A High Molecular Weight Melanoma-Associated Antigen–Specific Chimeric Antigen Receptor Redirects Lymphocytes to Target Human Melanomas

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Abstract

Immunotherapy, particularly the adoptive cell transfer (ACT) of tumor-infiltrating lymphocytes (TIL), is a very promising therapy for metastatic melanoma. Some patients unable to receive TIL have been successfully treated with autologous peripheral blood lymphocytes (PBL), genetically modified to express human leukocyte antigen (HLA) class I antigen–restricted, melanoma antigen–reactive T-cell receptors; however, substantial numbers of patients remain ineligible due to the lack of expression of the restricting HLA class I allele. We sought to overcome this limitation by designing a non–MHC-restricted, chimeric antigen receptor (CAR) targeting the high molecular weight melanoma-associated antigen (HMW-MAA), which is highly expressed on more than 90% of human melanomas but has a restricted distribution in normal tissues. HMW-MAA–specific CARs containing an antigen recognition domain based on variations of the HMW-MAA–specific monoclonal antibody 225.28S and a T-cell activation domain based on combinations of CD28, 4-1BB, and CD3ζ activation motifs were constructed within a retroviral vector to allow stable gene transfer into cells and their progeny. Following optimization of the HMW-MAA–specific CAR for expression and function in human PBL, these gene-modified T cells secreted cytokines, were cytolytic, and proliferated in response to HMW-MAA–expressing cell lines. Furthermore, the receptor functioned in both CD4+ and CD8+ cells, was non–MHC restricted, and reacted against explanted human melanomas. To evaluate this HMW-MAA–specific CAR in patients with metastatic melanoma, we developed a clinical-grade retroviral packaging line. This may represent a novel means to treat the majority of patients with advanced melanoma, most notably those unable to receive current ACT therapies.

Introduction

More than half of patients treated in protocols combining lymphodepletion and adoptive cell transfer (ACT) of autologous tumor-infiltrating lymphocytes (TIL) experienced objective tumor regression (1, 2). This potent melanoma therapy, however, has been limited by the requisite surgery to procure tumor-reactive TIL and by ex vivo identification and expansion of these cells. Therefore, an alternative strategy to insert tumor antigen–reactive T-cell receptor (TCR) genes into peripheral blood lymphocytes (PBL) was investigated.

TCR gene–modified T cells are capable of activation, cytokine secretion, and targeted lysis (3, 4). We reported the first clinical trials using autologous PBL modified to express a tumor antigen–reactive TCR in the treatment of patients with metastatic cancer that resulted in objective tumor regression (5, 6). These strategies, however, remain less effective than TIL, suggesting that further modifications are needed. Furthermore, only a minority of patients with advanced melanoma are eligible for current protocols, as they must express human leukocyte antigen (HLA)-Aα0201 and have tumors that express a common melanoma-associated antigen (MAA).

Unlike a conventional TCR, a chimeric antigen receptor (CAR) is capable of relaying excitatory signals to T cells in a non–MHC-restricted manner. These hybrid proteins, composed of an extracellular antigen recognition domain fused to an intracellular T-cell activation domain (7), may therefore be used in patients regardless of their HLA genotype. The non–HLA-restricted antigen recognition is achieved by harnessing the antigen-binding properties of monoclonal antibodies (mAb); this recognition is also independent of antigen processing, thus bypassing a potential mechanism by which tumor cells can evade the immune system in vivo (8). Several clinical trials using CAR-transduced T cells have been reported (9–12).
High molecular weight MAA (HMW-MAA; gene name CSPG4) is a cell-surface proteoglycan expressed on more than 90% of melanosomes. It is postulated that HMW-MAA contributes to the malignant phenotype of melanoma cells via enhancement of their spreading, invasion, and migration (13). The expression of HMW-MAA in adult tissues is restricted to hair follicles, basal cells of the epidermis, endothelial cells, and activated pericytes. Given its expression on the majority of melanoma lesions with limited inter- and intralesional heterogeneity and its restricted expression on normal tissues, HMW-MAA represents a clinically attractive target for CAR-based immunotherapy.

In this study, therefore, we developed a non–MHC-restricted CAR to target HMW-MAA. We next optimized this HMW-MAA–specific CAR for robust expression and function in human PBL. Finally, we generated a clinical-grade retroviral product for use in new ACT protocols for patients with metastatic melanoma. This mAb detected HMW-MAA in 59 of 59 melanoma specimens (14) and stained only the hair bulb and isolated basal cells in a panel of normal adult and fetal tissues (16). Further, radiolabeled forms of this mAb have safely imaged melanoma tumor deposits in more than 1,000 patients (13). Therefore, we constructed four 225.28S-based scFv cassettesthat differed by the order of the light-chain variable region (Vλ) and heavy-chain variable region (VH) and by one of two flexible linker peptides, GSTSGSKPGSSEQS (linker 218) or GGGSGGGGGGSGGGGS (linker G48). These scFv cassettes were cloned into an MSGV1-based retroviral vector containing the CD28 and CD3ζ signaling domains (28z) to generate four HMW-MAA–specific CARs (Supplementary Fig. S1). These four CARs were detected on the surface of transduced PBL and conferred reactivity against HMW-MAA–expressing tumors; the scFv with the Vλ-218-VH design (L2H) showed superior expression and function (Supplementary Table S2).

Next, we sought to optimize the intracellular T-cell activation domain while maintaining the L2H scFv cassette. Based on emerging evidence that 4-1BB (CD137) costimulation augments CAR function (15), two modifications that included the 4-1BB signaling motif were evaluated (Supplementary Fig. S3). As detailed in Supplementary Table S4, an initial screen of the three constructs identified superior transgene expression with L2H-28z and optimal function with the L2H-28z and L2H-CD28BBz constructs.

Function of HMW-MAA–specific CAR. A comprehensive comparison between the L2H-28z and L2H-CD28BBz HMW-MAA–specific CARs was done. Again, L2H-28z provided superior gene transfer in both percent and level of expression (Fig. 2A). PBL transduced with either CAR secreted cytokines when cocultured with a panel of HMW-MAA–expressing melanoma lines, but not those lacking HMW-MAA expression (Fig. 2B). Although qualitatively similar, L2H-28z–transduced PBL displayed modest enhancement in IFN-γ and tumor necrosis factor-α secretion, compared with L2H-CD28BBz–transduced PBL. The L2H-28z construct showed slightly greater cytolytic function (Fig. 2C) and proliferation (Fig. 2D) in response to melanoma targets than the L2H-CD28BBz construct. In contrast to our experience with an ErbB2-specific CAR (15), addition of the 4-1BB costimulatory motif did not improve any of the measured in vitro functions of this HMW-MAA–specific CAR. Inactivation of the CD28 internalization motif also failed to enhance expression or function (Supplementary Fig. S5). Therefore,
the L2H-28z format was selected as the optimal HMW-MAA–specific CAR. These data clearly show the ability of this HMW-MAA–specific CAR to redirect T cells to recognize melanoma lines and elicit effector functions, such as cytokine release, cytolysis, and proliferation.

**HMW-MAA–specific CAR functions in CD4+ and CD8+ T lymphocytes and confers non–MHC-restricted recognition of resected human melanomas.** The function of the HMW-MAA–specific CAR was evaluated in enriched populations of CD4+ and CD8+ T cells. Cytokine release was similar in the CD4+ and CD8+ cells (Fig. 3A), showing the efficacy of this receptor in both T-cell subsets. We also tested its efficacy against a panel of resected human melanomas with mixed HLA-A haplotypes. In generating single-cell tumor suspensions, cell-surface HMW-MAA expression was transiently lost, but reemerged after washing and overnight culture. Samples with viable tumor were assayed for HMW-MAA expression (Fig. 3B) and cocultured with HMW-MAA–specific CAR–transduced T cells in cytokine release assays (Fig. 3C). Of the four tumors tested, two had high expression (pt. T and pt. C) and two had moderate expression (pt. M and pt. K). HMW-MAA–specific CAR–transduced T cells recognized all four, and the IFN-γ secretion correlated with HMW-MAA expression ($P < 0.001$), as displayed in Fig. 3D. Furthermore, tumors from patients with disparate HLA-A haplotypes were recognized, supporting the non–MHC-restricted activation of the HMW-MAA–specific CAR.

**Clinical-grade HMW-MAA–specific CAR retrovirus redirects lymphocytes to target autologous melanoma.** A HMW-MAA–specific CAR retroviral packaging line was developed

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Figure 1. HMW-MAA was highly expressed on human melanoma lines and explanted melanoma tumors. A, HMW-MAA was detected on human, but not on murine (B16), melanoma lines by flow cytometry. B, in nonmelanoma lines, only a glioma line (SNB-75) expressed HMW-MAA. C, ex vivo activated PBL (7–83 d post-stimulation), endothelial cells (HUVEC), epidermal keratinocytes (NHEK), and bronchial epithelial cells (HBEC) did not express HMW-MAA; dermal fibroblasts (NHDF) displayed low HMW-MAA expression. D, eight explanted human melanomas expressed HMW-MAA.
Figure 2. PBL transduced to express an optimized HMW-MAA–specific CAR were redirected to target HMW-MAA–expressing melanoma lines. A, diagrams of optimized HMW-MAA–specific CARs and cell-surface expression of each construct in PBL transduced with the corresponding retroviral vector. HMW-MAA–specific CAR–transduced, but not untransduced, T cells were redirected to target HMW-MAA–expressing melanoma lines but not non–HMW-MAA–expressing cells, as shown by cytokine release (B), lysis (C), and proliferation assays (D).
for clinical-grade retrovirus production and screened to identify the highest-titer clone. Based on transduction efficiency (Fig. 4A) and cytokine release (Fig. 4B), clone D8 was selected. To test the ability of clone D8 transduced T cells to lyse tumor targets, PBL from two patients were transduced and subjected to cell lysis assay (Supplementary Fig. S6). HMW-MAA CAR–transduced cells specifically lysed melanoma line 1300 but not HMW-MAA–negative line H1299 or two normal human diploid fibroblast cultures. Clinical-grade supernatant harvested from clone D8 was then used to transduce PBL from a non–HLA-A*02 donor (pt. B) and showed efficient gene transfer (75% CAR⁺; Fig. 4C). As displayed in Fig. 4D, transduction with the HMW-MAA–specific CAR redirected this patient’s lymphocytes to effectively target his autologous tumor.

**Concluding remarks.** In this study, we developed a novel HMW-MAA–specific CAR that redirects lymphocytes to target human melanomas. Although two groups have reported...
early-generation CARs targeting HMW-MAA, they were not suitable for clinical use. The first used an scFv derived from an antibody (clone 763.74) of lower affinity and failed to show T-cell activation by HMW-MAA–expressing tumors in the absence of anti-idiotype antibody prestimulation (17). The second CAR (based on clone 225.28S) was limited by low expression and only shown to drive an EGFP reporter gene in a murine T-cell line (18). Following optimization for expression and function in human PBL, T cells expressing our HMW-MAA–specific CAR secreted cytokines, were cytolytic, and proliferated in response to HMW-MAA–expressing cell lines. Furthermore, the receptor functioned in both CD4+ and CD8+ cells, was non–MHC restricted, and reacted against explanted human melanomas.

Compared with intracellular melanocyte differentiation antigens such as gp100 and MART-1, HMW-MAA is
expressed in a higher percentage of human melanomas and has a more restricted pattern of normal tissue expression (14, 16). Other cell-surface melanoma-associated antigens are less frequently expressed on human melanomas (such as GD2 or GD3 ganglioside) or are more frequently expressed on normal tissues (such as GM3 or GM2 ganglioside; ref. 19). Despite these potential advantages for use in immunotherapy, and because on-target toxicity to normal tissues was observed using high-avidity TCRs against gp100 and MART-1 (6), care should be taken in the clinical application of any cell-based immunotherapy targeted to normal cell antigens and only patients with documented overexpression of HMW-MAA should be considered as candidates for ACT trials. Toxicology studies in small animals would not be useful because the HMW-MAA antibodies used in this and other studies do not cross-react with the murine HMW-MAA ortholog AN2. Although we did not observe lysis of normal diploid fibroblasts using HMW-MAA CAR transduced cells (Supplementary Fig. S6), we have on occasion observed lysis of melanoma cell line 888 that expressed a similar low level of HMW-MAA (data not shown), suggesting that different threshold levels of HMW-MAA recognition may be observed with specific patients’ T cells.

The optimized HMW-MAA–specific CAR described in this study represents a novel means to treat the large number of melanoma patients unable to receive current ACT therapies. HMW-MAA is also expressed on other tumors of neural crest origin (e.g., astrocytomas, gliomas, neuroblastomas, and sarcomas), as well as some forms of childhood leukemias and lobular breast carcinoma lesions. Furthermore, recent reports suggest that HMW-MAA may be up-regulated in pericytes associated with tumor neovascularature (20), making the potential application of HMW-MAA CAR–based cell therapies useful in a variety of malignancies.

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

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