Regression of Experimental Medulloblastoma following Transfer of HER2-Specific T Cells

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Abstract

Medulloblastoma is a common malignant brain tumor of childhood. Human epidermal growth factor receptor 2 (HER2) is expressed by 40% of medulloblastomas and is a risk factor for poor outcome with current aggressive multimodal therapy. In contrast to breast cancer, HER2 is expressed only at low levels in medulloblastomas, rendering monoclonal antibodies ineffective. We determined if T cells grafted with a HER2-specific chimeric antigen receptor (CAR; HER2-specific T cells) recognized and killed HER2-positive medulloblastomas. Ex vivo, stimulation of HER2-specific T cells with HER2-positive medulloblastomas resulted in T-cell proliferation and secretion of IFN-γ and interleukin 2 (IL-2) in a HER2-dependent manner. HER2-specific T cells killed autologous HER2-positive primary medulloblastoma cell lines and medulloblastoma cell lines in cytotoxicity assays, whereas HER2-negative tumor cells were not killed. No functional difference was observed between HER2-specific T cells generated from medulloblastoma patients and healthy donors. In vivo, the adoptive transfer of HER2-specific T cells resulted in sustained regression of established medulloblastomas in an orthotopic, xenogenic severe combined immunodeficiency model. In contrast, delivery of nontransduced T cells did not change the tumor growth pattern. Adoptive transfer of HER2-specific T cells may represent a promising immunotherapeutic approach for medulloblastoma. [Cancer Res 2007;67(12):5957–64]

Introduction

Medulloblastoma comprises up to 20% of all pediatric brain tumors and is currently treated with surgical resection, radiation therapy, and chemotherapy (1). However, only 60% to 70% of children with medulloblastoma will be cured with this aggressive multimodal therapy, and most of these will suffer long-term treatment-related complications (2). Given these results, new targeted therapies are necessary to improve outcome and reduce treatment-related morbidities. Immunotherapy has the potential to fulfill both of these needs. The human epidermal growth factor receptor 2 (HER2) is overexpressed in ~40% of medulloblastomas, and expression correlates with poor outcome (3). HER2 has been validated as a therapeutic target for breast cancers. However, the expression of HER2 is much lower on medulloblastoma tumor cells than on breast cancer cells, so they are not susceptible to HER2 monoclonal antibodies, like trastuzumab (Herceptin). Because trastuzumab not only interferes with HER2 signaling but also induces antibody-dependent cellular cytotoxicity, several strategies have been pursued to enhance the ability of HER2 monoclonal antibodies to recruit immune effector cells to HER2-expressing tumor cells. In preclinical studies, bispecific antibodies that were specific for both HER2 and CD3 or CD16, respectively, recruited CD3-positive T cells or CD16-positive natural killer (NK) cells to HER2-positive tumors. Alternatively, coadministrations of cytokines such as interleukin-12 (IL-12) resulted in enhanced antitumor activity of HER2-targeted therapies (4–6).

Recruiting the cellular arm of the immune response to HER2-positive tumor cells may also be accomplished by genetically engineering T cells to express a HER2-specific antibody as part of an artificial chimeric T-cell receptor (CAR; refs. 7, 8). These molecules combine the antigen binding property of monoclonal antibodies with the lytic capacity and potential longevity of T cells to provide an enhanced antitumor effect (9–11). HER2-specific CARs have been generated, and the antitumor activity of HER2-CAR–expressing T cells (HER2-specific T cells) has been shown in several xenogenic severe combined immunodeficiency (SCID) mouse models (8, 12, 13). However, the ability of HER2-specific T cells to kill HER2 low-expressing tumor cells, such as medulloblastoma, has not been evaluated.

In this study, we show that HER2-specific T cells recognize and kill medulloblastoma cell lines and autologous primary tumor cells ex vivo and have antitumor activity in an orthotopic, xenogenic SCID mouse model. Hence, adoptive transfer of HER2-specific T cells may represent a promising immunotherapeutic approach for HER2-positive medulloblastoma and other malignancies that express HER2 at low levels.

Materials and Methods

Blood donors, primary tumor cells, and cell lines. Studies using blood samples or primary tumor cells were done on Institutional Review Board–approved protocols, and informed consent was obtained from all donors. The medulloblastoma cell lines (Daoy, D283, and D341), the breast cancer cell lines (SK-BR-3 and MDA-MB-468), and 293T cells were purchased from the American Type Culture Collection. The generation of Daoy.2 cells, a Daoy derivative that overexpresses HER2 at high levels, was described previously (14). All cell lines were grown in DMEM (Invitrogen), supplemented with 2 mmol/L GlutaMAX-I, 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, and 1.0 mmol/L sodium bicarbonate.通常情况下，自然语言的阅读需要考虑文本的上下文、段落结构和句意逻辑。
pyruvate (all media supplements from Invitrogen). T cells were maintained in RPMI 1640 with 10% FCS containing 2 mmol/L GlutaMAX-I.

Tumor tissues were processed aseptically, and primary cell cultures were initiated using DMEM high-glucose medium (Invitrogen), supplemented with 15% heat inactivated FCS, 2 mmol/L GlutaMAX-I, 1% insulin-transferrin-selenium-X supplement, and 1% penicillin-streptomycin mixture (all media supplements from Invitrogen). Cells were used within 3 days of plating.

**Generation of retroviral constructs.** The HER2-specific single-chain variable fragment FRP5 was PCR amplified from pL-scFv(FRP5)-zeta-SN and subcloned in frame into a SFG retroviral vector containing a short hinge, and the transmembrane and signaling domains of the T-cell receptor ζ-chain (8, 15, 16). A LXSN retroviral vector was constructed containing the firefly luciferase gene and puromycin gene to generate firefly luciferase expressing Daoy cells for the in vivo study.

**Retrovirus production and transduction of T cells.** To produce retroviral supernatant, 293T cells were cotransfected with 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 GeneJuice transfection reagent (EMD Biosciences; refs. 16, 17). Supernatants containing the retrovirus were collected 48 and 72 h later. VSV-G pseudotyped viral particles were used to transduce the FLYRD18 producer cell line for the production of RD114 pseudotyped viral particles (18).

OKT3-activated T cells were transduced with retroviral vectors as described (17, 19). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep gradient centrifugation. About 5 × 10^6 PBMCs per well of a 24-well plate were activated with OKT3 (OrthoBiotech) at a final concentration of 1 μg/mL. On day 2, recombinant human IL-2 (Chiron) was added at a final concentration of 50 units/mL, and on day 3, cells were harvested for retroviral transduction. For transduction, we precoated a non-tissue culture–treated 24-well plate with a recombinant fibronectin fragment (FN CH-296: Retronectin; Takara Shuzo). Wells were washed with PBS (Sigma) and incubated twice for 30 min with retrovirus. Subsequently, 3 × 10^6 T cells per well were transduced with retrovirus in the presence of 50 units IL-2 per mL. After 48 to 72 h, the cells were removed and expanded in the presence of 50 to 100 units IL-2 per mL for 10 to 15 days before use. Nontransduced T cells used as controls were activated with OKT3 and expanded in the presence of 50 to 100 units IL-2 per mL for 10 to 15 days prior use in parallel to transduced T cells.

**Figure 1.** Medulloblastoma cell lines express HER2 at low levels, and their proliferation is not inhibited by trastuzumab. A, FACS analysis of cell lines: top row, medulloblastoma cell lines (D341, D283, Daoy); bottom row, breast cancer cell lines (MDA-MB-468, MCF-7, SK-BR-3) were stained with isotype control (open curves) or anti-HER2 (solid curves). Daoy and D283 expressed HER2 at low levels comparable to MCF-7. SK-BR-3 expressed HER2 ~25-fold higher where as D341 and MDA-MB-468 were negative for HER2 expression. B, HER2 is expressed in the CD133-positive progenitor population; CD133-positive progenitor cells from Daoy and D283 bulk cultures were isolated with anti-CD133 magnetic beads. HER2 expression was determined by FACS analysis demonstrating expression of HER2 on CD133-positive progenitor cells. C, trastuzumab does not inhibit the proliferation of Daoy and D283 cells: Daoy, D283, SK-BR-3, and MDA-MB-468 cells were cultured for 96 h in the presence of increasing trastuzumab concentrations before performing a proliferation assay. Trastuzumab significantly inhibited the proliferation of SK-BR-3 cells, whereas no inhibition was observed for Daoy, D283, and MDA-MB-468 cells; P value < 0.02.
Flow cytometry. For all flow-cytometric analyses, a FACScalibur instrument (BD Becton Dickinson) and CellQuest software (BD) were used. Data analysis was done on >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 [Sigma; fluorescence-activated cell sorting (FACS) buffer] 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, -CD4 PE, and -CD3 PerCP, and tumor cell lines with anti-HER2 PE. All monoclonal antibodies (except CD133, Miltenyi Biotec) were obtained from BD Biosciences. For determining the HER2 expression on CD133-positive progenitor cells, we sorted the cells with anti-CD133 magnetic beads as per manufacturer's instructions (Miltenyi Biotec). 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).

Analysis of cytokine production and T-cell expansion. Effector T cells (HER2CAR-expressing T cells or nontransduced T cells) from healthy donors or medulloblastoma patients were cocultured with HER2-positive and HER2-negative cell lines or primary medulloblastoma cells at a 1:1 effector-to-target ratio in a 24-well plate. After 24 to 48 h incubation, culture supernatants were harvested, and the presence of IFN-γ and IL-2 was determined by ELISA as per the manufacturer’s instructions (R&D Systems). T-cell expansion was determined by counting viable cells (trypan blue exclusion) 7 days after stimulation.

Proliferation assay. Tumor cells were cultured at 5,000 cells per well in a 96-well plate in the presence of increasing concentrations of trastuzumab. After 4 days, the cells were pulsed with 1 μCi (0.037 MBq) methyl-[3H]-thymidine and cultured for an additional 18 h. The cells were then harvested onto filters and dried; counts per minute were measured in a scintillation counter (TriCarb 2500 TR; Packard BioScience). The experiments were done in triplicate. Cells cultured without trastuzumab served as control.

Cytotoxicity assays. Cytotoxicity assays were done in triplicates as previously described (20). Briefly, 1 x 10^6 target cells were labeled with 0.1 mCi (3.7 MBq) ^51Cr and mixed with decreasing numbers of effector cells to give effector-to-target 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, supernatants were collected, and radioactivity was measured 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.

Orthotopic xenogenic SCID mouse model of medulloblastoma. All animal experiments were conducted on a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Recipient NOD-SCID mice were purchased from Taconic (C.B-17-129/f; Taconic), followed by an i.p. injection of 225 to 240 mg/kg Avertin solution and then maintained on isoflurane by inhalation throughout the procedure. The head was shaved, then the mice were immobilized in a Cunningham Mouse/Neonatal Rat Adaptor (Stoelting) stereotactic apparatus fitted into an E15600 Lab Standard Stereotactic Instrument (Stoelting), then scrubbed with 1% povidone-iodine. A 10-mm skin incision was made along the midline. The tip of a 31G 1/2-in needle mounted on a Hamilton syringe (Hamilton) 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 Daoy cells (2.5 × 10^5) 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. Five days after tumor cell injection, animals were treated with 2 × 10^6 effector cells in 5 µL to the same tumor coordinates. The incision was closed with two to three interrupted 7.0 Ethicon sutures (Ethicon, Inc.). A s.c. injection of 0.03 to 0.1 mg/kg buprenorphine (Buprenex RBH) was given for pain control.

Bioluminescence imaging. Isolourane-anesthetized animals were imaged using the IVIS system (IVIS, Xenogen Corp.) 10 min after 150 mg/kg d-luciferin (Xenogen) was injected i.p. (21). 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 and red most intense) was generated and superimposed by the same manufacturer. A pseudocolor image representing light intensity over the gray-scale reference image. Animals were imaged after injections every other day, then twice weekly for 2 weeks, 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 the Baylor College of Medicine’s Center for Comparative Medicine guidelines.

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 were stained by a standard H&E technique. HER2 expression in medulloblastomas was detected by a validated immunohistochemistry using a phospho-HER2 antibody (Upstate Biotech) as previously described (3, 22, 23).

Statistical analysis. For cytokine production, cytotoxicity, and proliferation assays, results are expressed in mean ± SE were indicated. Data were analyzed using compared using paired t tests or, when appropriate, the Wilcoxon signed-rank test. P values <0.05 were considered statistically different. 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

Medulloblastoma cell lines express HER2 at low levels and their proliferation is not inhibited by trastuzumab. Up to 40% of medulloblastoma express HER2. We used FACS analysis to determine the level of HER2 expression on the medulloblastoma cell lines Daoy and D283 in comparison to breast cancer cell lines that express HER2 at high (SK-BR-3) or at low levels (MCF-7; Fig. 1A). Daoy and D283 expressed HER2 at a low level comparable to MCF-7 cells; in contrast, D341 medulloblastoma cells were HER2 negative. To determine if HER2 expression is present on the medulloblastoma progenitor cell population, CD133-positive cells were isolated with magnetic beads and analyzed for HER2 expression (Fig. 1B; ref. 24). The progenitor cell population expressed similar levels of HER2 as unsorted cells, validating HER2 as a potential therapeutic target on medulloblastoma. The proliferation of the HER2 low-expressing cell lines, like MCF-7, is not inhibited by trastuzumab (25). To see whether low HER2 expression on Daoy and D283 cells is also too low for trastuzumab to be effective, cell proliferation assays were done. Trastuzumab did not inhibit the proliferation of HER2-positive Daoy and D283 cells at the highest concentration tested (2,000 µg/mL), which is 25-fold higher than the mean steady-state therapeutic serum concentration in humans (Fig. 1C). In contrast, trastuzumab inhibited the proliferation of SK-BR-3 cells at a concentration of 3.1 µg/mL, whereas no inhibition was observed for the HER2-negative cell line MDA-MB-468.

T cells expressing HER2-specific CAR secrete immunostimulatory cytokines and proliferate after exposure to HER2-positive medulloblastoma cells. For the generation of HER2-specific T cells, a SFG retroviral vector was constructed encoding a HER2-specific CAR consisting of a HER2-specific single chain variable domain. This CAR was shown to specifically target HER2-positive cells. The progenitor cell population, CD133-positive cells were isolated with magnetic beads and analyzed for HER2 expression (Fig. 1D; ref. 24). The progenitor cell population expressed similar levels of HER2 as unsorted cells, validating HER2 as a potential therapeutic target on medulloblastoma. The proliferation of the HER2 low-expressing cell lines, like MCF-7, is not inhibited by trastuzumab (25). To see whether low HER2 expression on Daoy and D283 cells is also too low for trastuzumab to be effective, cell proliferation assays were done. Trastuzumab did not inhibit the proliferation of HER2-positive Daoy and D283 cells at the highest concentration tested (2,000 µg/mL), which is 25-fold higher than the mean steady-state therapeutic serum concentration in humans (Fig. 1C). In contrast, trastuzumab inhibited the proliferation of SK-BR-3 cells at a concentration of 3.1 µg/mL, whereas no inhibition was observed for the HER2-negative cell line MDA-MB-468.

Figure 3. HER2-specific T cells kill HER2-positive medulloblastoma cell lines. A, HER2-specific T cells (HER2-T cells) killed HER2-positive medulloblastoma cells, Daoy and D283, in a 4-h 51Cr release cytotoxicity assay in contrast to HER2-negative cell lines, D341 (medulloblastoma) and autologous LCL. B, only HER2-specific T cells killed Daoy cells; nontransduced T cells did not.
fragment (FRP5) and the transmembrane and signaling domains of the T-cell receptor ζ-chain (HER2ζ.CAR; ref. 8). Mitogen-activated T cells from five healthy donors were transduced with RD114-pseudotyped retroviral vectors, and 10 to 14 days post-transduction, the expression of HER2ζ.CAR was determined by FACS analysis. On average, 42% (SD 15%) of T cells expressed HER2ζ.CAR, and both CD4- and CD8-positive T cells were transduced (Fig. 2A and B). To investigate if HER2-specific T cells recognized HER2-positive medulloblastoma cells, T cells were stimulated with HER2-positive and HER2-negative medulloblastoma cells. HER2-specific T cells only produced IFN-γ after exposure to HER2-positive targets, indicating that IFN-γ production depends on the presence of HER2 antigen (Fig. 2C). In addition to IFN-γ, HER2-specific T cells produced IL-2 and proliferated in an IL-2-dependent manner (Fig. 2D). T-cell subset analysis revealed that CD4- as well as CD8-positive HER2-specific T cells produced IFN-γ and IL-2 and proliferated after stimulation with Daoy cells (Supplementary Data Fig. S1).

**HER2-specific T cells kill HER2-positive medulloblastoma cells.** We next showed that HER2-specific T cells could kill HER2-positive medulloblastoma cell lines (Daoy and D283) in a standard 4-h 51Cr release assay. HER2-negative cells (D341; autologous lymphoblastoid cells) were not killed (Fig. 3A). In addition, nontransduced T cells did not kill Daoy cells, indicating that the killing of HER2-positive medulloblastoma cells depended on the expression on HER2ζ.CAR (Fig. 3B).

**HER2-specific T cells are activated after coculture with primary medulloblastoma cells.** Having shown that HER2-specific T cells recognize and kill HER2-positive medulloblastoma cell lines, we next validated HER2 as a target for T-cell therapies on primary medulloblastoma tumor samples. HER2-specific T cells were incubated with primary tumor cells from 10 patients, and after 24 to 48 h, IFN-γ production was determined. A total of 5 out of 10 tumor samples induced T-cell activation as judged by IFN-γ production (36–272 pg/mL), indicative of the presence of HER2 antigen (Fig. 4A). In contrast, nontransduced T cells produced only low levels of IFN-γ. To confirm the presence of HER2, paraffin-embedded sections of the 10 tumors were stained for phosphorylated HER2 (brown stain). Representative sections are shown of HER2-positive (patients 2, 3, 4, 7, 10) and HER2-negative (patients 1, 5, 6, 8, 9) tumors; magnification, ×400.

**HER2-specific T cells from patients with HER2-positive medulloblastoma recognize and kill autologous primary tumor cells.** HER2-specific T cells from healthy donors recognize and kill HER2-positive medulloblastoma cell lines as well as primary tumor cells. To exclude the possibility that a nonspecific, allogeneic component contributed to the observed HER2-specific T-cell activation, we generated HER2-specific T cells from two patients with HER2-positive tumors. HER2ζ.CAR expression was detected 7 to 10 days post-transduction in 31.5% of T cells from patient 3 and in 14.6% from patient 4, respectively. HER2-specific T cells recognized autologous tumor cells as judged by IFN-γ secretion (Fig. 5A). In addition, HER2-specific T cells killed primary autologous HER2-positive medulloblastoma cells, whereas autologous OKT3 blasts that do not express HER2 were not killed (Fig. 5B). Nontransduced patients’ T cells failed to exhibit a cytolytic function against HER-positive tumor cells (data not shown).
shown). As expected, patients’ HER2-specific T cells also killed HER2-positive medulloblastoma cell lines, whereas HER2-negative cell lines were not killed. These results indicate that, as in the allogeneic setting, HER2-specific T cells recognize and kill autologous HER2-positive medulloblastoma cells in a HER2-dependent fashion.

Regression of established medulloblastomas after adoptive transfer of HER2-specific T cells in an orthotopic xenogenic SCID model. Having established that HER2 expressed on medulloblastomas is a target for HER2-specific T cells ex vivo, we determined the in vivo antitumor activity of HER2-specific T cells in an orthotopic, xenogenic SCID mouse model. To allow serial imaging, Daoy cells were transduced with a retroviral vector encoding the firefly luciferase and puromycin-resistance genes (Daoy.Luc) and selected with puromycin. Luciferase expression was confirmed ex vivo. A total of $2.5 \times 10^5$ Daoy.Luc cells were injected into the forebrain of SCID mice, and after 5 to 7 days, the tumors of one group of mice were injected with $2 \times 10^6$ HER2-specific T cells ($n = 12$). Tumors of a second group of mice ($n = 10$) were not treated. In the untreated mice, the tumors grew exponentially as judged by bioluminescence imaging (Fig. 6A and B). In contrast, photon emission returned to baseline in all 12 mice after HER2-specific T cell injection, indicating tumor regression. Tumor regression was confirmed by histologic examination on a subset of animals (data not shown). To exclude nonspecific antitumor T cell activity, $2 \times 10^6$ nontransduced T cells were injected into tumor-bearing mice ($n = 5$). Injected tumors continued to grow at the same rate as untreated tumors as judged by bioluminescence imaging, indicating that the antitumor activity of injected T cells depends on the expression of HER2.CAR (Fig. 6B).

Treated and untreated mice were followed with bioluminescence imaging to determine tumor progression and long-term survival. Tumors continued to grow in untreated mice and in mice injected with nontransduced T cells. Mice from each control group had a mean survival of 67 ± 10 and 62 ± 8 days, respectively (Fig. 6C). Tumors injected with HER2-specific T cells remained undetectable for up to 55 days. Although they eventually recurred in all animals, tumor-bearing mice injected with HER2-specific T cells had a mean survival of 115 ± 5 days, which was significantly higher than for the two control groups ($P < 0.001$).

Discussion

HER2 is a validated target for cancer therapy; however, the majority of anti-HER2 targeted therapies are restricted to cancer cells that express HER2 at high levels. This excludes a large group of malignancies expressing HER2 at low levels, including several pediatric malignancies such as medulloblastoma. We show here that HER2-specific T cells, unlike HER2 antibody, can effectively inhibit the growth of and kill medulloblastoma cell lines and primary tumor cells ex vivo and have potent antitumor activity in an orthotopic, xenogenic SCID mouse model. These effector cells can be generated from medulloblastoma patients themselves. The biological function of HER2 has been extensively studied in breast cancers, and the presence of HER2 is associated with a significantly worse clinical outcome (26). HER2 signaling deregulates cell proliferation, inhibits apoptosis, and increases the metastatic potential of breast cancer cells (27). HER2 expression in medulloblastoma has been studied in more than 300 tumor samples, and expression has been documented by immunohistochemistry or Western blotting in ~40% of tumor samples (22, 23).
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In contrast, HER2 expression was not detected in normal cerebellum and other brain tissue (28). As in breast cancer, HER2 expression in medulloblastoma has been identified as a poor prognostic indicator, and aberrant HER2 signaling has been implicated in the pathogenesis and the metastatic potential of medulloblastoma (3, 29). In addition, we show here that HER2 is expressed on the CD133-positive medulloblastoma progenitor cell population. These findings suggest that HER2 is a promising candidate for targeted therapies for medulloblastoma.

Trastuzumab is only efficacious in cancer cells that express HER2 at high levels (30, 31). For example, MCF-7 cells express HER2 at low levels, and their growth is not inhibited by trastuzumab, whereas HER218 cells, a high HER2 expression derive of MCF-7, are inhibited by this antibody (25). In agreement with these results, we show here that the proliferation of HER2 low-expressing medulloblastoma cell lines (Daoy and D283) is not inhibited by trastuzumab. This result can be either explained by low trastuzumab binding or HER2-independent proliferation of medulloblastoma cells. Indeed, Calabrese et al. (32) recently showed that HER2 signaling in Daoy cells does not impact directly cell proliferation, apoptosis, or self-renewal.

T cells have the ability to detect antigens expressed at very low levels. In contrast to the lengthy process of generating conventional tumor antigen-specific T cells ex vivo whose frequencies are often <1 per 1,000 T cells, CAR-expressing T cells can be prepared readily in large quantities by mitogen activation of all T cells (10). Because CARs recognize antigens in an MHC unrestricted manner, they are immune to some of the major mechanisms by which tumors avoid MHC-restricted T-cell recognition, such as down-regulation of HLA class I molecules and defects in antigen processing (33). Here, we show that T cells expressing HER2-specific CARs with a ζ-signaling domain recognize and kill HER2-positive medulloblastoma cell lines and primary tumor samples in the allogeneic and autologous setting. HER2-specific T cells secreted not only IFN-γ but also IL-2 upon stimulation with Daoy cells. Although this may suggest costimulation by Daoy cells, these tumors cells are negative for the costimulatory molecules CD80, CD83, CD86, CD134, and CD137 as judged by FACS analysis (data not shown). Instead, we attribute costimulation by Daoy cells, these tumors cells are negative for the costimulatory molecules CD80, CD83, CD86, CD134, and CD137 as judged by FACS analysis (data not shown). Instead, we attribute this result to the presence of HER2 antigen, which was confirmed by immunohistochemistry for all five samples.

Direct injection of T cells into the tumor site is a potential treatment strategy for patients with incompletely resected or unresectable brain tumors. Local administration of autologous tumor-specific T cells into malignant gliomas in patients was safe and resulted in transient clinical responses in 5 out of 10 patients (36). The systemic delivery of brain tumor–specific T cells has also been reported in patients (37). However, at present, it is unclear how efficiently T cells home to tumor sites within the brain or whether the disruption of the blood-brain barrier is required (38). We therefore injected T cells directly into medulloblastomas in tumor-bearing mice. Tumors regressed in all animals treated with HER2-specific T cells, without the administration of exogenous cytokines, whereas untreated tumors and tumors treated with nontransduced T cells continued to grow. However, tumors recurred in all animals treated with HER2-specific T cells. Kahlon et al. (34) reported complete regression and no recurrence of U87 gliomas in an orthotopic, xenogenic SCID model after intratumoral...
Injection of T cell expressing a glioma-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. Medulloblastoma recurrence in our model may be due to nonpersistence of human HER2-specific T cells, a common problem of SCID mouse models. Several groups have reported enhanced proliferation and IL-2 secretion of T cells expressing CARs with additional signaling domains derived from costimulatory molecules such as CD28, CD134, and CD137 (39–41). The evaluation of “enhanced” CARs in our medulloblastoma model is currently in progress.

In summary, this study shows for the first time that HER2 is a target antigen for the immunotherapy of medulloblastoma. HER2-specific T cells not only recognized and killed HER2-positive medulloblastomas ex vivo, but also induced regression of experimental medulloblastomas in vivo. Hence, adoptive transfer of HER2-directed T-cells may represent a promising immunotherapeutic approach for medulloblastoma.

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References
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