Retroviral Transduction of Human Dendritic Cells with a Tumor-associated Antigen Gene

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ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells that can activate quiescent T lymphocytes. When pulsed with tumor-associated antigen (TAA) peptide or protein, murine DCs can provide antitumor immunity. We reasoned that DCs retrovirally transduced with TAA genes might have important advantages over peptide- or protein-pulsed DCs, including long-term TAA presentation in vivo, and presentation of important but undefined epitopes. Therefore, we attempted to retrovirally transduce human DCs with a melanoma TAA gene (MART-1) and determine whether these transduced DCs could raise a specific antitumor response from quiescent autologous T lymphocytes. After retroviral transduction, human CD34+ cells were differentiated into DCs in vitro using granulocyte macrophage colony-stimulating factor, tumor necrosis factor alpha, and stem cell factor. This method consistently yielded a population of DCs as analyzed by morphology, phenotype, and MLR. Flow cytometric analysis revealed that 22-28% of cells expressing the DC phenotype also expressed a transduced marker gene. When DCs were transduced with the gene encoding MART-1, they stimulated much higher levels of cytokine release by MART-1-specific tumor-infiltrating lymphocytes than control DCs transduced with an irrelevant gene. In vitro stimulation using MART-1-transduced DCs but not control-transduced DCs raised specific antitumor CTLs from autologous quiescent T cells. These results provide evidence that human DCs can be retrovirally transduced with a TAA gene and that these transduced cells can raise a specific antitumor immune response in vivo. Transduced DCs may be useful for in vivo immunization against TAA.

INTRODUCTION

DCs are highly potent APCs that are capable of activating quiescent T cells (1). Recent evidence indicates that DCs can play an important role in tumor immunity. Murine DCs pulsed with TAA peptide (2-4) or protein (5) or acid-stripped tumor cell peptides (6) can generate specific antitumor immunity in vitro and in vivo. Recent evidence also supports the role of human DCs in tumor immunity. DCs pulsed with idiotype protein, alternating with boosts of free idiotype protein, caused regression of established human lymphoma (7).

Because of their roles in antigen presentation and tumor immunity, DCs are an attractive target for genetic modification. Herpes simplex virus DNA was successfully transfected into murine DCs, which were then able to raise an anti-herpes simplex virus CTL response in vitro (8). There is, however, no convincing evidence that human DCs can be gene-modified or that any DCs can be gene-modified with a gene encoding a TAA.

We hypothesized that retroviral transduction with TAA genes might have important advantages over other methods of antigen delivery to DCs. Peptides pulsed onto DCs stay bound to MHC molecules only transiently because of dissociation and MHC turnover. However, retrovirally transduced DCs may be able to constitutively express and process TAA to produce long-term antigen presentation in vivo. In addition, TAA-transduced DCs may be able to present important but undefined MHC class I and II epitopes. Approaches that utilize peptide-pulsed DCs require that the peptides be clearly defined.

Several human melanoma TAA genes have now been cloned and sequenced (for reviews, see Refs. 9 and 10). One such TAA is MART-1, a 118-amino acid protein that is expressed by most human melanoma cells. It was cloned based on recognition by TILs that can cause tumor regression when adoptively transferred to patients with metastatic melanoma (11). In this study, human DCs were retrovirally transduced with MART-1 cDNA.

In developing a method for retroviral transduction of human DCs, we took advantage of two principles: (a) although mature hematopoietic cells are difficult to gene-modify, human CD34+ HPCs can be efficiently retrovirally transduced (12-15); and (b) human CD34+ HPCs can be differentiated into DCs by incubation in vitro with GM-CSF and TNF-alpha (16). The addition of SCF can dramatically increase the yield of DCs (17). Thus, our approach was to transduce CD34+ HPCs with a retrovirus encoding a TAA and then differentiate the transduced HPCs into DCs in vitro with GM-CSF, TNF-alpha, and SCF. We then tested whether the TAA-transduced DCs could express, process, and present TAA epitopes and whether they could raise specific antitumor CTLs by stimulating autologous quiescent T lymphocytes in vitro.

MATERIALS AND METHODS

Media and Cell Culture. CM is Iscove’s medium (Biofluids, Inc., Rockville, MD) supplemented with 10% human AB serum (male, heat-inactivated; Sigma Chemical Co., St. Louis, MO), 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (all from Biofluids, Inc.), and 25 µg/ml gentamicin sulfate (Life Technologies, Inc., Grand Island, NY). DC CM is CM supplemented with 100 ng/ml GM-CSF, 100 ng/ml TNF-alpha (both from Promega, Madison, WI), and 20 ng/ml SCF (R&D Systems, Inc., Minneapolis, MN). All retroviral producer cell lines (see below) were grown in DMEM with high glucose supplemented with 10% heat-inactivated FBS (both from Biofluids, Inc.), 2 mM glutamine, and antibiotics, which consisted of 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin sulfate, and 1.25 mg/ml amphotericin B (Fungizone; Life Technologies, Inc.). Human tumor cells used as targets in cytokine release, and cytolytic assays were grown in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and antibiotics. Human T2 cells (HLA-A2*, with "empty HLA" because of tap transporter deficiency) were grown in RPMI 1640 supplemented with 10% human AB serum, 2 mM glutamine, and antibiotics. Human 1235 TILs were grown in AIM-V (Life Technologies, Inc.) supplemented with 5% human AB serum and 6000 IU/ml IL-2 (Chiron, Emeryville, CA; TIL medium).

Retroviral Vectors and Producer Lines. The retroviral vectors SAM-MART-1-EN, SAM-mb7-1-EN, and SAM-p12-EN are based on the SAMEN vector (18). In this vector, both the 5' gene of interest and the 3' neomycin phosphotransferase gene (neoR) are translated from a single transcript, which is produced under the control of the long terminal repeat from Moloney murine leukemia virus. neoR is translated via an internal ribosomal entry site. SAM-
MART-1-EN was created by cloning the human melanoma TAA MART-1 cDNA into the pSAMEN multiple cloning site. SAM-mB7-1-EN and SAM-p12-EN were similarly created by cloning the murine B7-1 (mB7-1) cDNA and the irrelevant p12 (19) cDNA, respectively, into pSAMEN. Retroviruses were produced by either the PA317 packaging line (20), which expresses the amphotropic retroviral envelope, or the PG13 packaging line (21), which expresses the GALV envelope. PA317 cells expressing SAM-MART-1-EN (PA-MART), SAM-mB7-1-EN (PA-mB7-1), SAM-p12-EN (PA-p12), or SA-
MEN (PA-SAM) were created as described previously (22). PG13 cells were transduced with supernatant from the PA317 producer lines and selected with G418 (Geneticin; Life Technologies, Inc.) to obtain bulk cultures of the producer lines PG-MART, PG-mB7-1, PG-p12, and PG-SAM.

Collection of CD34+ Cells. CD34+ cells were obtained from HLA-A2+ patients on protocols for treatment of melanoma in the Surgery Branch, National Cancer Institute. CD34+ cells were mobilized into peripheral blood by five daily s.c. injections of 10 μg/kg granulocyte colony-stimulating factor (Neupogen; Amgen, Thousand Oaks, CA), followed by leukapheresis to obtain PBMCs on day 6. CD34+ cells were selected by an immunoaffinity column (CellPro, Inc., Bothel, WA) and cryopreserved. A single leukapheresis typically yielded 2—5 × 10^6 CD34+ cells.

Transduction and Differentiation of DCs from CD34+ cells. On day 0, CD34+ cells were thawed into CM, washed, and resuspended in DC CM in 6-well tissue culture plates at 5 × 10^5 cells/well. On days 1-3, the CD34+ cells were transduced three times in 24-h cycles. Each cycle consisted of coculturing the CD34+ cells with the adherent irradiated producer line (3000 cGy) in freshly harvested retroviral supernatant containing 8 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, WI), 100 ng/ml GM-CSF, 100 ng/ml TNF-α, and 20 ng/ml SCF for 6 h, followed by replacement of the medium with DC CM for 18 h. During the first 3 cycles, the cells were centrifuged at 1000 × g at 32°C. After the last transduction, the nonadherent CD34+ cells were replated in DC CM in new 6-well tissue culture plates. On day 8, half of the medium was replaced with fresh DC CM, and the nonadherent cells were transferred to new plates. On day 12, the nonadherent transduced population was harvested and used in experiments.

MLR. MLR was used to test the stimulatory function of DCs for allogeneic quiescent T cells. DCs were prepared, harvested, and irradiated (3000 cGy). Autologous PBMCs were collected by leukapheresis followed by centrifugation over a ficoll-hypaque gradient and irradiated (3000 cGy). Allogeneic T cells were prepared from a normal donor buffy coat by centrifugation over a ficoll-hypaque gradient, followed by negative selection with an immunoaf-

finity column that removes B lymphocytes and monocytes (R&D Systems, Inc.). T cells (1.5 × 10^6) were mixed with graduated numbers of either irradiated DCs or irradiated PBMCs in 200 μl of medium (RPMI, 10% FBS, 2 mM glutamine, and antibiotics) in flat-bottom 96-well tissue culture plates and incubated at 37°C in a 5% CO2 humidified incubator for four days. The cultures were then pulsed with 1.0 μCi [3H]thymidine (DuPont New England Nuclear, Boston, MA) for 8 h, and [3H]thymidine incorporation into DNA was quantitated (Beta plate; LKB Instruments, Gaithersburg, MD), and the percentage of lysis was calculated.

RESULTS

Retroviral Transduction of CD34+ HPCs and Differentiation into DCs. Our procedure for transduction of DCs is based on a method developed by Young and colleagues (17) for in vitro differ-
entiation of human CD34+ HPCs into DCs using GM-CSF, TNF-α, and SCF. In our studies, this method has consistently yielded a cell population that expanded 10—20-fold during differentiation and was 20—30% DCs by morphological (data not shown) and phenotypic analyses (Fig. 1). These cells also stimulated allogeneic MLR 50-fold better than autologous PBMCs (Fig. 2). Transduction with the MART-1 gene (see “Transduction of DCs with MART-1”) did not alter the ability of these cells to stimulate allogeneic MLR (Fig. 2).

In transduction optimization studies, a transduction marker was needed whose expression could be easily followed by double-stain flow cytometric analysis. This would allow quantitation of the number of cells that expressed both the transduction marker and a DC phenotype. Expression of MART-1 cannot be measured directly by flow cytometry without cell permeabilization, and specific antibodies are not available. Therefore, murine B7-1 was chosen as the transduction marker because: (a) it is a cell surface molecule; (b) it is not expressed on human DCs; and (c) a specific antibody against it is available, and this antibody does not cross-react with human DC phenotype markers, including human B7-1 (data not shown).

The transduction efficiency of DCs was optimized by varying the retroviral envelope and the transduction method. CD34+ HPCs were transduced with two retroviruses encoding mB7-1 that differed only in their viral envelope [one had the GALV envelope (PG-mB7-1), and the other had the amphotropic envelope (PA-mB7-1)]. In addition, the transduction was done with either producer cell supernatant or cocul-
ture with irradiated producer cells. The transduced CD34+ cells were then differentiated into DCs. Transduced DCs but not nontransduced controls exhibited surface mB7-1 expression at flow cytometric analysis. Lack of staining in the huB7-1+ nontransduced controls con-
firms the lack of cross-reactivity of the antibody between the human and murine moieties. Transduction and expression were more efficient with the GALV-enviroed retrovirus and with the coculture transduction method (Table 1). In fact, when the GALV envelope and coculture transduction method were used together, 22–28% of the cells that had a DC phenotype also expressed the transduction marker (Table 1). Similar results were obtained with other patients' cells (data not shown).

Transduction of DCs with MART-1. We then sought to determine if this method could be used to transduce DCs with the MART-1 melanoma TAA gene. The MART-1-transduced cells were analyzed for their ability to express, process, and present a MART-1 epitope using a MART-specific TIL, 1235 TIL. MART-1-transduced cells stimulated significantly higher levels of cytokine release from 1235 TIL than did control-transduced cells (Table 2). This was true of DCs at 6, 12, and 15 days after transduction, without G418 selection. This indicates that the MART-1-transduced cells were able to stably express, process, and present a TAA epitope. Because the CD34+ cells differentiated in vitro were heterogeneous, we sorted for the DC phenotype to determine whether purified DCs were transduced with MART-I. Coculture of 1235 TIL with sorted MART-transduced DCs resulted in high production of IFN-γ compared to coculture with sorted DCs transduced with a control gene (652 versus 46 pg/ml, respectively).

Stimulation of Autologous Peripheral Blood Lymphocytes with MART-1-transduced DCs. Because the CD34+ HPCs that were transduced with MART-1 and differentiated to DCs were strongly recognized by MART-specific lymphocytes, we asked whether the transduced DCs could stimulate quiescent autologous lymphocytes to raise a specific anti-MART CTL response. DCs were transduced with either the MART-1 or control SAM retrovirus, irradiated, and incubated with autologous quiescent T lymphocytes at a 1:10:10 stimulator:effector ratio. The T cells were restimulated with freshly transduced DCs every two weeks and were tested for MART-1 reactivity one week after each stimulation. Specific cytokine release by lymphocytes against MART-1-expressing targets was not evident after the first
restimulation. However, after two restimulations, the lymphocytes stimulated with MART-1-transduced DCs but not SAM-transduced DCs were highly MART-specific (Table 3) in one of three patients tested. Furthermore, they also released cytokine in response to HLA-A2* melanoma cells or HLA-A2* non-melanoma tumor cells that do not express MART-1. The lymphocytes stimulated with MART-DC also exhibited strong and specific lysis of HLA-A2* cells expressing MART-1 (Fig. 3). Both the MART-DC- and SAM-DC-stimulated lymphocytes proliferated well, expanding approximately 10-fold each week. These results indicate that CD34+ HPCs that are transduced with MART-I or control SAM retrovirus as described in "Materials and Methods," irradiated (1500 cGy), and incubated with autologous quiescent T cells pulsed either with the MART27-35 or irrelevant influenza M1 peptide, the HLA-A2* melanoma lines 624.38 mel and SK23 mel, the HLA-A2* melanoma line 586 mel, and the HLA-A2* breast cancer line MDA231, which does not express MART-1. OKT3 is a positive control in which the plate is coated with antibody against the T-cell receptor complex. One recent report presents data on the transient transfection of human DCs with the tyrosinase gene using cationic lipid (24). However, the

**DISCUSSION**

Because many malignancies, including melanoma, often respond poorly to conventional therapies such as surgery, radiation, and chemotherapy, specific immunotherapeutic approaches to cancer treatment are needed. DC therapy may offer such an approach. An emerging body of evidence indicates that murine DCs pulsed with TAA peptide or protein can treat or prevent tumor formation (2–6, 23). There is also evidence that human DCs may play a role in tumor immunity (7).

DCs stably gene-modified with TAA genes may provide important advantages over antigen-pulsed DCs (see "Introduction"). However, there is no published evidence that human DCs can be gene-modified with an antigen gene and specifically stimulate quiescent T cells. One recent report presents data on the transient transfection of human DCs with the tyrosinase gene using cationic lipid (24). However, the

Table 2 Cytokine release from MART-reactive TIL in response to DCs at various times after transduction with PG-MART

CD34+ HPCs from three patients (J, W, and M) were transduced with either the PG-MART or irrelevant PG-p12 retrovirus and then differentiated into DCs in vitro. At various times after transduction, the cells were tested for their ability to stimulate a MART-specific TIL. Transduced cells (1 × 10^6) were incubated with 1 × 10^5 1235 IL-2 for 24 h, and the supernatant was analyzed for IFN-γ content. OKT3 (plate coated with antibody against the T-cell receptor complex) is a positive control. Results are expressed as the mean ± SEM. ND, not determined.

Table 3 Cytokine release from lymphocytes stimulated with MART-transduced DCs

DCs were transduced with either the MART-1 or control SAM retrovirus as described in "Materials and Methods," irradiated (1500 Gy), and incubated with autologous quiescent T lymphocytes at a 1:10 stimulator:effector ratio. IL-2 (300 IU/ml) was added on day 2. The T cells were restimulated with freshly transduced DCs in this manner every two weeks and were tested for MART-1 reactivity one week after each stimulation. After two restimulations, the lymphocytes were tested for their ability to recognize various cells. These cells included T2 cells pulsed either with the MART27-35 or irrelevant influenza M1 peptide, the HLA-A2* melanoma lines 624.38 mel and SK23 mel, the HLA-A2* melanoma line 586 mel, and the HLA-A2* breast cancer line MDA231, which does not express MART-1. OKT3 is a positive control in which the plate is coated with antibody against the T-cell receptor complex. Results are expressed as the mean ± SEM.

Table 1 Transduction of human CD34+ HPCs differentiated into DCs

CD34+ HPCs were transduced with retroviruses encoding the transduction marker gene mB7-I with either the GALV (PG13) or amphotropic envelope (PA317). The transduction was done using either producer cell supernatant or coculture with irradiated producer cells. The transduced CD34+ HPCs were differentiated into DCs in vitro as described in "Materials and Methods." Cells were then stained with labeled antibodies against the transduction marker mB7-I and the DC phenotype molecules hB7-1 and hB7-2. Expression was determined by double-stain flow cytometric analysis.
transfected DCs stimulated only small amounts of cytokine release from activated tyrosinase-specific CTL, and there was no evidence that these DCs could raise specific anti-tyrosinase immunity.

The data presented here provide evidence that: (a) human DCs can be stably gene-modified by retroviral transduction; and (b) when transduced with a TAA, these cells can raise specific antitumor immune reactivity in vitro. These results may have implications for the use of transduced DCs in human tumor immunotherapy. Because the final DC population contains other cells, it is possible that non-DCs could be contributing to these observations. To determine whether DCs within the heterogeneous population of differentiated CD34+ cells were transduced with the MART-1 gene, cells with a characteristic DC phenotype were purified by cell sorting. These cells indeed expressed the MART-1 gene, based on IFN-γ release by MART-specific CTL (1235 TIL). Additionally, MART-1-transduced CD34+ cells differentiated to DCs could stimulate the generation of MART-1-specific T cells from PBMCs, whereas transduced CD34+ cells differentiated to monocytes using a macrophage colony-stimulating factor-containing cytokine combination (25) could not (data not shown). These findings suggest that the transduced DC fraction of differentiated CD34+ cells is responsible for the generation of MART-1-specific T-cell reactivity from quiescent lymphocytes.

One advantage of using DCs that are genetically modified with TAA genes is that they may present previously unknown MHC class I or II epitopes. Thus far, one immunodominant peptide epitope, MART27-35, has been identified from MART-1 (26). After stimulation with MART-transduced DCs, we tried to detect lymphocytes with reactivity against other MART-1 epitopes by testing for lymphocytes with reactivity against MART-DC or MART-expressing tumor cells, but without reactivity against T2 cells pulsed with MART27-35. However, no such epitope was found. This may be due, at least in part, to the fact that MART-1 is a relatively small protein. It is more likely that this method will find alternate epitopes in larger TAAs. We have made a retrovirus that encodes a larger melanoma TAA, gp100 (27), and are using this approach to determine if it has other unknown immunogenic epitopes.
The ability to gene-modify DCs may lead to additional immunotherapeutic approaches. Besides tumor antigen genes, DCs could be transduced with other genes, such as those encoding GM-CSF, IL-12, and other cytokines that may further enhance their ability to stimulate the immune response. It is possible that our current method of restimulating lymphocytes with MART-transduced DCs may not be the optimal way to use DCs to raise reactivity in vitro. Because DCs have high levels of costimulatory molecules (1), it is possible that they may actually inhibit proliferation of lymphocytes that have been activated in previous rounds of stimulation (28). Therefore, it might be more advantageous to stimulate lymphocytes once with TAA-transduced DCs, followed by restimulation with other nonprofessional APCs that have been engineered to express the TAA. In this study, only MART-transduced HPCs differentiated to DCs were used to stimulate and restimulate lymphocytes to show that these cells are able to raise anti-MART reactivity from quiescent lymphocytes. Perhaps because of this, only one of three patients tested thus far has consistently yielded specific antitumor lymphocytes. We are trying to solve this problem by developing methods to gene-modify nonprofessional APCs to use for restimulations, obtaining reagents from more patients, and further optimizing stimulation methods.

DCs transduced with TAA genes may have an important role in the immunotherapy of human cancer. The method outlined here can be directly applied to patient treatment without substantial modification. The entire cellular system is autologous, the transduced DCs do not require selection, and the necessary reagents are easily obtained by leukapheresis. Between 2–5 × 10^8 CD34^+ HPCs can be obtained from each leukapheresis, and this could give rise to approximately 5 × 10^9 cells after transduction and differentiation into DCs. Thus, all of the reagents necessary to produce a substantial dose of TAA-transduced DCs for adoptive transfer to patients could be obtained from a single leukapheresis.

In summary, we present here the first published evidence that human DCs can be gene-modified with a TAA by retroviral transduction of CD34^+ HPCs followed by differentiation into DCs. Furthermore, these transduced cells are capable of raising antitumor reactivity from autologous quiescent lymphocytes in vitro. This method may be useful for the immunotherapy of human cancer.

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