HER-2, gp100, and MAGE-1 Are Expressed in Human Glioblastoma and Recognized by Cytotoxic T Cells

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ABSTRACT

It has recently been demonstrated that malignant gliomas express certain known tumor-associated antigens, such as HER-2, gp100, and MAGE-1. To further determine the possible utilization of these antigens for glioma immunotherapy and as surrogate markers for specific tumor antigen cytotoxicity, we characterized the presence of mRNA and protein expression in 43 primary glioblastoma multiforme (GBM) cell lines and 7 established human GBM cell lines. HER-2, gp100, and MAGE-1 mRNA expression was detected in 81.4%, 46.5%, and 39.5% of the GBM primary cell lines, respectively. Using immunoreactive staining analysis by flow cytometry, HER-2, gp100, and MAGE-1 protein expression was detected in 76%, 45%, and 38% of the GBM primary cell lines, respectively. HLA-A1-restricted epitope specific for MAGE-1 peptide (EADPTGHSY) CTL clone B07 and HLA-A2-restricted epitope specific for HER-2 peptide (KIFGSLAFL) CTL clone A05 and gp100 peptide (ITDQVPFSV) CTL clone CK3H6 were used in this study. The specificity of CTL clone was verified by HLA/peptide tetramer staining. Three CTL clones could efficiently recognize GBM tumor cells in an antigen-specific and MHC class I-restricted manner. IFN-γ treatment can dramatically increase MHC class I expression of GBM tumor cells and significantly increase CTL recognition of tumor cells. Treatment with the DNA hypomethylating agent 5-aza-2′-deoxycytidine induced and up-regulated the mRNA expression of MAGE-1 and epitope presentation by autologous MHC. These data indicate that HER-2, gp100, and MAGE-1 could be used as tumor antigen targets for surrogate assays for antigen-specific CTLs or to develop antigen-specific active immunotherapy strategies for glioma patients.

INTRODUCTION

The incidence of primary brain tumors in the United States is approximately 17,400 per year. The most common and deadly brain tumor type is glioblastoma multiforme (GBM). The survival rate for this disease per year has not changed substantially over the past 50 years, despite advances in surgery, radiotherapy, and chemotherapy (1, 2). Clearly, novel strategies are needed to significantly affect the outcome for these patients. In melanoma patients, adoptive transfer of autologous tumor-infiltrating lymphocytes with interleukin (IL)-2 resulted in tumor regression, suggesting that the immune system plays a critical role in fighting tumors (3). To further understand the molecular nature of tumor antigens recognized by T cells in vivo, much effort has been devoted to the identification of tumor antigens that may serve as important immune targets.

MAGE-1 was initially analyzed from melanomas and became the first identified tumor antigen recognized by T cells (4). The dominant epitope, EADPTGHSY, was subsequently identified and recognized by CTLs in the context of HLA-A1 (5). MAGE-1 is expressed in 48% of metastatic melanomas and in many different histological tumor types (6–9). MAGE-1, however, is silent in normal cells except for testis and placenta (4). Thus far, at least 11 MHC class I epitopes and two MHC class II epitopes have been identified (10). The vaccination of MAGE-1 peptide-pulsed dendritic cells (DCs) is capable of inducing clinical and systemic tumor-specific immune responses without provoking major side effects in melanoma patients (11). Melanoma patients immunized with melanoma cell vaccine induce antibody responses to recombinant MAGE-1 antigen (12).

Human melanoma-associated antigen, gp100, is a melanocyte differentiation antigen recognized in patients with melanoma by HLA-restricted CTLs and antibody (13, 14). It appears to be a promising target antigen. Several clinical trials indicated that gp100 was a highly immunogenic antigen in melanoma patients and also found a strong correlation between T-cell recognition of the gp100 antigen and clinical responses (15, 16). Thus far, at least 16 MHC class I-restricted gp100 epitopes and several MHC class II epitopes have been identified by CTLs derived from different patients (10). gp100 (9209–217) is the most well characterized and most commonly used epitope in preclinical and clinical melanoma studies.

HER-2, also called HER-2/neu or c-erbB2, encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity and extended homology in structure and sequence to epidermal growth factor receptor (17). The HER-2 oncoprotein is a well-defined tumor antigen. Gene amplification and overexpression of HER-2 have been demonstrated with high frequency in a number of human malignancies including breast and ovarian tumors (18), as well as in colon carcinoma (19) and renal cell carcinoma (20). Patients with HER-2 protein-overexpressing breast, ovarian, non-small cell lung, colon, and prostate cancers have been shown to have a pre-existent immune response to HER-2. HER-2-specific vaccine has been tested in human clinical trials. Early results demonstrate that the immunity elicited is durable even after vaccinations have ended (21). A number of HER-2-specific CTLs have been isolated that could recognize not only HER-2-overexpressing ovarian and breast carcinoma but also other epithelial tumors. Due to the overexpression of HER-2 in tumor cells and the immunogenicity of HER-2, it represents an excellent target for T-cell-mediated immunotherapy (22). Thus far, at least 13 HLA class I epitopes and several HLA class II epitopes have been identified (10). The dominant HLA-A2-restricted epitope derived from HER-2 extracellular domain (9360–377) KIFGSLAFL (23) will be investigated in this study.

Although there were reports of HER-2 protein expression (24–26) and gp100 and MAGE-1 mRNA expression (27–30) in brain tumor, the immunogenicity of HER-2, gp100, and MAGE-1 and regulation in GBM are still unknown. In this study, we analyzed HER-2, gp100, and MAGE-1 mRNA expression by reverse transcription-PCR (RT-PCR) and protein level by flow cytometry analysis. We also investigated whether HER-2, gp100, and MAGE-1 could be naturally processed and could then present their dominant epitopes to CTLs in vitro. We demonstrate that the majority of GBMs express these antigens and process the dominant epitope, thus allowing CTL recognition of these peptides. Finally, we investigated whether IFN-γ or DNA hypomethylating agent treatment could affect CTL recognition of tumor cells. These findings may allow surrogate assay determination of specific tumor antigen cytotoxicity for glioma immunotherapy. The characterization of these tumor-associated antigens will also...
allow development of antigen-specific active and adoptive immunotherapy strategies.

MATERIALS AND METHODS

Tumor Specimens and Primary Cell Culture. Brain tumor specimens from patients were obtained from the Cedars-Sinai Institutional Review Board-approved Brain Tumor Registry after being reviewed and released by a pathologist in the operating room. Classification of tumor type and grade was made by independent pathologists in accordance with the WHO histological typing of central nervous system tumors. All tumor specimens were obtained from patients who had signed institutional review board-approved informed consent forms. The glioma tissues were processed under sterile conditions in a laminar flow hood by certified technicians. Tumor cells were recovered from Ficoll-Hypaque gradients (Invitrogen, Carlsbad, CA), washed in tissue culture medium, and aliquoted for tissue culture. For the establishment of primary tumor cell line culture, tumor cells were seeded in a flask in the following culture medium: Ham’s F-12/DMEM with high glucose (Irvine Scientific, Santa Ana, CA), 10 mM HEPES (Invitrogen), 0.1 mg/ml gentamicin (Invitrogen), and 10% heat-inactivated fetal bovine serum (Irvine Scientific). Primary tumor cells were subcultured for three to four passages. Each passage yielded a 2–4-fold increase in cell number. Tumor cell culture followed the Standard Operating Procedure approved by Cedars-Sinai Biosafety Committee. Four patients’ cells were randomly selected for chromosomal karyotype analysis at the Cytogenetics Laboratory Facility, Cedars-Sinai Medical Center. At least 20 cells were analyzed from the cultures of each of these four patients. Four of these karyotypes displayed gain of chromosome 7. The criteria of karyotypic analysis was based on the International System of Cytogenetic Nomenclature in 1991 (31).

Cell Lines. GBM cell lines (IR-801, IR-802, and IR-803) were provided by Immune Response Inc. (San Diego, CA). T2 cell line and GBM cell lines, including U-373MG, U-118MG, U-138MG, and U-87MG, were supplied by American Type Culture Collection (Manassas, VA). The COS-7 A1 cell line was provided by the National Cancer Institute (Bethesda, MD). The cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Irvine Scientific), 10 mM HEPES buffer, 100 units/ml penicillin-streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), and 0.05 mM β-mercaptoethanol (Sigma, St. Louis, MO).

In Vitro Treatment of Tumor Cells with 5-Aza-2'-deoxycytidine (DAC). Primary glioblastoma cells were seeded at a density of 3–4 × 10^5 cells/ml in a T75 tissue culture flask. When cells became firmly adherent to plastic, the medium was replaced with fresh medium containing 1 μM DAC (Sigma) every 12 h for 3 days (six pulses). At the end of treatment, the medium was replaced with fresh culture medium without DAC for an additional 48 h and used for molecular and functional assays. Control cultures were treated under similar experimental conditions in the absence of DAC.

Synthetic Peptides and HLA Typing. All of the peptides using in this study were synthesized by Macromolecular Resource (Fort Collins, CO). The identity and purity of each of the peptides were confirmed by mass spectrometer and high-performance liquid chromatography analysis. Peptides were dissolved in DMSO at 1 mM concentration for future use. GBM cells were stained with biotin-conjugated HLA-A2- or HLA-A1-specific monoclonal antibody (US Biological, Swampscott, MA) or biotin-conjugated isotype control antibody. After streptavidin-perCP (BD PharMingen, San Diego, CA) staining for 30 min, the mean fluorescence intensity of HLA-A2 staining was analyzed by flow cytometry.

RNA Isolation and cDNA Synthesis. Total cellular RNA was extracted from the primary glioblastoma cell lines (passage 3–4) using the RNA4PCR kit (Ambion, Austin, TX) according to the manufacturer’s protocol. For cDNA synthesis, ~1 μg of total RNA was reverse transcribed into cDNA using random oligo(dT) and reverse transcriptase. cDNA was stored at −20°C for PCR.

Detection of the Expression of MAGE-1, gp100, and HER-2 mRNA by RT-PCR. The PCR mixture consisted of 5 μl of 10× thermophilic DNA polymerase reaction buffer, 4 μl of 10 mM deoxynucleotide triphosphate, 3 μl of MgCl2 (25 mM), 100 pm each primer, 5 units of AmpliTag DNA polymerase, and 2 μl of reverse transcription mixture. DNase- and RNase-free water was added to bring the reaction volume to 50 μl. The PCR program was set as follows: 1 cycle of 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. Primers for β-actin, gp100, and MAGE-1 used for this study were identical to those used in our previous report (32). HER-2 primers and product were as follows: forward, 5'-TCTGAGCTTCATCATGCTTGC-3', reverse, 5'-AGGCGATA-AGCTGTTGTCACC-3', and amplification product, 458 bp.

A 15-μl aliquot of each reaction was electrophoresed through a 1.5% agarose gel and stained with ethidium bromide for digital photograph under UV light, and the expected band size was assessed.

Flow Cytometry Analysis for Protein Expression. Immunostaining for HER-2, gp100, and MAGE-1 protein expression was performed using an intracellular staining kit (BD Pharmingen) according to the manufacturer’s recommendations. Appropriate dilutions of anti-HER-2, anti-Pmel (gp100) HMB-45 clone, anti-MAGE-1 monoclonal antibody from Lab Vision (Fremont, CA), and isotype control mouse IgG1 (BD Pharmingen) were incubated with tumor cells for 45 min at 4°C in 2% heat-inactivated fetal bovine serum/PBS. Secondary staining consisted of FITC-conjugated goat antimouse IgG1 (DAKO, Carpinteria, CA). The relative expression of tumor antigen in the different cell lines was determined by fluorescence intensity ratio (r), which was obtained by dividing tumor antigen mean fluorescence intensity by individual isotype control mean fluorescence intensity (33). We set the following criteria for evaluating the levels of tumor antigens: r < 1.6 denotes no detectable protein expression; 1.6 ≤ r < 3.0 denotes weak expression; and r ≥ 3.0 denotes strong expression.

Tumor Cell Recognition by CTL Assay. CTL cells were plated with target cells in 96-well round-bottomed plates in 200 μl of complete medium. After 18–24 h of incubation at 37°C, the supernatant was harvested for detection of IFN-γ release using ELISA kits (Endogen, Cambridge, MA). To determine whether cytokotic activity was MHC I dependent, tumor cells were incubated with W6/32 (anti-HLA class I) monoclonal antibody from American Type Culture Collection at a final concentration of 50 μg/ml for 1 h at 37°C before the addition of CTLs. Coefficient of variation of intra- and interassay of ELISA assay is <10%.

Generation and Cloning of HER-2- and MAGE-1-Specific CTLs by in Vitro Stimulation with Peptides. Peripheral blood mononuclear cells (PBMCs) obtained from HLA-A2+ or HLA-A1+ GBM patients were provided by Ficoll-Paque (Invitrogen) density gradient centrifugation. Cells were seeded (1 × 10^5 cells/3 ml/well) into 6-well plates (Costar, Cambridge, MA) in RPMI 1640 supplemented with 10% human AB blood phenotype serum, 2 mM L-glutamine, 10 mM HEPES, and antibiotics. After 2 h of incubation at 37°C, adherent cells were used for DC generation as described previously (32). Mature DCs were incubated with 10 μg/ml HER-2 or MAGE-1 peptide at 37°C for 2 h. Some 2 × 10^5 peptide-pulsed DCs were cultured with 2 × 10^6 autologous T cells in 2 ml of RPMI 1640 containing 5% human AB serum, IL-6 (1000 units/ml; R&D Systems, Minneapolis, MN), and IL-12 (5 ng/ml; R&D Systems) in 10 wells of 24-well culture plates (Falcon; Becton Dickson, San Jose, CA). T-cell cultures were restimulated weekly with 2 × 10^5 peptide-pulsed DCs in the presence of 100 IU/ml IL-2 (Proleukin; Chiron, Emeryville, CA) and 5 ng/ml IL-7 (R&D Systems). Cultures were maintained at <1.5 × 10^5 T cells/ml. Six days after the third stimulations, an aliquot of each T-cell culture was examined to evaluate the antigen-specific T cells by IFN-γ release. T cells from polyclonal cultures containing specific T cells were cloned by limiting dilution (34). Wells with the highest antigen-specific IFN-γ secretion were identified for further expansion.

Rapid Expansion of HER-2- and MAGE-1-Specific Clones. After specificity testing, 1–2 × 10^5 specific T cells were resuspended in 50 ml of RPMI 1640 containing IL-2 (300 IU/ml), OKT3 (30 ng/ml), IL-7 (10 ng/ml), 10 × 10^6 irradiated allogeneic feeder cells from three randomly chosen individuals, and 5 × 10^5 irradiated autologous PBMCs pulsed with HER-2 or MAGE-1 peptide for 4 h at room temperature. The rationale for using the three pooled irradiated allogeneic feeder cells was that these may secrete additional cytokines that support the rapid expansion of CTLs. The cells were cultured in the smallest ledge of a 25-cm² flask for optimal density (45-degree angle). On day 10, the flask was placed upright; the cells were harvested or restimulated and given half-fresh medium with replenishment of cytokines. On day 14, cells were harvested and either prepared for additional expansion cycles or cryopreserved. In general, one cycle expansion of CTL clones resulted in a 50–100-fold increase in cell number.
Table 1: Expression of HER-2, gp100, and MAGE-1 mRNA in GBM \textsuperscript{a} cells and normal brain tissue.

Total RNA was isolated from 43 primary GBM cell lines (p3–4 level), 7 GBM tumor cell lines, and 6 normal brain tissue samples and reverse transcribed into cDNA. RT-PCR was performed using primers specific for HER-2, gp100, and MAGE-1 (see “Materials and Methods”).

<table>
<thead>
<tr>
<th>GBM cell line</th>
<th>HER-2</th>
<th>gp100</th>
<th>MAGE-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-87MG</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U-118MG</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>U-1373MG</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U-138MG</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IR-801</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IR-802</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IR-803</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Normal brain tissue (n = 6)  
HER-2: 66.7\%; gp100: 50\%; MAGE-1: 0\%

Primary tumor cell lines (n = 43)  
HER-2: 81.4\%; gp100: 46.5\%; MAGE-1: 39.5\%

\textsuperscript{a} GBM, glioblastoma multiforme; RT-PCR, reverse transcription-PCR.

**Tetramer Staining.** HER-2-, gp100-, and MAGE-1-specific peptide tetramer (phycoerythrin-peptide loaded HLA tetramer complexes) were synthesized and provided by Beckman Coulter (San Diego, CA). Specific CTL clone CD8 cells were resuspended at 10^6 cells/50 \( \mu \)l fluorescence-activated cell-sorting buffer (phosphate buffer plus 1% inactivated fluorescence-activated cell-sorting buffer). Cells were then incubated at 30 min at room temperature, and incubation was then continued for 30 min at 4°C with 10 \( \mu \)l of anti-CD8 monoclonal antibody (Becton Dickinson). Cells were washed twice in 2 ml of cold fluorescence-activated cell-sorting buffer before analysis by fluorescence-activated cell sorting (Becton Dickinson).

**Statistical Analysis.** Spearman rank correlation coefficients (\( R \)) were evaluated to determine whether there was an association between antigen expression and CTL recognition determined by IFN-\( \gamma \)-cytokine release assays. Paired \( t \) tests were used to determine the effects of IFN-\( \gamma \) and DAC tumor cell treatment on cytokine release by CTL clones. A difference of \( P < 0.05 \) was considered significant.

**RESULTS**

HER-2, gp100, and MAGE-1 mRNA Expression in Primary Cultured GBM Cells. Forty-three primary cultured GBM cell lines, seven GBM tumor cell lines, and six fresh normal brain tissues from trauma patients were used to examine the mRNA expression of HER-2, gp100, and MAGE-1 by RT-PCR. The distribution of tumor antigen mRNA expression in seven GBM cell lines is listed in Table 1. HER-2, gp100, and MAGE-1 mRNA expression was detected in 81.4\%, 46.5\%, and 39.5\%, respectively, of the 43 GBM primary cell lines. In normal brain tissue, MAGE-1 was not detected, although HER-2 and gp100 were detected in 66.7\% and 50\% of the samples, respectively.

Expression of HER-2, gp100, and MAGE-1 Protein in GBM Tissue and Primary Cultured Tumor Cells. To determine whether HER-2, gp100, and MAGE-1 proteins were expressed within GBM cell lines, primary cultured cell lines were processed for immunofluorescence staining with specific monoclonal antibody and analyzed by flow cytometry. Based on the criteria of fluorescence intensity ratio (\( r \); see “Materials and Methods”), HER-2, gp100, and MAGE-1 protein expression was found in 76\%, 45\%, and 38\%, respectively, of the 43 primary tumor cell lines tested. Representative samples are shown in Fig. 1. Two independent experiments were performed on seven established GBM cell lines; data are shown in Table 2. We did not find protein expression in cell lines apparently lacking mRNA expression.

Characterization of MAGE-1, gp100, and Her-2 CTL Clones. To analyze whether HER-2-, gp100-, and MAGE-1-derived epitopes are presented by GBM tumor cells, we generated tumor antigen-specific CTL clones. Based on previous reports of HER-2, gp100, and MAGE-1, one dominant epitope was chosen for each antigen to generate CTL clones: HLA-A2-restricted HER-2 (9\textsuperscript{369–377}) peptide (KIFGSLAFL); HLA-A1-restricted MAGE-1 (9\textsuperscript{161–169}) antigen peptide (EADPTGHSY); and HLA-A2-restricted gp100 (9\textsuperscript{209–217}) peptide (ITDQVFPSV). The gp100 CTL clone CK3H6 derived from melanoma tumor-infiltrating lymphocyte culture was provided by Dr. Mark E. Dudley (National Cancer Institute, NIH). Its specificity and activity have been well studied and published previously (16, 35). We used PBMCs from GBM patients and autologous DCs pulsed with peptide to generate HER-2 and MAGE-1 CTL clones. Autologous DCs from a single HLA-A2\(^+\) patient and a single HLA-A1\(^+\) patient were used to generate HER-2- and MAGE-1-specific CTL clones, respectively. PBMCs were stimulated by autologous DCs pulsed with...
Effects of IFN-γ Treatment on Recognition of GBM Cells by HER-2-, gp100-, and MAGE-1-Specific CTL Clones. IFN-γ treatment increased MHC class I expression on GBM cells (data not shown). The ratio of fluorescence intensity was increased by 5.2 ± 1.3-fold (P < 0.05). As shown in Tables 3 and 4, after IFN-γ treatment, the recognition of tumor cells by CTL clone A05, CK3H6, and B07 was significantly increased (P < 0.05).

**Table 2: Expression of HER-2, gp100, and MAGE-1 protein in GBM cell lines**

<table>
<thead>
<tr>
<th>GBM cell line</th>
<th>HER-2</th>
<th>gp100</th>
<th>MAGE-1</th>
<th>HER-2</th>
<th>gp100</th>
<th>MAGE-1</th>
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<tbody>
<tr>
<td>U-87MG</td>
<td>2.0</td>
<td>1.7</td>
<td>5.4</td>
<td>2.3</td>
<td>1.8</td>
<td>5.1</td>
</tr>
<tr>
<td>U-118MG</td>
<td>2.3</td>
<td>1.3</td>
<td>3.9</td>
<td>2.4</td>
<td>1.2</td>
<td>3.8</td>
</tr>
<tr>
<td>U-373MG</td>
<td>1.4</td>
<td>1.4</td>
<td>1.8</td>
<td>1.4</td>
<td>1.4</td>
<td>1.9</td>
</tr>
<tr>
<td>U-138MG</td>
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<td>2.6</td>
<td>2.3</td>
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a GBM, glioblastoma multiforme; mAb, monoclonal antibody.

**Recognition of Primary Cultured GBM Cell Line by HER-2-, gp100-, and MAGE-1-Specific CTL Clones.** CTL clone cells (1 x 10⁵) were incubated with 1 x 10⁵ primary cultured GBM cells (p3–4 level) or GBM cells in 200 µl of complete medium. After incubation for 24 h, the recognition of the tumor cells by CTLs was assessed by the secretion of IFN-γ in supernatant. As shown in Tables 3 and 4, HER-2 CTL clone A05, gp100 clone CK3H6, and MAGE-1 clone B07 only recognized HLA-A2- or HLA-A1-matched GBM cells where specific tumor antigen expression was detected. The observation that these three CTL clones recognized tumor antigen in HLA class I restriction was further confirmed by HLA class I antibody staining and specific tetramer. No staining was seen with nonspecific tetramer (Fig. 2).

**Table 3: Recognition of primary cultured GBM cells by HER-2 and gp100 CTL clone**

<table>
<thead>
<tr>
<th>GBM cell code</th>
<th>HER-2-specific CTL clone A05</th>
<th>gp100-specific CTL clone CK3H6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2 + gp100 (9209-217)</td>
<td>+</td>
<td>HER-2</td>
</tr>
<tr>
<td>T2 + HER-2 (9209-217)</td>
<td>+</td>
<td>1 µM</td>
</tr>
<tr>
<td>66</td>
<td>1248</td>
<td>1950</td>
</tr>
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<td>43</td>
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<td>43 + W6/32d</td>
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<td>U-87MG</td>
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a GBM, glioblastoma multiforme.

**Table 4: Recognition of GBM cells by MAGE-1-specific CTL clone B07**

CTL clone B07 cells (1 x 10⁵) were incubated with 1 x 10⁵ target cells for 24 h, and release of IFN-γ (pg/ml) was analyzed by ELISA.

<table>
<thead>
<tr>
<th>GBM cell code</th>
<th>HLA-A1 expression</th>
<th>MAGE-1 protein expression</th>
<th>Control IFN-γ</th>
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<tr>
<td>Unpulsed COS-7A</td>
<td>+</td>
<td>+</td>
<td>159</td>
</tr>
<tr>
<td>COS-7.A1 + MAGE-1 (9220-217)</td>
<td>+</td>
<td>1 µM</td>
<td>&gt;2500</td>
</tr>
<tr>
<td>79</td>
<td>+</td>
<td>+</td>
<td>1499</td>
</tr>
<tr>
<td>4</td>
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<td>+</td>
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<tr>
<td>169</td>
<td>+</td>
<td>+</td>
<td>1259</td>
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<tr>
<td>43 + W6/32d</td>
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<td>+</td>
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a GBM, glioblastoma multiforme.
b MAGE-1 protein expression was stained and analyzed by flow cytometry. –, no detectable expression; +, weak expression; ++, strong expression.
c Tumor cells treated with 1000 pg/ml IFN-γ for 48 h. d Tumor cells were incubated with W6/32 (anti-HLA class I) antibody at a final concentration of 50 µg/ml for 1 h at 37°C before the addition of CTLs. Results are representative of two individual experiments, and each value represents the mean from triplicate wells.

As the results show in Tables 3 and 4, the levels of tumor antigen
protein determine the sensitivity of target cells to recognition by CTLs. As shown in Fig. 4, after treatment with DAC, GBM could be efficiently recognized by HLA-A1-restricted MAGE-1-specific CTL clone B07, and IFN-γ release by B07 T cells was significantly increased compared with that of the control (without treatment; P < 0.01). DAC treatment generates sufficient amounts of immunogenic peptides to be loaded on preexisting MHC class I molecules, which provides cell surface copies of HLA class I complexes above the threshold level required for efficient CTL recognition of targets cells (36). Our data have shown that treatment of MAGE-1-negative GBM tumor cells with DAC induced MAGE-1 expression and increased their recognition by MAGE-1-specific CTLs.

**DISCUSSION**

In this study, we demonstrated that HER-2, gp100, and MAGE-1 are expressed in human glioblastoma cells and also recognized by cytotoxic T cells. Antigen recognition is determined by both antigen expression and MHC expression on the cell surface. Our results demonstrated that tumor antigen expression in GBM cells correlates with tumor cell recognition by CTLs, which is in agreement with other reports on melanoma (11, 23). Our studies showed that IFN-γ treatment could significantly increase MHC class I expression on GBM tumor cells, further enhancing the sensitivity of recognition by CTLs. The demonstration of MHC up-regulation in human glioma cell lines leading to increased recognition by antigen-specific CTLs further supports the incorporation of IFN-γ in the effector phase of glioma immunotherapy.

MAGE-1 is a cancer/testis antigen, which is a novel family of immunogenic proteins. MAGE genes were initially analyzed from melanoma and shown to exhibit a nearly exclusive neoplasm-specific expression pattern. Our results showed that MAGE-1 was exclusively expressed in GBM tissues and cells and was not detected in normal brain. A study of childhood astrocytomas reported the immunohistochemical presence and cellular localization of the MAGE-1 antigen in all specimens of malignant gliomas including glioblastoma. No MAGE-1 expression was detected, however, in the lowest grade, pilocytic astrocytoma (4). Previous studies have defined the regulatory role of DNA methylation in the constitutive expression of cancer/
testis antigens by melanoma cells and renal cell carcinoma and showed that in vitro treatment with the DNA hypomethylating agent DAC induced and/or up-regulated functional cancer/testis antigen expression in neoplastic cells (36). In our studies, MAGE-1-negative GBM tumor cells, after exposure to 1 μM DAC for 72 h, demonstrated MAGE-1 mRNA expression by RT-PCR. Furthermore, DAC-treated GBM cells were recognized by the MAGE-1-specific MHC-restricted cytotoxic T-cell clone B07, whereas untreated control cells were not recognized. This finding suggests that production of the antigen encoded by the MAGE-1 gene was induced by DAC and that it was presented in association with MHC class I molecules at the cell surface for T-cell recognition. Normally, about 40% of GBM tumor cells are MAGE-1 positive, so it would be important to induce its expression before MAGE-1 immunization. Successful up-regulation of the MAGE-1 tumor antigen by DAC might be combined with a strategy to up-regulate MHC levels by IFN-γ to augment antigen recognition by T cells (37).

Rimoldi et al. (29) were the first to document that melanoma-associated antigen-specific CTL lines could recognize HLA-matched glioma cells in vitro. Then, Chi et al. (27), Scarchella et al. (28), and Sahin et al. (30) reported that gp100 and MAGE-1 mRNA was expressed in glioma tumor cells and tumor tissue by RT-PCR, and HER-2 was found in brain tumor by immunohistochemical staining (25, 26). More recently, Prins et al. (38) validated melanoma-associated antigen gp100 and TRP-2 as immunotherapeutic targets in a murine glioma model (38). Very importantly, IL-13 receptor α2 has been identified as a glioma-specific antigen (39), and a HLA-A2.1-restricted CTL epitope (WLPFGFLI) was defined by Okano et al. (40). Recently, we reported on TRP-2 as a CTL target in malignant glioma, which demonstrated that TRP-2 antigen can be naturally processed and recognized by TRP-2-specific CTLs. We also found that TRP-2-specific cytotoxic T-cell activity was detected when PBMCs were stimulated with autologous DCs pulsed with irradiated GBM tumor cells in vitro and in patents’ PBMCs after DC-pulsed autologous tumor lysate vaccinations (41). The goal of tumor antigen identification study is to translate the finding from bench side to bedside. In our DC-based clinical trial, we found that vaccinations with DCs pulsed with tumor lysates were able to induce HER-2-, gp100-, and MAGE-1-specific CTLs in patients’ PBMCs after vaccinations as determined by tetramer analysis.3 Our demonstration of the presence of HER-2, gp100, and MAGE-1 expression on GBM cells and the generation of peptide-specific, MHC-restricted CTLs that recognized GBM cells lay the groundwork for the use of relevant surrogate assays to determine the generation of antigen-specific cytotoxicity.

Although the presence of gp100 and HER-2 in normal brain could be a concern, previous melanoma clinical trials with gp100 peptide and breast cancer clinical trials with HER-2 peptide did not elicit autoimmune responses, despite the generation of CD8+ and CD4+ T cells responsive to gp100 and HER-2. In adults, the HER-2 gene is present as a single copy in normal cells; however, amplification of the gene and resultant protein overexpression are seen in various cancers. It is possible that the epitopes expressed on normal tissues are below the threshold level for T-cell recognition, whereas their overexpression in tumor cells can trigger an anticancer response (10). Even if normal brain tissue expresses HER-2 and gp100 mRNA, most neural cells do not express MHC, preventing CTL recognition (42). In the past decade, HER-2, gp100, and MAGE-1 as attractive brain tumor therapy target candidates. Our studies demonstrate that these tumor antigens can be effective targets for both CD8 and CD4 T-cell adoptive transfer and active specific immunotherapy in malignant glioma.

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