Cancer Therapy: Preclinical

HER2-Specific T Cells Target Primary Glioblastoma Stem Cells and Induce Regression of Autologous Experimental Tumors

Nabil Ahmed1,2,3, Vita S. Salsman1,2,3, Yvonne Kew6, Donald Shaffer1,2, Suzanne Powell6,7, Yi J. Zhang8, Robert G. Grossman8, Helen E. Heslop1,2,3,4, and Stephen Gottschalk1,2,3,5

Abstract

Purpose: Glioblastoma multiforme (GBM) is the most aggressive human primary brain tumor and is currently incurable. Immunotherapies have the potential to target GBM stem cells, which are resistant to conventional therapies. Human epidermal growth factor receptor 2 (HER2) is a validated immunotherapy target, and we determined if HER2-specific T cells can be generated from GBM patients that will target autologous HER2-positive GBMs and their CD133-positive stem cell compartment.

Experimental Design: HER2-specific T cells from 10 consecutive GBM patients were generated by transduction with a retroviral vector encoding a HER2-specific chimeric antigen receptor. The effector function of HER2-specific T cells against autologous GBM cells, including CD133-positive stem cells, was evaluated in vitro and in an orthotopic murine xenograft model.

Results: Stimulation of HER2-specific T cells with HER2-positive autologous GBM cells resulted in T-cell proliferation and secretion of IFN-γ and interleukin-2 in a HER2-dependent manner. Patients' HER2-specific T cells killed CD133-positive and CD133-negative cells derived from primary HER2-positive GBMs, whereas HER2-negative tumor cells were not killed. Injection of HER2-specific T cells induced sustained regression of autologous GBM xenografts established in the brain of severe combined immunodeficient mice.

Conclusions: Gene transfer allows the reliable generation of HER2-specific T cells from GBM patients, which have potent antitumor activity against autologous HER2-positive tumors including their putative stem cells. Hence, the adoptive transfer of HER2-redirection T cells may be a promising immunotherapeutic approach for GBM.

Clin Cancer Res; 16(2); 474–85. ©2010 AACR.

Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor in adults (1). Currently, the best therapy consists of gross total surgical resection, which is frequently not possible due to location in eloquent areas of the brain, followed by radiotherapy and chemotherapy. Long-term survival is occasionally seen in rare cases that occur in young adults, generally under age 30. However, in the majority of cases, which occur in middle and old age, radiotherapy and chemotherapy only slow but do not stop tumor growth, resulting in 5-year survival rates of <4% (2, 3). The recent identification of chemotherapy- and radiotherapy-resistant CD133-positive tumor stem cells in GBMs (4, 5) may help explain why conventional therapies are ineffective. Although the exact mechanism of tumor stem cell resistance to conventional therapies remains elusive, their quiescent state and increased capacity to eliminate cytotoxic drugs and repair damaged DNA are thought to be key contributing factors (4, 5). Immunotherapy may be able to benefit GBM patients because immune-mediated killing relies neither on tumor cell proliferation nor on the aforementioned cytotoxic pathways.

Results from completed phase I/II immunotherapy clinical trials with tumor cell or dendritic cell vaccines were encouraging, showing disease stabilization and suggesting prolonged patient survival (6–9). However, these trials have also highlighted some of the limitations of dendritic cell vaccines (10), particularly their failure to reproducibly and effectively expand tumor antigen–specific T cells, which may be present at low frequency or are anergized. One means of overcoming this limitation is adoptive T-cell transfer, in which tumor-specific T cells are prepared ex vivo and then transferred to affected individuals. Genetic modification of T cells with chimeric antigen receptors (CAR) can reliably generate tumor-specific T cells ex vivo.
Materials and Methods

Blood donors, primary tumor cells, and cell lines. Blood samples and primary tumor cells were obtained from subjects with GBM on a protocol approved by the Institutional Review Board of Baylor College of Medicine at Baylor College of Medicine and The Methodist Hospital. The GBM and medulloblastoma cell lines (U373 and Daoy) and the breast cancer cell line (MDA-MB-468) were purchased from the American Type Culture Collection. All cell lines were grown in DMEM (Invitrogen) supplemented with 15% heat-inactivated FCS, 2 mmol/L GlutaMAX-I, 1% insulin-transferrin-selenium-X supplement, and 1% penicillin-streptomycin mixture (all medium supplements from Invitrogen). Cells were used within 7 d of plating or established as primary cell lines.

Immunohistochemistry. Mice were euthanized by CO2 inhalation and fixed with intracardiac perfusion of 4% paraformaldehyde. The brain tissue was postfixed overnight and embedded in paraffin, and histology was done on 10-μm serial horizontal sections. Tissue sections obtained from mouse xenografts and from paraffin-embedded surgical excision samples were stained by a standard H&E technique. HER2 expression in GBM xenografts was detected by phosho-HER2 immunohistochemistry as previously described (17).

Generation of retroviral constructs. The HER2-specific CAR with a CD28-ζ signaling domain was constructed by subcloning the HER2-specific single-chain variable fragment FRP5 into a SFG.CD28.ζ retroviral vector as previously described (18). A retroviral vector encoding the fusion protein eGFP-firefly luciferase (eGFP.FFLuc) was used to generate firefly luciferase–expressing GBM cells for the in vivo studies (18).

Retrovirus production and transduction of T cells. To produce retroviral supernatant, 293T cells were cotransfected with an FRP5.CD28.ζ retroviral vector containing plasmid, Peg-Pam-e plasmid encoding the sequence for MoMLV gag-pol, and plasmid pMEVSVg containing the sequence for VSV-G using GeneJuicer transfection reagent (EMD Biosciences; ref. 19). Supernatants containing the retrovirus were collected 48 and 72 h later. VSV-G pseudotyped viral particles were used to transduce the PG-13 producer cell line for the production of viral particles.

OKT3/CD28 activated T cells were transduced with retroviral vectors as described (19). Briefly, peripheral blood mononuclear cells were isolated by Lymphoprep (Greiner Bio-One) gradient centrifugation. Peripheral blood mononuclear cells (5 × 10⁵ per well) in a 24-well plate were activated with OKT3 (OrthoBiotech) and CD28 monoclonal antibodies (BD Biosciences) at a final concentration of 1 μg/mL. On day 2, recombinant human interleukin (IL)-2 (Chiron) was added at a final concentration of 100 units/mL, and on day 3, cells were harvested for retroviral transduction. For transduction, we precoated a nontissue culture–treated 24-well plate with a recombinant fibronectin fragment (FN CH-296; RetroNectin; Takara Bio USA). Wells were washed with PBS (Sigma) and incubated twice for 30 min with retrovirus. Subsequently, 3 × 10⁵ T cells per well were transduced with retrovirus in the presence of 100 units/mL IL-2. After 48 to 72 h, cells were removed for clinical use (11, 12). CARs are synthetic molecules that consist of an extracellular antigen binding domain that usually contains the heavy and light chain variable regions of a monoclonal antibody joined to transmembrane and cytoplasmic signaling domains derived from the CD3-ζ chain and from costimulatory molecules such as CD28. CARs recognize antigens expressed on the surface of tumor cells, and in this study, we targeted the human epidermal growth factor receptor 2 (HER2), a tumor-associated antigen that is expressed by up to 80% of GBMs but not by normal postnatal neurons or glia (13–16).

We now show that T cells from GBM patients can readily be modified with HER2-specific CARs to produce effector cells, which release immunostimulatory cytokines in response to, and kill, autologous primary HER2-positive GBM tumor cells, including CD133-positive GBM stem cells. These HER2-specific T cells also had a potent antitumor activity against autologous tumors in an orthotopic xenogeneic severe combined immunodeficient mouse model. Hence, the adoptive transfer of HER2-directed T cells may be an attractive immunotherapeutic approach for GBM.
and expanded in the presence of 50 to 100 units/mL of IL-2 for 10 to 15 d before use.

Flow cytometry. We used a FACScalibur instrument (Becton Dickinson) and CellQuest software (Becton Dickinson) for all flow cytometric analyses, analyzing >10,000 events; in all cases, negative controls included isotype antibodies. Cells were washed once with PBS containing 2% fetal bovine serum and 0.1% sodium azide [fluorescence-activated cell sorting (FACS) buffer; Sigma] before addition of antibodies. After 15 to 30 min of incubation at 4°C in the dark, the cells were washed once and fixed in 0.5% paraformaldehyde/FACS buffer before analysis.

T cells were analyzed with anti-CD8 FITC, anti-CD4 phycoerythrin, and anti-CD3 perdinin chlorophyll protein, and tumor cell lines with anti-HER2 phycoerythrin. All monoclonal antibodies (except CD133; Milleniy Biotech) were obtained from BD Biosciences. To determine cell surface expression of the HER2 CAR transgene, a recombinant HER2-Fc fusion protein (R&D Systems) was used. Bound HER2-Fc was detected with a goat anti-Fc FITC secondary antibody (Chemicon; ref. 19).

Cytotoxicity assays. Cytotoxicity assays were done as previously described (20). Briefly, 1 × 10⁶ target cells were labeled with 0.1 mCi (3.7 MBq) ⁵¹Cr and mixed with decreasing numbers of effector cells to give E:T ratios of 40:1, 20:1, 10:1, and 5:1. Target cells incubated in complete medium alone or in 1% Triton X-100 were used to determine spontaneous and maximum ⁵¹Cr release, respectively. After 4 h, we collected supernatants and measured radioactivity in a gamma counter (Cobra Quantum; Perkin-Elmer). The mean percentage of specific lysis of triplicate wells was calculated according to the following formula: (test release – spontaneous release)/(maximal release – spontaneous release) × 100.

Analysis of cytokine production and T-cell expansion. Effector T cells (FRP5.CD28.CAR-expressing T cells or nontransduced T cells) from GBM patients or healthy volunteers were cocultured with primary autologous GBM cells in short-term culture (<14 d). HER2-positive and HER2-negative cell lines at a 1:1 E:T ratio were plated in 2×10⁶ autologous HER2-specific or nontransduced T cells (5 × 10⁴ per mouse). On day 8, mice were treated with 2 × 10⁶ agarose gel with 6% formaldehyde. Total RNA (0.5 μg) was used to synthesize biotinylated cRNA using TotalPrep RNA Amplification kit (Ambion, Inc.), and 1.5 μg of biotinylated cRNA were applied to the HumanWG-6 v3 BeadChips and processed according to the vendor's instruction. The BeadChips were scanned using Beadstation 500 GX scanner. The image files were imported into the BeadStudio software version 3.2.7 (Illumina), and the data were processed using the quantile normalization algorithm. This method assumes that the distribution of the expression values does not change dramatically between arrays. All arrays were adjusted so that they had an almost identical overall intensity distribution. All 42,620 genes were used to calculate the correlation coefficient between samples in the BeadStudio software. All samples that contained genes with detection P value of <0.001 were subjected to a differential analysis using Illumina's own test with false discovery correction.

Orthotopic xenogenic SCID mouse model of GBM. All animal experiments were conducted on a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Recipient nonobese diabetic-SCID mice were purchased from Taconic (C.B-Lgh⁻¹/Icr-Tac-Prkdcscid, FOX CHASE CB-17 SCID ICR). Nine- to 12-wk-old male mice were anesthetized with rapid sequence inhalation isoﬂurane (Abbot Laboratories) followed by an i.p. injection of 225 to 240 mg/kg of Avertin solution and then maintained on isoflurane by inhalation throughout the procedure. The head was shaved, and then the mice were immobilized in a Cunningham Mouse/Neonatal Rat Adaptor (Stoelting) stereotaxic apparatus fitted into an E15600 Lab Standard Stereotaxic Instrument (Stoelting) and then scrubbed with 1% povidone-iodine. A 10-mm skin incision was made along the midline. The tip of a 31-gauge, 0.5-inch needle mounted on a Hamilton syringe served as the reference point. A 1-mm burr hole was drilled into the skull, 1 mm anterior to and 2 mm to the right of the bregma.

Firefly luciferase–expressing primary GBM cells (5 × 10⁴ in 2.5 μL) from patients 2, 3, or 5 were injected 3 mm deep to the bregma, corresponding to the center of the right caudate nucleus over 5 min. The needle was left in place for 3 min, to avoid tumor cell extrusion, and then withdrawn over 5 min. All GBMs engrafted and started to grow as judged by exponential increments in their bioluminescence signals. Six days after tumor cell infection, mice containing malignant cells derived from each individual patient were randomly assigned to receive no therapy, HER2-specific T cells, or nontransduced T cells. T-cell therapy consisted of 2 × 10⁶ autologous HER2-specific or nontransduced T cells injected locally in 5 μL to the same tumor coordinates. The incision was closed with two to three interrupted 7.0 Ethico sutures (Ethicon, Inc.). A s.c. injection of 0.03 to 0.1 mg/kg of buprenorphine (Buprenex RBH) was given for pain control. For the experiment with CD133-positive GBM stem cells, cells were isolated by high-speed cell sorting and injected into mice as described above (1 × 10⁴ in 2.5 μL per mouse).
autologous HER2-specific or nontransduced T cells in 5 μL to the same tumor coordinates.

**Bioluminescence imaging.** Isoflurane-anesthetized animals were imaged using the IVIS system (Xenogen Corp.) 10 min after 150 mg/kg D-luciferin (Xenogen) was injected i.p. The photons emitted from luciferase-expressing cells within the animal body and transmitted through the tissue were quantified using "Living Image," a software program provided by the same manufacturer. A pseudocolor image representing light intensity (blue, least intense; red, most intense) was generated and superimposed over the grayscale reference image. Animals were imaged every other day for 1 wk after injections, then twice weekly for 2 wk, and then weekly thereafter. They were regularly examined for any neurologic deficits, weight loss, or signs of stress and euthanized according to preset criteria in accordance with the Baylor College of Medicine Center for Comparative Medicine guidelines.

**Statistical analysis.** For the bioluminescence experiments, intensity signals were log transformed and summarized using mean ± SD at baseline and multiple subsequent time points for each group of mice. Changes in intensity of signal from baseline at each time point were calculated and compared using paired t tests or Wilcoxon signed-ranks test.

**Results**

**Generation of functional HER2-specific T cells from subjects with GBM.** To redirect patients’ lymphocytes to HER2, we used a second-generation HER2-specific CAR consisting of an extracellular domain derived from the high-affinity HER2 monoclonal antibody, FRP5 (21), and signaling domains from the costimulatory molecule CD28 and the CD3ζ-chain (FRP5.CD28,ζ CAR; Supplementary Fig. S1A). CD3/CD28 activated T cells from 10 subjects with GBM patients in triplicates are shown.
consecutive newly diagnosed GBM patients were transduced with RD114-pseudotyped retroviral vectors encoding FRP5.CD28.ζ CAR, and 4 to 7 days after transduction, we determined the expression of HER2-specific CARs by FACS analysis. On average, 79% (SD, ±15%) of GBM patients' T cells expressed HER2-specific CARs, and both CD4- and CD8-positive T cells were transduced (Supplementary Fig. S1B and C).

To evaluate the in vitro function of patients' HER2-specific T cells, we used standard cytotoxicity assays and coculture experiments with both HER2-positive targets (GBM cell line U373 and medulloblastoma cell line Daoy) and HER2-negative controls (MDA-MB-468). All patients' HER2-specific T cells killed HER2-positive target cells and secreted immunostimulatory Th1 cytokines (IFN-γ and IL-2), whereas nontransduced T cells from the same patients did not (Fig. 1A and B). In addition, HER2-negative target cells were not killed and did not induce cytokine production. Hence, genetic modification of T cells from GBM patients allows rapid

---

**Fig. 2.** HER2 protein expression on primary GBM. A, FACS analysis. Primary GBM cells from freshly excised tumors in short-term culture were stained for HER2 expression. Open curves, isotype control; solid curves, HER2. Nine of 10 tumor cell lines expressed HER2 on the cell surface. B, using the HER2-specific mouse monoclonal antibody NCL-L-CB11 (Novocastra), HER2 expression was confirmed on the corresponding paraffin-embedded sections. One tumor had no detectable HER2 protein expression using both methods (patient 8). Magnification, ×200.

---

**Fig. 3.** HER2-specific T cells kill autologous HER2-positive GBM and are activated in coculture. A, the cytolytic activity of T cells expressing HER2 CAR was determined in a standard 4-h chromium release assay. There was always an increase of cytolytic activity of HER2-specific T cells above background (nontransduced T cells) against autologous HER2-positive GBMs. As controls, the HER2-positive GBM cell line U373 and the HER2-negative MDA-MB-468 were used. HER2-specific T cells from all patients killed U373 cells, whereas MDA-MB-468 was not killed (shown for patient 4). B, HER2-specific T cells (black columns) or nontransduced T cells (white columns) from GBM patients were cocultured with autologous tumor cells or HER2-negative control cells (MDA-MB-468; hatched columns) in a 1:1 ratio. Twenty-four to 48 h after stimulation, the cytokine concentration in the medium was determined by ELISA. HER2-specific T cells produced IFN-γ and IL-2 after stimulation with eight of nine HER2-positive tumor samples. No cytokine release was seen with nontransduced T cells. Median cytokine levels for all patients are shown for U373 (HER2-positive control) and MDA-MB-468 (HER2-negative control). Results of experiments done in duplicates are shown.
and reliable generation of functional HER2-specific T cells.

HER2-specific T cells recognize and kill autologous GBM cells. To investigate if patients' HER2-specific T cells recognize and kill autologous HER2-positive GBM cells, we used short-term cultured autologous GBM cells as targets. Nine of 10 tumor cell lines expressed HER2 on the cell surface as judged by FACS analysis (Fig. 2A).
HER2 expression of the corresponding primary tumor was confirmed by immunohistochemistry (Fig. 2B). Although we observed moderate heterogeneity of HER2 expression within individual GBMs, there was concordance between FACS analysis and immunohistochemistry, with one tumor having no detectable HER2 protein expression using both methods (patient 8).

In standard 4-hour \(^{51}\)Cr release assays, there was always an increase of cytolytic activity of HER2-specific T cells in comparison with nontransduced T cells against autologous HER2-positive GBMs, confirming HER2 specificity (Fig. 3A). Besides tumor cell killing, cytokine production of T cells is critical for their activation and sustained antitumor activity. In coculture experiments, HER2-specific T cells from eight of nine patients with HER2-positive GBMs secreted substantial amounts of Th1 cytokines (IFN-\(\gamma\) and IL-2) denoting T-cell activation (Fig. 3B). No IFN-\(\gamma\) or IL-2 production was detected when HER2-specific T cells were cultured with HER2-negative targets (MDA-MB-468) or from cocultures with autologous nontransduced T cells. These results indicate that HER2-specific T cells recognize and kill autologous HER2-positive GBM cells in a HER2-specific manner.

**HER2-specific T cells kill autologous CD133-positive GBM stem cells.** We next evaluated if HER2-specific T cells also kill CD133-positive GBM stem cells, which are resistant to current standard therapies, including radiotherapy and chemotherapy. We separated the CD133-positive and CD133-negative cell populations from primary GBM cell lines of three patients by high-speed cell sorting. Three percent to 5% of the total cell population was CD133 positive (Fig. 4A). This GBM stem cell population expressed 2.8- to 4-fold higher levels of HER2 in comparison with their CD133-negative counterparts (Fig. 4B). In a 4-hour \(^{51}\)Cr release assay, HER2-specific T cells of all three patients killed autologous CD133-positive as well as CD133-negative cells (Fig. 4C). Autologous nontransduced T cells induced no significant killing of either cell populations. These results show that HER2-specific T cells are able to target the treatment-resistant CD133-positive compartment, which may contribute to tumor recurrence in GBM.

**Regression of primary GBM xenografts after administration of autologous HER2-specific T cells.** To determine the in vivo antitumor activity of HER2-specific T cells, we used an orthotopic autologous GBM xenograft model. Primary GBM cell lines from GBM patients were established in vitro, and their identity with the primary tumor was confirmed using a 48,000-probe gene chip array (Pearson correlation coefficient \((r^2) = 0.77-0.83\); Supplementary Fig. S2). To allow serial bioluminescence imaging in vivo, we transduced primary GBM cell lines from three patients with a retroviral vector encoding an eGFP-firefly luciferase fusion gene. All cells were green fluorescent protein (GFP) positive as judged by FACS analysis, and firefly luciferase expression was confirmed in vitro (data not shown). The tumorigenicity of the three patients' eGFP.FFLuc-expressing GBM cell lines was confirmed by injecting \(5 \times 10^4\) cells stereotactically into the right frontal cortex of SCID mice. Bioluminescence imaging of tumor xenografts showed progressive and exponential growth of tumors in all experimental animals (Supplementary Fig. S3). Untreated animals had a median survival of 17 days (range, 14-22).

To test the effector function of autologous HER2-specific T cells, 5 \(\times 10^4\) eGFP.FFLuc-expressing GBM cells from all three patients were injected stereotactically into the right frontal cortex of SCID mice. On day 6 after tumor cell injection, mice received an intratumoral injection of 2 \(\times 10^6\) autologous HER2-specific T cells or nontransduced T cells. A subset of animals was not treated. We quantified tumor growth by serial bioluminescence. In untreated animals, and in animals treated with autologous nontransduced T cells (Fig. 5A), tumors grew exponentially over time. In contrast, photon emission decreased significantly in all eight mice after autologous HER2-specific T-cell injection, indicating tumor regression (Fig. 5B). GBM xenografts from patients 2 and 3 regressed completely, whereas the xenografts from patient 5 had a partial response. The light signal continued to be undetectable after 6 months in four of eight animals, indicating tumor eradication. This was confirmed by histologic examination.

Kaplan-Meier survival studies 60 days after tumor injection showed that untreated mice and mice receiving autologous nontransduced T cells had a median survival of 14 and 15 days, respectively. In contrast, mice treated with autologous HER2-specific T cells had a median survival of 90 days, with 50% of the mice surviving >100 days \((P < 0.001;\) Fig. 5C).

To provide direct evidence that HER2-specific T cells also kill autologous CD133-positive GBM stem cells in vivo, CD133-positive cells from the eGFP.FFLuc-expressing GBM cell lines of patient 2 were sorted and injected stereotactically into the right frontal cortex of SCID mice \((1 \times 10^5\) cells; \(n = 8\)). Mice were imaged daily until the bioluminescence signal was comparable to that of xenografts obtained from unsorted cells. On day 8, mice were injected with autologous HER2-specific T cells or nontransduced T cells. Although tumors in animals treated with nontransduced T cells continued to grow exponentially, all animals treated with HER2-specific T cells had a measurable response, including two complete remissions, on bioluminescence imaging (Fig. 5D). This result further supports the conclusions of our in vitro experiments (Fig. 4) that HER2-specific T cells have antitumor activity against CD133-positive glioma cells.

**Discussion**

In this study, we investigated HER2-targeted T cells as a potential therapeutic agent for GBM and showed that T cells from GBM patients can be readily genetically engineered to be rendered HER2 specific. These effector cells recognized autologous HER2-positive GBMs including their CD133-positive stem cells in vitro and had potent antitumor activity in an orthotopic xenograft model.
It has proved difficult to generate tumor-specific T cells in cancer patients using dendritic cell vaccines, most likely because tumor-specific T cells are present at low frequency and in an anergic state due to the immunosuppressive environment induced by malignancies such as GBMs (10, 22). Adoptive T-cell transfer of \textit{ex vivo} expanded tumor-specific T cells may overcome these limitations, and forced expression of antigen-specific CARs or transgenic \(\alpha/\beta\) T-cell receptors (TCR) has been used to generate such effector T lymphocytes (23–26). CARs combine the antigen-binding property of monoclonal antibodies with the lytic and self-renewal capacities of T cells and have several advantages over transgenic \(\alpha/\beta\) TCR receptors (11, 12). CAR-expressing T cells recognize and kill tumor cells in an MHC-nonrestricted fashion so that target cell recognition by CAR T cells is unaffected by some of the major mechanisms by which tumors avoid MHC-restricted T-cell (\(\alpha/\beta\) TCR) recognition, such as downregulation of HLA class I molecules and defective antigen processing (11, 12). In addition, most tumors do not express costimulatory molecules so that \(\alpha/\beta\) TCR engagement is followed by incomplete T-cell activation. We (27) and others (28, 29) have

Fig. 4. HER2-specific T cells target primary GBM stem cells. A, primary GBM cells from three patients (GBM patients 2, 3, and 5) were stained for CD133 and isolated using high-speed sorting. Approximately 3% to 5% of the total primary GBM cell population was CD133 positive. B, this CD133-positive cell compartment was uniformly HER2 positive. Moreover, in all three tumors analyzed, the CD133-positive GBM stem cells expressed higher levels of HER2 in comparison with the CD133-negative tumor cell population. C, in a 4-h \(^{51}\text{Cr}\) release assay, HER2-specific T cells from these three patients killed autologous CD133-positive cells as well as their CD133-negative counterparts. Autologous nontransduced T cells induced no appreciable killing.
Fig. 5. Adoptively transferred HER2-specific T cells induce regression of autologous GBM xenografts in vivo. Primary GBM cells (5 × 10⁴) from patient 2 (two mice per group), patient 3 (three mice per group), and patient 5 (three mice per group) were injected stereotactically into the caudate nucleus of 9- to 12-wk-old SCID mice followed by intratumoral injection of 2 × 10⁶ autologous HER2-specific or nontransduced T cells 6 d after tumor inoculation. A, tumors grew progressively in untreated mice as shown for two representative animals (top row) and in mice receiving nontransduced T cells (middle row), whereas tumors regressed over a period of 2 to 5 d in response to a single injection of autologous HER2-specific T cells generated from the same patient (bottom row). B, quantitative bioluminescence imaging. Autologous HER2-specific T cells induced tumor regression when compared with nontransduced T cells (two-tailed P = 0.002, Mann-Whitney U test). Solid arrows, time of T-cell injection; open arrows, background luminescence (mean, ∼10⁵ photons/s/cm²/sr); n, number of animals tested in each group. C, Kaplan-Meier survival curve. Survival analysis done 60 d after tumor establishment. Mice treated with autologous HER2-specific T cells had a significantly longer survival probability (P < 0.001) in comparison with untreated mice and mice that received nontransduced T cells. D, 1 × 10⁴ CD133-positive GBM cells from patient 2 were injected as described above followed by intratumoral injection of 2 × 10⁶ autologous HER2-specific or nontransduced T cells 8 d after tumor inoculation. Whereas tumors in animals treated with nontransduced T cells (n = 4) continued to grow exponentially, all of the animals treated with autologous HER2 T cells (n = 4) regressed, with two of these animals having no detectable tumors within 6 d after T-cell injection.
shown that CARs can overcome this limitation if they incorporate costimulatory endodomains within the chimeric receptor sequence or if they are expressed in virus-specific CTLs. Our current HER2-specific CAR with a CD28 signalizing domain induces immunostimulatory cytokine release, including IL-2, from transduced cells of eight of nine HER2-positive GBM patients following CAR engagement.

Several cell surface antigens have been identified as potential targets for GBM-directed CAR T-cell therapies, including HER2, IL-13Ra2, EPH receptor A2, and EGFRvIII (14, 30–32). We chose HER2 because this antigen is expressed by a high percentage of tumors (>70%), and signaling through HER2 deregulates cell proliferation, inhibits apoptosis, and increases the metastatic potential of cancer cells (33, 34). Moreover, HER2 expression increases with the degree of anaplasia in glial tumors, and mutations in the HER2 signaling pathway have been identified in gliomas (35). Our own results confirmed a high frequency of HER2 expression, showing the antigen on 9 of 10 GBM tumors by two independent methods (immunohistochemistry and FACS analysis). As with other tumor-associated antigens expressed by GBMs, there was moderate heterogeneity in the expression pattern of HER2 within individual tumors (36, 37), and optimum killing in a clinical setting may require targeting more than one tumor-associated antigen or the induction of epitope spreading beyond the original targets to prevent immune escape (20, 38).

Despite the biological significance of HER2 signaling, the expression of HER2 on GBMs is low in comparison with breast cancer, rendering HER2 monoclonal antibodies ineffective (19, 30). HER2-specific T cells allow targeting of the relatively low levels of HER2 expressed by GBMs because the overall avidity of receptors arrayed on a T cell is greater than the avidity of a bivalent antibody, and engagement of even a limited number of TCR molecules is sufficient to trigger a cytotoxic effector response (39, 40). One concern of targeting HER2-expressing malignancies with T cells is “off-target” effects because the administration of HER2 monoclonal antibodies has been associated with side effects, the most concerning of which is the poorly understood cardiac toxicity. If HER2-specific T cells are long lived, this problem could be accentuated in severity and persistence. Potential cardiac toxicities are difficult to model in mice because T cells that are specific for human HER2 do not recognize murine HER2. Nonetheless, we have shown that HER2-negative cells are not killed by HER2-specific T cells. In addition, primary endothelial and epithelial cells (Supplementary Fig. S4) do not activate HER2-specific T cells and five patients who received HER2-specific T cells had no dose-limiting toxicities (41, 42). Finally, HER2 vaccines are well tolerated, and no cardiac toxicities were observed in patients who developed HER2-specific T-cell responses (43).

CD133 expression has identified a population with stem cell–like properties in normal and cancerous tissues of the central nervous system (4, 5). In GBMs, these cells are chemotherapy and radiotherapy resistant and most likely contribute to the ineffectiveness of conventional therapies. For example, although temozolomide was reported to preferentially deplete CD133-positive stem cells in primary GBMs in vitro, this effect was absent in O6-methylguanine-DNA methyltransferase–nonmethylated tumors, which represent 50% to 70% of primary GBMs and carry a worse prognosis (44). The use of temozolomide in a randomized prospective clinical trial in patients with GBM has thus only resulted in a marginal survival advantage (14.6 versus 12.1 months; ref. 3). We show here that CD133-positive GBM stem cells express HER2 and are killed by autologous HER2-specific T cells in vitro and in vivo similar to CD133-negative GBM cells. Hence, immune-targeted therapies may eradicate malignant stem cells that are resistant to conventional therapy.

Several studies have reported the infusion of activated T lymphocytes systemically or into resection cavities of recurrent or progressive malignant gliomas (45–47). These infusions were safe and resulted in disease stabilization and, in some instances, in partial regression. We injected HER2-specific T cells directly into autologous GBM xenografts in tumor-bearing mice. Tumors from patients 2 and 3 regressed completely without the need for exogenous cytokines, whereas untreated tumors and tumors treated with nontransduced T cells continued to grow. Tumors from patient 5 only had a partial response. Initial analysis revealed no significant differences in the level of HER2 expression as well as the in vitro effector function of HER2-specific T cells from these three patients. We are now investigating other biological differences between the tumors that may account for variability in effector cell sensitivity.

Tumors recur in several animals most likely due to limited T-cell persistence in vivo. Kahlon et al. (48) reported complete regression and no recurrence of U87 gliomas in an orthotopic xenogeneic SCID model after intratumoral injection of T cells expressing an IL-13Ra2-specific CAR. In their model, the U87 glioma cell line was genetically modified to secrete IL-2, a cytokine critical for T-cell survival and expansion in vivo. Tumor recurrence in our model may be due to inadequate persistence of human HER2-specific T cells in the xenograft, a known limitation of SCID mouse models.

In summary, this study shows that HER2-specific T cell can be readily generated from GBM patients by gene transfer with HER2-specific CARs. HER2-specific T cells recognized and killed autologous HER2-positive GBM, including CD133-positive stem cells, ex vivo and induced regression of experimental GBMs in vivo. Hence, the adoptive transfer of HER2-redirected T cells may be an attractive immunotherapeutic approach for GBM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Malcolm K. Brenner for the helpful discussion and advice, Awateef Akrabi for assistance with
FACS analysis, Christopher Threeton for assistance with flow sorting, and Drs. Richard L. Harper and James Rose (The Methodist Hospital neurosurgeons).

Grant Support

Clayton Foundation for Research, Dana Foundation, and American Brain Tumor Association. H.E. Heslop is the recipient of a Doris Duke Distinguished Clinical Scientist Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 5/29/09; revised 10/23/09; accepted 10/27/09; published OnlineFirst 1/12/10.

References

HER2-Specific T Cells Target Primary Glioblastoma Stem Cells and Induce Regression of Autologous Experimental Tumors


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-09-1322

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2010/01/18/1078-0432.CCR-09-1322.DC1

Cited articles
This article cites 48 articles, 19 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/16/2/474.full.html#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
/content/16/2/474.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.