, immunization generates potent antitumor immunity against tumor cells in HHD mice and designed active immunization as proper immunotherapeutic protocols. Our results suggest a potential role for peptide 3-5 as tumor-associated antigen peptides in colon cancer.

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