Determination of Cellularly Processed HLA-A2402-Restricted Novel CTL Epitopes Derived from Two Cancer Germ Line Genes, MAGE-A4 and SAGE

Yoshihiro Miyahara,1 Hiroaki Naota,1 Lijie Wang,1 Atsunori Hiasa,1 Megumi Goto,1 Masato Watanabe,3 Shigehisa Kitano,1 Satoshi Okumura,1 Tetsushi Takemitsu,1 Atsushi Yuta,2 Yuichi Majima,2 François A. Lemonnier,4 Thierry Boon,5 and Hiroshi Shiku1

Abstract Purpose: For identification of CTL epitopes useful for cancer vaccines, it is crucial to determine whether cognate epitopes are presented on the cell surface of target cancer cells through natural processing of endogenous proteins. For this purpose, we tried to use the cellular machinery of both mice and humans to define naturally processed CTL epitopes derived from two “cancer germ line” genes, MAGE-A4 and SAGE.

Experimental Design: We vaccinated newly produced HLA-A2402 transgenic mice with DNA plasmids encoding target antigens. Following screening of synthesized peptides by splenic CD8+ T cells of vaccinated mice, we selected candidate epitopes bound to HLA-A2402. We then examined whether human CD8+ T cells sensitized with autologous CD4+ PHA blasts transduced by mRNA for the cognate antigens could react with these selected peptides in an HLA-A2402-restricted manner.

Results: After DNA vaccination, murine CD8+ T cells recognizing MAGE-A4143-151 or SAGE715-723 in an HLA-A2402-restricted manner became detectable. Human CTLs specific for these two peptides were generated after sensitization of HLA-A2402-positive CD8+ T cells with autologous CD4+ PHA blasts transduced with respective mRNA. CTL clones were cytotoxic toward tumor cell lines expressing HLA-A2402 and cognate genes. Taken together, these CTL epitopes defined in HLA-A24 transgenic mice are also processed and expressed with HLA-A2402 in human cells. The presence of SAGE715-723-specific precursors was observed in HLA-A2402-positive healthy individuals.

Conclusions: Two novel HLA-A2402-restricted CTL epitopes, MAGE-A4143-151 and SAGE715-723, were identified. Our approach assisted by cellular machinery of both mice and human could be widely applicable to identify naturally processed CTL epitopes.

A number of cancer genes appropriate for immunotherapy have been identified by a wide variety of genomic approaches and immunologic analyses (1). For designing cancer vaccines that target these genes, it is important to determine that peptides encoded by these antigens actually elicit CTL response. In the strategy known as “reverse immunology,” multiple synthesized peptides expected to bind to particular HLA type are screened for their capacity to prime CD8+ T cells possessing the corresponding HLA type. This strategy has been extended to HLA transgenic mice, mostly HLA-A0201 (2, 3). However, whereas many CTL epitopes that could elicit CTL responses have been determined, it sometimes remains unclear whether the identified epitopes are presented on the cell surface of target cancer cells through natural processing of endogenously produced proteins.

To overcome this problem, we developed in the present study a novel approach with two steps: (a) selection of candidate CTL epitopes by the use of DNA vaccinated (4) HLA-A2402 transgenic mice and (b) confirmation of these epitopes being immunogenic and cellularly processed in human by the use of a newly developed system where CD4+ PHA blasts (5) transduced with the mRNA of cognate antigens are used as antigen presenting cells. We focused on two clinically attractive cancer germ line genes, MAGE-A4 (6) and SAGE (7), and identified HLA-A2402-restricted CTL epitopes encoded by both antigens.
Materials and Methods

Mice. β2-Microglobulin knockout mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were purchased from Shizuka Animal Laboratory Center (Shizuoka, Japan). All mice were maintained at the Animal Center of Mie University School of Medicine (Tsukuba, Japan) under conditions fulfilling the institutional regulations governing the treatment of laboratory animals.

HHDA2402 construct. The chimeric HHDA2021 construct cloned into pBluescript KS(−) vector (Stratagene, La Jolla, CA) was used as template DNA (3). At first, we site mutagenized to introduce BamH I sites in the fragment encompassing α1 and α2 domains of the HHDA2021 gene by using the QuikChange site-directed mutagenesis kit (Stratagene). BamH I site in a (Gly4Ser)3 linker region was also site mutagenized without amino acid change. The fragment encompassing α1 and α2 domains of HLA-A2402 genomic DNA (a kind gift from Dr. M. Takiguchi, Kumamoto University, Japan) was amplified by PCR with 5′ BamH I site and 3′ Bgl I site. After restriction enzyme digestion, we finally substituted the fragment encompassing α1 and α2 domains of HHDA2021 for α1 and α2 domains of HLA-A2402. The final HHDA2402 construct was verified by DNA sequencing (ABI PRISM 310 capillary DNA sequencer).

Plasmids. Full-length MAGE-A4 and SAGE cDNAs were cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA), and full-length EBNA3A cDNA cloned into pcDNA3.1 was a kind gift from Dr. Kuzushima (Aichi Cancer Center, Japan). These plasmids were purified by using Qiagen EndoFree Plasmid Mega kit (Qiagen, Hilden, Germany). The truncated SAGE cDNA, encoding COOH-terminal 289 amino acids, was reconstructed and cloned into pcDNA3.1 for the purpose of more convenient preparation of mRNA synthesis.

Cells lines. P1.HTR is a subline of P815 mastocytoma cell line of DBA/2 origin. T2A24 cell lines were derived from the T2 cell line, which is deficient in TAP transporter proteins, upon transfection with HLA-A2402 cDNA. The breast carcinoma cell line R27 (A2402 negative) and lung carcinoma cell line 11-18 (A2402) were provided by Dr. Y. Ichinose (Osaka, Japan). No expression of MAGE-A4 or SAGE was detected in the esophageal carcinoma cell lines KE-4 (A2402), T2A24 (A2402 negative) and the truncated SAGE plasmids

Preparation of mRNA. Before in vitro mRNA synthesis, the MAGE-A4 plasmid was linearized with XhoI and the truncated SAGE plasmids were linearized with HinIII. The in vitro transcription was done with T7 polymerase according to the instructions provided by the manufacturer (mMESSAGE mMACHINE T7 Kit; Ambion, Austin, TX). Then, in vitro – transcribed RNA was polyadenylated using poly[A] polymerase [Poly(A) Tailing Kit, Ambion] according to the instructions supplied by the manufacturer. The resulting capped and tailed RNA was resuspended in water and stored at –80°C before use in transfection to LCLs or CD4+ PHA blasts.

Electroporation. CD4+ PHA blasts or LCLs were harvested and washed twice in PBS and resuspended in the specified electroporation buffer. Next, 10 μg of mRNA was mixed with 100 μL of the cell suspension, containing up to 1 × 106 cells, transferred to an electroporation cuvette, and nucleofected with an Amaxa Nucleofector apparatus (Amaxa, Cologne, Germany). Protocol T23 and human T-cell solution were used for CD4+ PHA blasts and protocol U-15 and human B-cell solution were used for LCLs. After electroporation, the cells were immediately transferred to 2.0 mL of complete medium and cultured overnight in 6-well plates at 37°C in a CO2 incubator until use.

Immunization. Six- to 8-week-old female HHDA2402 mice received abdominal delivery of plasmid DNA-coated gold particles by using a Helios Gene Gun System (Bio-Rad, Hercules, CA) at a helium discharge pressure of 350 to 400 p.s.i.. The gold particles were prepared according to the instructions provided by the manufacturer. Each mouse received 2 to 2 × 106 cells per well in 1 mL RPMI 1640 supplemented with 25 mmol/L HEPES, 10% heat-inactivated human AB serum from healthy donors, 2 mmol/L i-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. On day 0, 10 μg/mL phytohemagglutinin (PHA-P, Sigma-Aldrich, St. Louis, MO) were added to the culture. On day 3, half of the medium was replaced with complete medium containing interleukin-2 (IL-2; 20 units/mL) and IL-7 (40 ng/mL), and this was repeated every 3 days. The activated CD4+ T cells were electrophorated and used as antigen presenting cells around days 14 to 28 of the culture. Purified recombinant human IL-2 was provided by Takeda Pharmaceutical (Osaka, Japan).

Table 1. Candidate HLA-A24-restricted peptides (9 mer) derived from SAGE and MAGE-A4

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<th>Protein</th>
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<tr>
<td>SAGE</td>
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<td></td>
<td>143-151</td>
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Preparation of murine CD8+ T cells. CD8+ splenic T cells were obtained by positive enrichment using MACS system (Miltenyi Biotec). In brief, splenocytes were labeled with CD8 α (Ly2) microbeads in PBS containing 0.5% bovine serum albumin. After washing, the cells were prepared as described previously (7). In brief, CD4+ T cells, freshly isolated by positive enrichment using MACS CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), were seeded into 24-well plates (Nunc, Roskilde, Denmark) at a density of 1 to 2 × 106 cells per well in 1 mL RPMI 1640 supplemented with 25 mmol/L HEPES, 10% heat-inactivated human AB serum from healthy donors, 2 mmol/L
applied on MS columns equipped with a MiniMACS magnet as specified by the supplier (Miltenyi Biotec). T-cell fractions were confirmed to contain more than 95% CD8+ T cells by flow cytometric analysis.

Enzyme-linked immunospot assay. Murine IFN-γ enzyme-linked immunospot (ELISPOT) assays were done as described with minor modifications (11). Briefly, 96-well nitrocellulose ELISPOT plates (MAHA S4510; Millipore, Bedford, MA) were coated overnight at 4 °C with 2 μg/mL anti-mouse IFN-γ monoclonal antibody (mAb, clone R4-6A2; PharMingen, San Diego, CA). The wells were washed with 0.05% Tween 20 (PBS/Tween) and blocked with FCS-containing culture medium for 2 hours at 37 °C. Freshly isolated CD8+ T cells (1 × 10^5 per well) from immunized mice and peptide-pulsed CD8+ cells (1 × 10^5 per well) were plated into each well at a final volume of 200 μL. After incubation for 22 hours at 37 °C in a CO2 incubator, the plate was then washed thoroughly with PBS/Tween, supplemented with 1.25 μg/mL biotinylating enzyme BirA. Monomeric HLA/2-microglobulin/peptide complexes were folded in vitro using a ... biotinylating enzyme BirA. Monomeric HLA/2-microglobulin/peptide complexes were folded in vitro using a...
Ethical considerations. The experimental protocols described in the present study were approved by the Ethics Review Committee for Animal Experimentation of Mie University School of Medicine. CD4+ PHA blasts, LCLs, and CD8+ T cells were obtained from healthy volunteers with written informed consent. The experimental protocol was approved by Institutional Review Board in the Mie University School of Medicine.

Results

Production of HHDA2402 transgenic mice. The starting point of our construct was the HHDA0201 gene (3), coding for a chimeric construct containing the leader sequence of HLA-A0201, the human β2-microglobulin, peptide linker, and the HLA-A0201 and H-2Dβα3, transmembrane, cytoplasmic domains. Briefly, the HLA-A0201 α1 and α2 domains were substituted by HLA-A2402 α1 and α2 domains (Fig. 1A).

To verify whether this chimeric gene could be correctly expressed on the cell surface, the mammalian expression vector pcDNA3.1 encoding this gene was constructed and transfected to P1.HTR. The transfected cells were positively stained by anti-HLA-A24 antibody (data not shown). The HHDA2402 construct was injected into C57BL/6 mouse embryos to obtain transgenic mice. Mice with high expression of HLA-A2402 in PBMN were selected and crossed with murine h2-microglobulin C57BL/6 knockout mice (h2m-/-/C0/C0). Figure 1B shows phenotypic characterization of wild-type, murine h2m+/C0, HHDA2402+/C0 murine h2m+/C0, and HHDA2402+/C0 murine h2m+/C0 transgenic mice. HHDA2402+/C0 murine h2m+/C0 transgenic mice expressed low levels of H-2.

Determination of HLA-A2402-restricted CTL epitopes of cancer germ line genes. We first examined the utility of the transgenic

![Graph A: EBNA3A](image)

![Graph B: MAGE-A4](image)

![Graph C: SAGE](image)

Fig. 3. Immunogenicity of prepared MAGE-A4-derived peptides in human host. A, IFN-γ ELISPOT assays of human bulk CTL lines sensitized twice by MAGE-A4 mRNA transduced autologous CD4+ PHA blasts. As antigen-presenting cells, we used mRNA of MAGE-A4 or truncated SAGE-transduced autologous LCLs. B, bulk CTLs from No. 2 well (A) were expanded with mRNA-transduced autologous LCLs and autologous PBMCs in IL-2 (20 IU/mL). As antigen-presenting cells, we used a series of T2:A24 cells pulsed with each peptide. Thus, the expanded cell line predominantly released IFN-γ when MAGE-A4 peptide-pulsed T2A24 were used as antigen-presenting cells.
mice to present an antigen peptide known to bind to HLA-A2402. The expression plasmid encoding the EBV-derived EBN3A gene was used. HHDA2402 mice were immunized with EBNA3A expression plasmid DNA. After two immunizations, splenic CD8+ T cells were prepared and examined by ELISPOT assay with splenic CD8+ cells pulsed with the respective prepared peptides as targets. CD8+ T cells specific for the predicted EBN3A 246-254 peptide were obtained (Fig. 2A; refs. 8, 9).

The HLA-A2402 transgenic mice were vaccinated with the plasmids coding for the MAGE-A4 or the SAGE genes. The CD8+ T cells were then examined with the MAGE-A4 or SAGE peptides selected for predicted bindings to HLA-A2402 based on BioInformatics and Molecular Analysis Section HLA Peptide Binding Prediction. For MAGE-A4, two 9-mer peptides (MAGE-A4 239-247 and MAGE-A4 143-151) were positive in ELISPOT assay (Fig. 2B). Likewise, the SAGE 776-784 and SAGE 715-723 peptides were detected (Fig. 2C). As shown in Fig. 1B, H-2 was also weakly expressed in HHDA2402 mice so that it remained unclear whether these four peptides were present on the cell surface with H-2 or with HLA-A2402. We therefore conducted similar experiments in wild-type C57BL/6 mice. Approximately equally positive spots were visible in MAGE-A4 239-247 and SAGE 776-784, whereas MAGE-A4 143-151 and SAGE 715-723 were negative in wild-type mice. These results indicate that MAGE-A4 143-151 and SAGE 715-723 are presented in the context of HLA-A2402.

Generation of HLA-A2402-restricted peptide-specific human CTLs by the use of CD4+ PHA blasts transduced with mRNA. To determine whether MAGE-A4 143-151 and SAGE 715-723 are immunogenic and present on the cell surface through the protein processing machinery of human host, we induced two peptide-specific CTLs in HLA-A2402-positive individuals. CD8-positive cells obtained from healthy volunteers were sensitized in vitro with autologous CD4+ PHA blasts transduced by mRNA for the cognate molecules. After double stimulation with CD4+ PHA blasts transduced by whole mRNA of MAGE-A4, MAGE-A4-specific bulk CTL lines were induced as shown Fig. 3A. After expansion of these bulk lines through sensitization with mRNA-transduced autologous LCLs, autologous PBMC, and IL-2, they showed specific reactivities to MAGE-A4 143-151 peptide-pulsed T2A24 cells (Fig. 3B). MAGE-A4 143-151-specific clone, clone 2-28, was established by limiting dilution. Clone 2-28 was positively stained by MAGE-A4 143-151 A24 tetramer but not by control tetramer and was cytotoxic to MAGE-A4 143-151 peptide pulsed target cells with HLA-A2402-restricted fashion (Fig. 4A and B, left). Furthermore, this clone was cytotoxic to tumor cell lines that expressed both MAGE-A4 and HLA-A2402 (Fig. 4B, right). These results indicate that MAGE-A4 143-151 peptide is cellularly processed and present on mRNA transduced CD4+ PHA blasts as well as on tumor cells expressing HLA-A2402 and MAGE-A4 molecule.

SAGE 715-723-specific bulk CTLs were also induced after double stimulation with CD4+ PHA blasts transduced by truncated mRNA of SAGE (Fig. 5A). Fluorescence-activated cell sorting analysis of this bulk line revealed it contained SAGE 715-723-positive CD8+ T cells (Fig. 5B). We also established SAGE 715-723-specific HLA-A2402-restricted clone, named clone 22. The clone was positively stained by SAGE 715-723 HLA-A2402 tetramer (Fig. 6A) and secreted IFN-γ when cultured with 293-A2402 transfectants.
cells transfected by plasmid DNA encoding whole SAGE gene (Fig. 6B). Clone 22 was cytotoxic to both K562A24 and R27A24, both of which express HLA-A2402 and SAGE. In addition, this clone was cytotoxic to SAGE positive esophageal cancer cell line TE-10 but not to SAGE-negative esophageal cancer cell line TE-8, both of which express HLA-A2402. This clone also showed specific killing activity against A2402-positive lymphoblastoid cell line transduced with mRNA of truncated SAGE gene (Fig. 6C).

Recent induction of SAGE715-723 A24 tetramer-positive T cells after a single stimulation of CD8+ T cells with SAGE715-723-pulsed CD8+ peripheral blood mononuclear cells in A2402-positive healthy individuals. HLA-A2402-restricted SAGE715,723-specific CTLs were frequently generated after a single in vitro stimulation of CD8+ T cells with SAGE715,723-pulsed CD8+ PBMNs in A2402-positive healthy volunteers. In three of six HLA-A2402-positive healthy volunteers, SAGE715,723 A24 tetramer-positive T cells were detected after 10 days in vitro mixed lymphocyte culture. Representative outcomes of the flow cytometric analysis of individual culture wells from one healthy volunteer are shown in Fig. 7.

Discussion

For worldwide application of peptide-based cancer vaccines, determination of many CTL epitopes restricted to various HLA alleles is indispensable. Ideally, these epitopes are tumor restricted, immunogenic, and frequently and stably expressed in many types of cancers. "Cancer germ line" genes such as MAGE and NY-ESO-1 (15) code for such epitopes. In the present study, we have focused on CTL epitopes restricted to HLA-A2402, because HLA-A2402 is frequently observed in Asians including 60% of Japanese and in 17% of Caucasians (16, 17). To our knowledge, only a few cancer germ line gene encoded CTL epitopes restricted to HLA-A2402 have been determined. These include MAGE-A1 (18), MAGE-A2 (19), MAGE-A3 (20), and NY-ESO-1 (21). The present study was designed to identify CTL epitopes encoded by two cancer germ line antigens, MAGE-A4 (6) and SAGE (7), which would be potentially useful for cancer vaccine trials. MAGE-A4 belongs to MAGE family and seems an extremely attractive antigen because of its expression in a wide variety of tumors and also its immunogenicity (ref. 22; e.g., esophageal carcinoma [60%], head and neck carcinoma [50%], non—small cell lung carcinoma [24%], gastric tumor [33%], and Hodgkin's disease [21%]; refs. 23–26). In addition, immunohistochemical analysis revealed that MAGE-A4 expression inversely correlated with patient survival in serous ovarian cancer and in transitional cell carcinoma of urinary bladder (21, 27). Importantly, CTL epitopes restricted to HLA-A2 (24), HLA-A1 (28), and HLA-B37 (29) have been reported in addition to the HLA-A2402-restricted CTL epitope defined in the present study, various peptides seem immunologically recognizable in the context of various HLA-types. In our preliminary analyses of sera from patients with malignancies, we also observed the presence of antibodies for MAGE-A4 protein in the sera of some patients with MAGE-A4-expressing tumors. Considered together, these evidences indicate that MAGE-A4 is a particularly
attractive antigen with immunogenic properties in a proportion of patients.

SAGE, initially identified by representational difference analysis, is also expressed in a variety of tumors, such as bladder (24%), squamous cell lung cancers (32%), and head and neck carcinoma (20%) at mRNA level (7). The present findings that HLA-A2402-restricted SAGE715-723-specific precursors are frequently found in the circulating peripheral blood of HLA-A2402-positive individuals suggests that the cognate CTL epitope, SAGE 715-723, being highly immunogenic in many hosts. The surprising phenomenon of the appearance of tetramer-positive CTL specific to cancer germ line gene products after a single stimulation of CD8+ T cells with peptide-pulsed CD8+ PBMC has been rarely reported previously apart from Melan-A/MART-1-specific CD8+ T cells (30), a melanocyte differentiation antigen. The origin and nature of these SAGE-specific CD8+ T cells and their relevance to escape mechanism from deletion and anergy in T-cell development are currently under investigation in our laboratories.

It is critical to know that identified immunogenic peptides are actually presented on the cell surface of target cancer cells through cellular processing. Ayyoub et al. (31) reported the successful identification of naturally processed HLA-A2-restricted CTL epitope derived from SSX-2 antigen using the in vitro proteasomal digestion method. This method actually served as a tool to predict candidate epitopes passed through endogenous processing machinery. In the present study, we applied a novel approach using HLA-transgenic mice combined with DNA vaccination. After vaccinations, we successfully identified two HLA-A2402-restricted CTL epitopes encoded by cancer germ line genes, MAGE-A4 and SAGE. This approach depends on natural processing of cognate antigen molecules in murine antigen-presenting cells in response to DNA vaccination. Murine CTL are naturally sensitized by HLA-A2402-bound peptides presented by these antigen-presenting cells of HLA-A2402 transgenic mice. Street et al. (32) previously reported some limitation of use of HLA-A2.1 transgenic mice exemplified by the different processing of human papillomavirus type
in vitro.6 These mRNA-transduced cells can be used as antigen-presenting cells to sensitize CTL specific for mRNA derived peptides and also as target cells in ELISPOT assays as well as CTL assays. Preparation of mRNA transduced CD4+ PHA blasts is relatively simple and stable. We were also successful in inducing MAGE-A4-specific CTL restricted to HLA-B allele and also CD4+ T cells from PBMC of MAGE-A4-positive patient.7 The use of HLA transgenic mice with mRNA-transduced CD4+ PHA human blasts may allow the screening and determination of a variety of cancer antigens suitable for use in cancer vaccines.

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References


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