Cancer Therapy: Preclinical

Immunotherapy of Metastatic Melanoma Using Genetically Engineered GD2-Specific T cells
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Abstract

Purpose: Genetic engineering of human T lymphocytes to express tumor-directed chimeric antigen receptors (CAR) can produce antitumor effector cells that bypass tumor immune escape mechanisms that are due to abnormalities in protein-antigen processing and presentation. Moreover, these transgenic receptors can be directed to tumor-associated antigens that are not protein-derived, such as the ganglioside GD2, which is expressed in a high proportion of melanoma cells.

Experimental Design: We generated chimeric T cells specific for the ganglioside GD2 by joining an extracellular antigen-binding domain derived from the GD2-specific antibody sc14.G2a to cytoplasmic signaling domains derived from the T-cell receptor ζ-chain, with the endodomains of the costimulatory molecules CD28 and OX40. We expressed this CAR in human T cells and assessed the targeting of GD2-positive melanoma tumors in vitro and in a murine xenograft.

Results: Upon coincubation with GD2-expressing melanoma cells, CAR-GD2 T lymphocytes incorporating the CD28 and OX40 endodomains secreted significant levels of cytokines in a pattern comparable with the cytokine response obtained by engagement of the native CD3 receptor. These CAR-T cells had antimelanoma activity in vitro and in our xenograft model, increasing the survival of tumor-bearing animals.

Conclusion: Redirecting human T lymphocytes to the tumor-associated ganglioside GD2 generates effector cells with antimelanoma activity that should be testable in subjects with disease. (Clin Cancer Res 2009;15(18):5852–60)

The rising incidence of cutaneous melanoma and the failure to significantly improve outcomes in metastatic disease have led to increasing interest in immunotherapeutic approaches, because these can be remarkably effective. Several investigators have focused on targeting tumor-associated antigens that fall into the cancer testis antigen group, including MAGE, BAGE, GAGE, and NY-ESO-1, or the melanocyte differentiation protein group, including gp100, Melan-A/MART-1, and tyrosinase, which are widely present on melanoma cells. These studies have used cytotoxic T cell lines (4, 5), clones with native (6) or transgenic αβ T cell receptors (7) specific for cancer testis antigen–derived peptides that are recognized in association with human leukocyte antigen (HLA) class I antigens on the tumor cell surface. It is clear, however, that the heterogeneity of protein antigen expression and presentation in melanoma is a characteristic that helps limit the proportion of patients who are able to respond to such targeted strategies (8). One means of increasing the effectiveness of targeted T cell therapy of melanoma, therefore, may be to use artificial chimeric receptors derived, for example, from the antigen binding domain of a monoclonal antibody (9). When coupled to appropriate intracellular signaling domains, T cells expressing these chimeric antigen receptors (CAR) can kill tumor cell targets (10). They have the advantage of acting in a MHC unrestricted manner,
Translational Relevance

T lymphocytes expressing chimeric antigen receptors directed against GD2 can recognize and kill primary melanoma cells, in vitro and in vivo. Administration of such cells to patients with metastatic melanoma may therefore produce benefits.

Materials and Methods

Establishment of cell lines. After informed consent, tumor biopsies (from metastatic skin lesions) were obtained from five patients with stage III or IV melanoma. The tumor tissue was minced and the fragments resuspended in 30 mL of digestion medium containing DNAse at 30 U/mL, hyaluronidase at 0.1 mg/mL, and collagenase at 1 mg/mL. The dissociation was performed in a shaking water bath at 37°C. The resultant cell suspension was filtered through a 70 μm cell strainer, washed in complete T-cell medium containing 45% RPMI and 5% FCS, and then cultured at 37°C in 5% CO2. Culture medium was reduced to 100 IU/mL penicillin and 100 mg/mL streptomycin, and 1% FCS after 48 h incubation.

Cytotoxicity assays. The cytotoxic activity of the nontransduced and CAR-GD2 T lymphocytes was evaluated in a standard 51Cr release assay, as previously described (18, 22). We evaluated isotope release at 6 and 18 h after contact with effector-to-target (E:T) ratios of 40:1, 20:1, 10:1, and 5:1, using a gamma counter (Perkin-Elmer).

Coculture experiments. Seven days after transduction, nontransduced and CAR-GD2 cells were collected, counted, and plated at 5 × 103 cells/well in a 24-well plate at 20:1 ratio with eGFP-expressing (>98%) tumor cells. Tumor cell cytolysis was measured using the CBA array (BD Bioscience) and the percent of CD3-positive and CD8-positive melanoma cells allowed to target tumor cells in which antigen processing or presentation pathways are disrupted. Moreover, they can be directed to nonpeptide antigens on the cell surface, broadening the range of target structures that can be recognized on malignant cells. Hence, CAR-expressing T cells could complement MHC restricted cytotoxic T cells, and increase the overall effectiveness of this cellular immunotherapy.

Many melanoma cells express a range of gangliosides, including GD2, GM2, GM3, and GD3, that may be a good choice for target for CAR-T cells, because their expression is highly tissue-restricted (11, 12). Although these carbohydrate antigens are expressed by both normal melanocytes and melanoma cells, expression is significantly up-regulated after malignant transformation of melanocytes (13, 14), and is associated with changes in the proliferation, migration, and metastatic potential of the tumor cells (15). Moreover, natural or vaccine-induced antibodies to gangliosides in melanoma patients have been correlated with improved disease relapse-free survival (16, 17).

We now show that the overexpression of GD2 by human primary melanoma cells allows these cells to be targeted in vitro and in vivo by GD2 CAR-expressing primary T cells, and that incorporation of endodomains from both CD28 and OX40 molecules (18) mediates costimulation of the T lymphocytes, inducing T cell activation, proliferation, and cytotoxicity against GD2-positive melanoma cells. The 4405M, CLB, SENMA, and P1143 melanoma cell lines were transfected with retroviral vectors encoding either eGFP or FF-Luc. We plated 1 × 105 tumor cells in 1 well of a 6-well plate and the cells were grown to 60% to 70% confluency. Culture medium was replaced by the appropriate retroviral supernatant (1.5 mL/well) and 1 μg of polybrene was added. When the tumor cells reached confluency, they were trypsinized and plated in a T25 flask. The FF-Luc–transduced cells were then selected with puromycin (Sigma-Aldrich) at 1 μg/mL. The eGFP-transduced tumor cell lines did not require selection as >98% of the cells were eGFP-positive postretroviral transduction.

Flow cytometry. FITC-, phycoerythrin (PE), or peridinin chlorophyll protein (perCP)-conjugated anti-CD4, -CD8, -CD80 and -CD86 monoclonal antibodies (all from Becton-Dickinson) were used to label lymphocytes, whereas anti-MCSF-Pe (Milenyi-Biotech Inc.) and a purified anti-GD2 monoclonal antibody (Becton-Dickinson Pharmingen) were used to stain the melanoma cells. We added a secondary antibody (RAM-IgG2a-b-PE; Becton-Dickinson) to detect the anti-GD2 (IgG2a) antibody by indirect immunofluorescence. CAR expression by transduced T lymphocytes was detected using a monoclonal anti-idiotypic, 1A7 (TriGem, Titan), followed by staining with the secondary antibody RAM-IgG1-PE (Becton-Dickinson; ref. 23). The proliferation of nontransduced and transduced T cells, in the presence or absence of tumor cells, was evaluated by FACs analysis after labeling T cells with CFSE (Invitrogen) according to the manufacturer’s instructions.

K562 cell line was obtained from the American Type Culture Collection. All cell lines were maintained in RPMI (Hyclone) supplemented with 10% heat inactivated FCS, 100 IU/mL penicillin, 100 mg/mL streptomycin, 1 mmol/L sodium pyruvate (Invitrogen), and 2 mmol/L GlutaMAX. Six established melanoma cell lines, isolated from surgical specimens at Istituto Nazionale Tumori, Milan, were also used to screen GD2 expression.

Mononuclear cells. Peripheral blood, obtained after informed consent from normal donors, was processed over Ficoll gradients, and the resulting peripheral blood mononuclear cells (PBMC) were cultured in complete T-cell medium containing 15% RPMI and 15% Clik’s medium supplemented with 10% heat inactivated FCS, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L GlutaMAX.

Retroviral constructs. The 14g2a scFv sequence was cloned in the SFG retroviral backbone in frame with the human IgG1-CH2CH3 domain, followed by the CD28 and OX40 endodomains and the ζ-chain of the T-cell receptor/CD3 complex, to form the 14g2a-CD28-OX40-ζ (CAR-GD2) construct as previously described (18). We also used vectors encoding the Firefly Luciferase gene (FF-Luc-) or the eGFP protein to track cell survival and proliferation in vivo, as previously described (21). The RD114 retrovirus envelope (RDF plasmid) and the MoMLV gag-pol (PegPam3-e plasmid) were used to engineer the retroviral vectors.

Recommandation of production and transduction. Transient retroviral supernatants were produced by cotransfection of 293T cells with the PegPam-e, RDF, and the desired SFG vectors (CAR-GD2, eGFP, or FF-Luc) using the Fugene6 transfection reagent (Roche), and used to transduce OKT3 (Ortho Biotech) activated PBMCs, as previously described (22).

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Xenogeneic SCID mouse model of melanoma. To assess the in vivo antitumor activity of the CAR-GD2 T lymphocytes, we used a SCID mouse model and the P1143 or 4405 M melanoma line expressing FF-Luciferase. SCID mice (8 to 9 weeks old) were sublethally irradiated (250 rad) and injected i.v. with $2 \times 10^6$ tumor cells. Tumor cell engraftment was monitored using the IVIS 100 imaging system (Caliper Life-sciences), and on days 4 and 21, $1 \times 10^7$ nontransduced or CAR-GD2 T lymphocytes were injected i.v. The animals were imaged weekly to evaluate tumor growth, and photon emission from luciferase-expressing cells was quantified using the “Living Image” software provided with the IVIS system (Caliper Life-sciences). Briefly, after drawing a region of interest over the tumor region, the intensity of the signal measured was expressed as total photons/s/cm² (p/s/cm²/sr).

Statistical analysis. For cytotoxicity and cytokine production, results were presented as mean ± SD and paired Student’s t test was used to determine statistical significance. For the bioluminescence results, the signal intensity was 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. $P < 0.05$ was considered statistically significant.

Results

Expression of GD2 by primary melanoma cells. To confirm that GD2 would be a potential target for CAR-directed T cell therapy, we dissociated primary melanoma cells from five patients after biopsy of cutaneous metastatic melanoma and used an additional six established cell lines in our study. Cells from the five patients and four of the established lines expressed GD2 on immunofluorescence staining, and between 17% and 95% of the cells were positive, with variable intensity of expression (Fig. 1). We also measured expression of GD2 on a normal skin fibroblast cell line and confirmed it did not express the ganglioside. To confirm the absence of nonneoplastic cells in the primary culture, we looked at the expression of MCSP on the cells (Supplementary Table S1), and to compare the frequency of expression of GD2 with that of other known melanoma tumor associated antigens, we also measured expression of gp100, MART1, and MAGE-1. The percentage range of GD2-positive cells was comparable with the range of malignant cells expressing these three other melanoma-associated antigen cells (Supplementary Table S1). All cell populations studied were negative for expression of the costimulatory molecules CD80 or CD86 (data not shown).

T cells expressing a GD2-specific chimeric receptor kill GD2-positive melanoma cell lines. T cells from four healthy donors were transduced with a vector encoding the 14g2a single-chain antibody linked to $\zeta$ and to the endodomains of the two costimulatory molecules CD28 and OX40, which enhance the activation, proliferation, and cytotoxicity of T cells produced by the CAR after antigen engagement (18). Five days after transduction, we measured the expression of GD2-specific CAR by flow cytometry using the anti-14g2a idiotypic antibody 1A7 and found that 95% of cells transduced with the 14g2a-CD28-OX40-ζ retroviral vector were CAR positive (range, 93-97%; Fig. 2A). The CAR-GD2 construct transduced CD4-positive and CD8-positive T cell populations with equal efficiency (Fig. 2B).

To mimic the range of GD2 expression seen on primary melanoma cells, we measured the ability of CAR-GD2 T cells to kill...
three melanoma cell lines with different GD2 expression. P1143 was a high expressor [95% positive, mean fluorescence intensity (MFI) = 838]; SENMA was intermediate (45% positive, MFI = 884); CLB was low (19% positive, MFI = 182), and finally, the melanoma cell line 4405 M was used as a GD2-negative tumor cell control. At 6 hours and 18 hours, 51Cr release assays showed that the antitumor activity was proportional to the level of GD2 antigen expression (Fig. 3). As anticipated, the CAR-GD2 T cells had little activity against the GD2-negative tumor cell line (4405 M), the GD2-NK-cell target line K562 (data not shown), or against normal skin fibroblasts or PBMCs that are also GD2 negative (Supplementary Fig. S1). CAR-GD2 T cells were, however, able to kill a mesenchymal stem cell line positive for GD2 (95% positive, MFI = 799; Supplementary Fig. S1). Of note, such cross-reactivity with GD2-positive normal mesenchymal stem cells has not produced discernible adverse effects in any clinical trial of GD2 monoclonal antibodies in patients with neuroectodermal tumors (24, 25) or in a phase I study of CAR-GD2 T cells in patients with neuroblastoma (26).

Fig. 2. T lymphocytes can be genetically modified to express CARs targeting GD2. Activated T lymphocytes were genetically modified to express CAR-GD2. A, expression of CAR-GD2 as assessed by FACS analysis using a specific 14,2a anti-idiotype antibody (1A7). Graph is a representative expression of CAR-GD2 from four different transduced T cell lines. B, both CD4-positive and CD8-positive T lymphocytes expressed the CAR-GD2 after gene transfer.
CAR-GD2–expressing T cells secrete cytokines upon stimulation with GD2-expressing melanoma cells. Functional activation of CAR-GD2–expressing T cells following their exposure to GD2-positive melanoma cells was measured by cytokine release assay. As described above, GD2-positive target cells were killed by T cells expressing the CAR, and significant interleukin 2 (IL-2), IL-5, IFN-γ, and tumor necrosis factor α (TNF-α) release occurred during coculture with the three melanoma lines. As Fig. 4 shows, the quantity of IL-2, IL-5, IFN-γ, and TNF-α secreted by the CAR-GD2 T cells after 24 hours of culture correlated with the level of GD2 expression on the target cells, and was highest for P1143 and lowest for CLB. Neither IL-4 nor IL-10 was detected in the supernatants of stimulated cells.

CAR-GD2 induces sustained killing and clonal expansion in coculture experiments. We next determined whether the killing and cytokine release mediated by CAR-GD2 T cells could lead to CAR-T cell proliferation and tumor cell eradication in vitro in a 5-day coculture experiment. We used CFSE-labeled control or CAR-GD2 T cells, to determine whether CAR stimulation by CAR-GD2–expressing T cells induces effector T cell proliferation. Nontransduced T cells proliferated only in the presence of exogenous IL-2 (100 U/mL), whereas proliferation of CAR-expressing T cells increased in response to all three GD2-expressing tumor cell lines, irrespective of whether these tumor cells expressed high, intermediate, or low levels of GD2 (Supplementary Fig. S2). To discover if these expanded CAR-GD2 T cells were functional, we labeled tumor cells with eGFP and cocultured them at the CAR-T cell:tumor cell ratio of 20:1 in the absence of IL-2. After 5 days of culture, we enumerated viable GFP-positive cells by flow cytometric analysis. Figure 5 shows that viable tumor cells were eradicated in cocultures with T cells expressing CAR-GD2 but not in cocultures with nontransduced T cells. Hence CAR-GD2 T cells proliferate in vitro in response to the GD2 antigen and eradicate melanoma cells that express the antigen. As expected, the GD2-negative cell line 4405 M was not killed in the 5-day coculture experiment, showing GD2 antigen recognition is essential for the activity of CAR-GD2–expressing T cells.

Adoptive transfer of GD2-specific T cells provide antitumor effect in a xenogeneic SCID model. We next measured the antitumor activity of CAR-GD2 T cells in vivo. To monitor tumor cells in vivo, we expressed the firefly luciferase (FFLuc) gene in 4405 M and P1143 cells, together with the puromycin-resistance gene. After puromycin selection, we injected $2 \times 10^6$ FFLuc-P1143 or 4405 M tumor cells i.v. into SCID mice. After 4 days, FFLuc expression was evaluated by bioluminescence imaging and the mice were divided into three groups which received nontransduced T cells or T cells expressing CAR-GD2 at $1 \times 10^7$ i.v. and finally a group that received tumor cells alone. A second injection of nontransduced or CAR-GD2 T cells was given at day 21 and we measured luciferase signal every week in the 10 mice of each of the groups. Figure 6A shows five representative mice from the nontransduced and CAR-GD2 T cells group, and shows that tumor grew rapidly in the lungs of mice receiving nontransduced T cells. By contrast, the tumors in mice receiving T cells expressing CAR-GD2 diminished within 48 to 72 hours of injection, and luciferase
derived remained largely absent in the group receiving CAR-GD2 T cells. Although the survival of the mice receiving the tumor cells alone or tumor cells plus nontransduced T cells was 68 ± 6 days and 72 ± 12 days, respectively ($P = 0.03$), 80% of the mice from the group receiving CAR-GD2 T cells were still alive at day 100 and showed a significant survival advantage when receiving CAR-GD2-specific T cells ($P = 0.006$; Fig. 6B). Finally, we observed no tumor regression when CAR-GD2 T cells were infused in mice bearing GD2-4405 M tumor cells (Fig. 6A).

Discussion

We have shown that the ganglioside antigen GD2 is expressed on the majority of primary melanoma cell lines, and that T cells engineered to express a CAR directed to this antigen are able to recognize and lyse GD2-positive melanoma target cells in vitro and in a SCID mouse model in vivo. The transgenic receptor construct included the signaling endodomains of the CD28 and OX40 costimulatory molecules, and redirected T cells showed activation, proliferation, and cytokine release after T-cell receptor engagement by GD2.

Melanoma has long been a target of cellular immunotherapies directed to the tumor-associated antigens expressed by the malignant cells. Although earlier clinical research focused on reinfusion of expanded tumor infiltrating lymphocytes, efforts have recently been directed against the cancer testis series of antigens such as MAGE and the melanocyte differentiation proteins such as MART-1, by generation of T cells expressing conventional αβ-T-cell receptors specific for these antigens. These receptors recognize peptide fragments in association with MHC class I molecules, and are therefore restricted in their patient range to individuals with the appropriate MHC polymorphism. Moreover, they are unable to recognize tumor subclones in which the antigen processing machinery is deficient. T cells that express synthetic or chimeric receptors that recognize unprocessed structures on the cell surface may thus have an advantage over T cells whose tumor reactivity is mediated through their native receptor.

It has been known for some time that the ganglioside GD2 is expressed by tumors derived from neuroectoderm, including neuroblastoma, sarcoma, and small lung cancer. This tumor-associated carbohydrate antigen is also expressed by many melanoma cells (27), in which it is involved in cell adhesion and may contribute to metastasis (28). Although GD2 is present on the surface of many melanoma cells, it is absent on most normal tissue, with only limited expression in brain and on peripheral nerves, making this ganglioside an attractive target for adoptive cell therapy in metastatic melanoma (29).

GD2 monoclonal antibodies have already been used with benefit in patients with other GD2-positive malignancies, such
as neuroblastoma, but melanoma cells have more variable (and usually lower) expression of the antigen. Hence, the benefits of GD2 antibody infusion in melanoma have been limited (24, 25). The level of GD2 expression on melanoma cells, however, is evidently sufficient to produce a cytotoxic response from T cells expressing the same monoclonal antibody binding site in the form of a chimeric T-cell receptor. We saw complete killing of the tumor cells even when GD2 expression was low, consistent with previous observations that even tumor cells with dim expression of the targeted antigen can be eliminated by CAR-modified T cells (22). The killing of cells that are resistant to antibodies of the same specificity may be related to the improved avidity of multiple antibody-derived binding domains when they are arrayed on a cell surface rather than existing as bivalent molecules in solution, or it may reflect a superior cytolytic activity of T effector cells compared with antibody (30). Hence, tumor cells with dim antigen expression can be completely eliminated after coculture experiments, even when short term assays based on 51Cr release assay may produce lower intermediate cytotoxicity than tumor cells with high antigen expression. Like most malignancies, melanoma cells lack expression of the T-cell costimulatory molecules required for complete activation of T lymphocytes that engage tumor-associated antigens through their native or chimeric receptors. Hence, to optimize T cell triggering and effector function, we coupled the chimeric receptor to costimulatory endodomains to increase T cell survival and expansion. Following CAR engagement, endodomains from single costimulatory molecules, such as CD28, 4-1BB, or OX40, into the CAR may be sufficient to activate the cellular components of the killing machinery and to produce IL-2 release and T cell proliferation (31, 32). We have previously shown, however, that the simultaneous expression in cis of two endodomains such as CD28 and OX40 within a GD2-CAR produces superior T cell proliferation and effector function than expression of a single costimulatory endodomain (18). This benefit likely occurs because both CD28 and OX40 signals are both functional, and produce greater activation of NF-κB than either endodomain alone, because they act through two independent pathways (18).

If adoptive transfer of CAR-modified T cells in melanoma is to be of clinical value, it will be essential to be able to treat metastatic disease. Accordingly, we studied the effects of the CAR-GD2 T cells in a xenograft lung metastatic model. Human T cells expressing the 14g2a-CD28-OX40-ζ CAR produced significant...
antitumor activity in this model, but were unable to completely eradicate the disease. This incomplete benefit may reflect the difficulties of sustaining human T cell function and trafficking in a xenogeneic environment, or it may also represent the limitations of even the combination of CD28 and OX40 endodomains, which on their own cannot completely recapitulate the temporospatial features of the costimulatory events required to sustain T cell activation physiologically (26,33).

In summary, we have shown that GD2 on melanoma cells is a potential target for CAR-T cells. We believe that administration of such cells may usefully complement other cellular immunotherapies and biotherapies for this disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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