Efficient and Nontoxic Adenoviral Purging Method for Autologous Transplantation in Breast Cancer Patients

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ABSTRACT

Tumor cell contamination of clinical grafts is a major concern in autologous hematopoietic stem cell transplantation because these contaminating cells can contribute to relapse. In the present work, we use a suicide gene therapy approach that successfully accomplishes the two main goals of any purging strategy: highly efficient elimination of contaminating tumor cells and preservation of the engraftment capability of the hematopoietic progenitor cells. Human CD34+ cells spiked with breast cancer cells were infected with an adenoviral vector encoding the cytokine deaminase transgene (Ad-CMV-CD). In vitro, transduction with Ad-CMV-CD followed by exposure to 400 µM 5-fluorocytosine resulted in complete elimination of clonogenic contaminating tumor cells without affecting the clonogenic potential of the human hematopoietic CD34+ cells. Transplantation of nonobese diabetic/LtSz-scid/severe combined immunodeficient (NOD/SCID) mice with unpurged contaminated grafts and unpurged contaminated grafts, allowed us to test the safety and efficacy of our procedure in two independent purging experiments. Hematopoietic engraftment kinetics as well as the quantity and quality of human engraftment were not affected by the purging therapy. Results showed a significant difference in survival between the nonpurged group (28%) and the purged group (100%; P = 0.012). Moreover, highly sensitive histological and molecular analyses confirmed the absence of tumor cells in the recipients of purged marrow. In contrast, metastatic tumors were detected in animals that received unpurged transplants. We anticipate that this strategy will result in a safe and efficacious hematopoietic graft product for autologous transplantation for patients with multiple forms of epithelial cancers.

INTRODUCTION

PBSCs have replaced BM as the primary source of hematopoietic progenitors for autologous transplant after high-dose chemotherapy in most cancer patients. Although tumor burden may be lower in PBSCs than in BM, malignant cells can be detected in 10–40% of PBSCs from patients with metastatic breast cancer and in 5–20% of PBSCs from patients with stage II and III breast cancer (2–6). Genetic marking experiments have proven that tumor cells contaminating the graft can contribute to posttransplant relapse in patients with neuroblastoma (7) and myeloid leukemias (8, 9). Although analogous data are not available for breast cancer, indirect data suggest a similar role for contaminating tumor cells in relapsed disease. Detection by PCR of a breast cancer-specific sequences in the apheresis products of breast cancer patients undergoing autologous PBSC transplant is associated with an increased probability of relapse and decreased disease-free survival (4). PCR data would correlate with the presence of contaminating BCCs, which have been estimated to be as high as 2.5–7 × 10^5 BCCs in a typical PBSC graft for a 60-kg breast cancer patient (1).

Many procedures have been designed to eradicate residual tumor cells from autologous harvests. These procedures include the use of monoclonal antibodies either against HPC antigens for positive selection or against tumor-specific cell surface molecules for negative selection. Although immunomagnetic selection of CD34+ cells achieves a 2–4 log depletion of tumor cells, highly sensitive assays to detect tumor cells have shown that some clonogenic tumor cells copurify with the HPCs (10, 11). Chemical purging, in contrast, can accomplish higher rates of tumor cell killing but can compromise the engraftment capacity of the graft (12, 13). In this study, we have developed and validated a method to eliminate contaminating tumor cells from autologous grafts, while preserving the functional capability of the HPCs to reconstitute hemopoiesis in the patient after myeloablative therapy has been administered. We designed a strategy for purging hematopoietic cell grafts from BCCs using an Ad carrying the prodrug-converting enzyme CD (14). In the presence of CD, cells convert the nontoxic drug 5-FC into its metabolite, the toxic drug 5-fluorouracil. Unlike epithelial tumor cells, hematopoietic cells are not good targets for adenoviral infection. Therefore, the therapeutic action of the CD transgene is restricted to the contaminating BCCs. In the present study, we tested whether this purging strategy eliminates the contaminating BCCs, while preserving the ability of the SRCs to reconstitute the human hemopoiesis in a preclinical xenotransplantation model, which resembles the clinical situation of autologous transplantation with tumor-contaminated PBSC grafts. The results presented in this work demonstrate that tumor cells are completely eliminated although the short- and long-term engraftment capacity of the SRCs is maintained. Our strategy will provide a safe and efficacious purging method, as well as relevant information about the role of tumor contamination in the posttransplant relapse.

MATERIALS AND METHODS

PBSC Samples. Mobilized leukapheresis samples from healthy donors were obtained from the Cryopreservation Unit of Hospital General Universitario Gregorio Marañón under hospital guidelines. CD34+ cells were positively selected using an ISOLEX 300i device (Baxter, Deerfield, IL) according to the manufacturer’s specifications.

Cell Lines. The MDA-MB-231 (15) and MDA-MB-468 BCC lines and the embryonic kidney cell line 293 (16) were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with 10% FBS, glutamine, and antibiotics (Life Technologies, Inc., Grand Island, NY; growth medium).

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4 The abbreviations used are: PBSC, peripheral blood stem cell; BM, breast cancer cell; Ad, adenoviral vector; CMV, cytomegalovirus; pCMV, (human) CMV promoter (enhancer region); CD, cytokine deaminase; BM, bone marrow; HPC, hematopoietic progenitor cell; 5-FC, 5-fluorocytosine; SCID, severe combined immunodeficient; SRC, SCID repopulating cell; EGF, enhanced green fluorescent protein; CK-19, human cytokeratin-19; RT-PCR, reverse transcription-PCR; NOD, nonobese diabetic; NOD/SCID mice, NOD/LtSz-scid/scid mice; PI, propidium iodide; FBS, fetal bovine serum; pfu, plaque-forming unit(s).
Chemicals. PI, 4,6-bis(hydroxethyl)-2-aminoethanesulfonic acid (BES), and 5-FC were purchased from Sigma Chemical Co. (St. Louis, MO).

Plasmid Vectors. The plasmid containing the EGFP was purchased from Clontech Laboratories Inc (Palo Alto, CA). The pcMV, polycloning site and bovine growth hormone polyadenylation signal (BGHpa) were obtained from the pcDNA3.0 vector from Invitrogen (Barcelona, Spain), pαs1pα and pBHGI0 (17) were purchased from Microbiz (Microbiz Biosystems Inc; Toronto, Ontario, Canada).

Recombinant Adenovirus Construction. CD gene from Escherichia coli (Gene Bank accession no. S56903) was amplified using the following primers: sense primer, 5’-GTCAagcctAGGCTAaAaaaTGTCG-3’, and antisense primer, 5’-GTACgggcccTGGCGAGACAGCC-3’. Denoted in lowercase are the HindIII and ApaI restriction sites introduced to facilitate insertion into the eukaryotic expression vector pcdNA3. To check sequence integrity and the insertion of eukaryotic translation start signal (shown in bold), the amplified CD gene was sequenced. The expression cassette containing the CMV promoter, the CD gene, and the bovine growth hormone polyadenylation signal was subcloned into the pαs1pα plasmid, resulting in the CD shuttle vector. Adenoviral rescue was accomplished by cotransfection of the CD shuttle vector and the adenoviral backbone pBHG10 plasmid into 293 cells using calcium phosphate precipitation. Large-scale production of adenovirus was made by infecting 293 cells and purifying crude viral lysates by cesium chloride centrifugation followed by overnight dialysis. Aliquots of the virus were stored at −80°C. The viral titer was calculated using the plaque assay in 293 cells. Incorporation of the expression cassette into the Ad-CMV-CD recombinant adenovirus was confirmed using viral DNA PCR amplification of the CD gene. Ad-CMV-EGFP was constructed as described above. Briefly, EGFP protein was excised from the pEGFP-1 vector and inserted into pcdNA3, followed by insertion into pαs1pα shuttle vector. Cotransfection into 293 cells was made as described above.

Flow Cytometry Analysis of Infectivity of BCCs and PBSCs. To minimize antibody blocking of adenoviral receptors, BCCs and PBSCs were infected in serum-free medium. BCCs were detached from the flasks using calcium phosphate precipitation. Large-scale production of adenovirus was made by infecting 293 cells and purifying crude viral lysates by cesium chloride centrifugation followed by overnight dialysis. Aliquots of the virus were stored at −80°C. The viral titer was calculated using the plaque assay in 293 cells. Incorporation of the expression cassette into the Ad-CMV-CD recombinant adenovirus was confirmed using viral DNA PCR amplification of the CD gene. Ad-CMV-EGFP was constructed as described above. Briefly, EGFP protein was excised from the pEGFP-1 vector and inserted into pcdNA3, followed by insertion into pαs1pα shuttle vector. Cotransfection into 293 cells was made as described above.

Evaluation of the Sensitization of BCCs and PBSCs to 5-FC. MDA-MB-231 BCCs were infected in suspension with 100 pfu/cell or mock infected for 1 h. After adenovirus exposure, cells were plated in triplicate in growth medium supplemented with different concentrations of 5-FC. Toxicity to 5-FC was assayed using trypan blue exclusion after 4 days in culture. After the 4-day incubation, serial dilutions of MDA-MB-231 cells were seeded in 5-FC-free medium and incubated for 7 days to allow colony formation. The colonies were stained with methylene blue (PANREAC, Barcelona, Spain) for 1 h, and after washing with PBS, the plates were dried at room temperature, and the colonies were counted using an inverse microscope. Toxicity of PBSCs to 5-FC was assayed as described above. Clonogenic capacity of the PBSCs after the 4-day culture was evaluated by plating 2500 cells in 1 ml of MethoCult GF 4434 (Stem Cell Technologies, Vancouver, British Columbia, Canada), and scoring the hematopoietic colonies after 14 days.

Animals. NOD/SCID mice were used in the experiments. Breeding pairs, originally obtained from Jackson Laboratory (Bar Harbor, ME), were bred at the Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas Animal Facility (Registration number 28079-21A). All of the animals were handled under sterile conditions, maintained in microisolator individually ventilated cages, allowed γ-irradiated food and UV-irradiated water ad libitum, and routinely screened for pathogens, in accordance with FELASA (Federation of European Laboratory Animal Science Associations) procedures. The experiments were carried out according to the Spanish and European regulations. Before transplantation, 6-8-week-old mice were total-body irradiated with 2.2 Gy of X-rays (300 kV, >10 mA; Philips MG-32A, Hamburg, Germany).

Transplantation. Artificial mixtures of human CD34+ cells and 2% of MDA-MB-231 BCCs were either mock infected or infected with Ad-CMV-CD as previously described. After adenovirus exposure or mock infection, 5 × 10^6 CD34+ cells and 1 × 10^5 BCCs per mouse were transplanted by tail-vein injection into NOD/SCID. The animals transplanted with the infected grafts (purged group) were i.p. treated with 5-FC (1g/kg/day; two i.p. shots/day), whereas mice receiving noninfected grafts (unpurged group) were untreated. Two independent experiments were carried out, with a total of seven mice in the control group and six mice in the purged group. Moribund mice responding to the unpurged group of mice from the assay 1 were euthanized according to the Spanish and European regulations.

Human Hematopoietic Engraftment. At periodic intervals after transplantation, marrow samples were aspirated from one femur by puncture through the knee joint, as described previously (18). At the end of the experiments, mice were killed, and samples from their bone marrow, lungs, liver, and brain were obtained. Human hematopoietic engraftment was evaluated by flow cytometric analysis of marrow samples. Aliquots of 1-5 × 10^6 cells/tube were stained for 25 min at 4°C with antihuman CD45-PECy5 (Clone J33; Immunotech, Marseille, France) in combination with either anti-human-CD34-PE (Anti-HPCA-2; Becton Dickinson Immunocytometry, San Jose, CA), anti-human-CD38-FITC (Clone T16; Immunotech), anti-human-CD19-PE (Anti-Leu-12; Becton Dickinson) or anti-human CD13-FITC (Clone SJ1D1; Immunotech). RBCCs were lysed in 2.5 ml of lysis solution (0.155 mol/liter NH4Cl + 0.01 mol/liter KHCO3 + 10^-4 mol/liter EDTA) for 10 min at room temperature. Cells were then washed in PBA (phosphate buffered salt solution with 0.1% BSA and 0.01% sodium azide), suspended in PBA + 2 μg/ml PI and analyzed by flow cytometry. Cells labeled with conjugated nonspecific isotypic antibodies were used as controls. At least 10,000 PI-negative events were analyzed using an EPICS XL cytometer.

Histological Examination of Metastatic Cancer. One of the two lungs infected in 10% buffered formalin followed by paraffin embedding. Sections of different levels were stained with H&E and examined microscopically.

RNA Isolation, RT-PCR Amplification. Total cellular RNA was isolated from the organs processed (lung, liver, kidney, brain) using TRizol Reagent (Life Technologies, Inc.) following the manufacturer’s instructions. RT-PCR amplification of a human CK-19-specific sequence was carried out using primers described elsewhere (19). The One-Step RT-PCR system (Life Technologies, Inc.) was used following the manufacturer’s instructions. The conditions for the RT-PCR were as follows: 50°C, 60 min for cDNA synthesis (from 2 μg of RNA), followed by 35 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 60 s. The conditions for the nested PCR were 30 cycles of 94°C for 30 s and 72°C for 60 s. A 745-bp band was visualized after electrophoresis on 1% agarose gels.

Statistical Analysis. Data are presented as means ± SE. Survival probability was calculated by the Kaplan-Meier method. Comparison of survival probability between groups was calculated with the log-rank test. The processing and statistical analyses of the data were performed using Stata Statistical Software, Release 5.0 (Stata Corporation, College Station, TX).

RESULTS

Efficient and Selective in Vitro BCC Killing after Ad-CMV-CD Transduction and 5-FC Exposure. The infectivity of BCCs and mobilized human CD34+ hematopoietic progenitors by Ads was assessed using an Ad containing the green fluorescent protein (Ad-CMV-EGFP). BCCs were exposed to increasing numbers of pfu/cell (0–100 MDA-MB-231, 0–20 MDA-MB-468). Experiments in Fig. 1 show that 1-h infection of MDA-MB-231 (Fig. 1A) and MDA-MB-468 (Fig. 1B) cells with Ad-CMV-EGFP/cell resulted in more than 99% of cells being positive for EGFP expression. However, when CD34+ cells were exposed to the highest adenoviral dose tested for BCCs (100 pfu/cell), an average of only 8.5% hematopoietic CD34+ cells were transduced (Fig. 1C). On the basis of these observations, 1-h infection with 100 pfu/cell was established as a standard transduction condition.

Experiments in Fig. 2A show the viability of MDA-MB-231 cells that were either mock-infected or infected with Ad-CMV-CD after incubation with 5-FC. The viability of mock-infected MDA-MB-231 cells was maintained even at concentrations as high as 1 mM. On the
Fig. 1. Differential efficiency of adenoviral gene transfer between peripheral blood CD34⁺ cells and BCCs by the Ad-CMV-EGFP Ad vector. BCCs and PB CD34⁺ cells were mock infected (unfilled plots) or infected with a different number of pfu/cell (filled plots). A, MDA-MB-231 (100 pfu/cell); B, MDA-MB-468 (20 pfu/cell); C, CD34⁺ cells (100 pfu/cell). EGFP expression was assayed using flow cytometry 24 h postinfection as described in “Materials and Methods.”

Fig. 2. In vitro sensitization of MDA-MB-231 to 5-FC. A, MDA-MB-231 cell killing because of bacterial CD expression; B, clonogenic capability of MDA-MB-231 cells after the 4-day incubation period; C, clonogenic capability of peripheral blood CD34⁺ cells after the in vitro manipulation. In A, MDA-MB-231 cells were infected with 100 pfu/cell (■), or mock infected (○) for 1 h and then cultured in the presence of different concentrations of 5-FC for 4 days. At the end of culture, cells were counted and the percentage of survival was plotted against noninfected cells. In B, surviving cells at the end of the 4-day period were plated in 5-FC-free medium to allow colony formation. Black bars, cells cultured in the absence of 5-FC; white bars, cells cultured in the presence of 400 μM 5-FC. In C, peripheral blood CD34⁺ cells were mock infected (black bars), infected with 100 pfu/cell Ad-CMV-CD (dotted bars), infected with 100 pfu/cell Ad-CMV-CD, and cultured with 400 μM 5-FC (white bars), and mixed with 1% of MDA-MB-231 infected with 100 pfu/cell Ad-CMV-CD, and cultured with 400 μM 5-FC (hatched bars).

contrary, the viability of MDA-MB-231 cells infected with Ad-CMV-CD was sharply decreased in the presence of the prodru. Clonogenic assays performed with MDA-MB-231 cells after exposure to Ad-CMV-CD and 5-FC (400 μM), showed the complete elimination of their clonogenic potential (Fig. 2B). Similar experiments were conducted to test the cytotoxic effect of the purging procedure into human HPCs. Human CD34⁺ cells were infected with Ad-CMV-CD and were maintained in the presence of 400 μM 5-FC for 4 days. The clonogenic capacity of CD34⁺ cells after the 4-day culture was tested using the granulocyte-macrophage colony-forming unit colony assay. Fig. 2C shows that the clonogenic capacity of CD34⁺ cells was preserved in all instances, even after infection with Ad-CMV-CD and incubation with 400 μM of 5-FC.

Preservation of the in Vivo Repopulating Capability of Human Contaminated Hematopoietic Grafts after the Gene Therapy Purging. A major concern in any purging protocol is whether or not the manipulation of the graft results in a significant impairment of the repopulating properties of the hematopoietic cells. To evaluate this issue, NOD/SCID mice were transplanted with artificial mixtures of mobilized CD34⁺ and MDA-MB-321 cells as described in “Materials and Methods.” Human hematopoietic engraftment was measured, from day 30 to day 120 posttransplantation, in BM samples. Results from two independent purging assays (Fig. 3, A and B) showed that the purging procedure did not affect the engraftment capacity of the SRCs, as measured by the level of human hematopoietic cells in the BM of both groups of transplanted mice. In addition to the engraftment kinetics studies, effects on the lineage composition in the human hematopoiesis of the engrafted animals were investigated. As shown in Fig. 3C, similar cytometric profiles of human hematopoietic cells (CD34⁺ versus CD38⁺ and CD13⁺ versus CD19⁺) were observed in BM samples analyzed at 120 days after transplant.

Efficient Purging of BCCs in Contaminated Hematopoietic Samples. To establish the relationship between the degree of graft contamination and survival, mice were transplanted with human 5 × 10⁶ CD34⁺ cells artificially contaminated with increasing concentrations of the BCC line MDA-MB-231 (0.0002–0.2%) or with uncontaminated CD34⁺ cells. As shown in Fig. 4A; 100% survival was observed in mice transplanted with either uncontaminated CD34⁺ cells or the lower dose of MDA-MB-231 cells (0.0002%). A progressive reduction in the survival rate was observed in groups transplanted with higher doses of tumor contamination (up to 0.2%). To evaluate the presence and the localization of tumoral residual disease present in every group of transplanted mice, RT-PCR for the human CK-19 gene was performed. One-third of the mice transplanted with only 10 MDA-MB-231 cells, representing 0.0002% of graft contamination, were found positive for CK-19, mainly located in the lungs of these animals (data not shown). The examination of the tissues from mice transplanted with the higher numbers of contaminating MDA-MB-231 cells revealed not only a CK-19-positive signal in all cases but also evidence of microscopic and macroscopic disease (data not shown).
To evaluate the effectiveness of the purging procedure, we used a tumor contamination burden 10 times higher (2%) than the highest dose previously tested (0.2%). Results in Fig. 5A show that there was a significant reduction in the survival of mice transplanted with unmanipulated grafts (5 × 10^6 human CD34^+ cells, unpurged group), when compared with the purged group (28 versus 100%; \( P < 0.012 \)). Macroscopic and histological examination of the organs of moribund animals corresponding to the unpurged group revealed the presence of tumoral nodules in the lungs of these animals. In contrast, the lungs of animals corresponding to the purged group did not show any evidence of disease (Fig. 5B). To corroborate histological analyses, a CK-19 RT-PCR assay was performed in lung samples from moribund mice in the unpurged group and mice in the purged group. As shown in Fig. 5C, CK-19-positive signal was found only in the unpurged group.

**DISCUSSION**

Preliminary results from high-dose chemotherapy protocols for high-risk breast cancer treatment were promising. However, recent reports show that there is still a high rate of patient relapse. Although relapse can be attributed to nondetectable residual disease in the...
patients and, thus, help in the design of new chemotherapy treatment procedures. However, currently available purging procedures lack the efficiency and safety of the purging procedure described here is based on the differential susceptibility of BCCs to adenovirus infection. As reported previously, solid tumors [neuroblastoma (20), breast cancer (14, 21)] and multiple myeloma (22) can be efficiently targeted using Ads. HPCs, on the other hand, are poor targets for Ads and, as mentioned by Wroblewski et al. (23), only under special infection conditions [preincubation with cytokines and prolonged exposure to high multiplicities of infection (24, 25)] are they transduced with Ads. In recent years, several groups, including ourselves, have reported excellent in vitro results using Ads containing different therapeutic genes for the eradication of tumoral cells, while preserving human CD34+ clonogenic potential (14, 22, 26, 27). However, these in vitro assays used in these works did not estimate the in vivo behavior of either the tumor cells (28) or the hematopoietic cells (29). To our knowledge, there is no report about the maintenance of the in vivo engraftment capacity of hematopoietic grafts after adenovirus-based purging procedures as well as the effectiveness of the tumor eradication.

The analysis of the human engraftment kinetics in the xenotransplantation model allowed us to evaluate the functionality of the hematopoietic cells exposed to the Ad and 5-FC. The level of human hematopoiesis found in both groups of animals (purged versus unpurged) was similar suggesting that the procedure did not affect the capacity of the SRCS to engraft into NOD/SCID mice. In the same way, the quality of the human hematopoiesis was also unaffected by the purging protocol. Consistent with our previous reported experience (18, 30), human myeloid and B-lymphoid cells were present at the same level in the marrow of both groups of mice. In addition, a significant population of human CD34+ cells were still present 3–4 months after transplant, which suggests that the progenitor compartment was maintained in vivo.

The results obtained from the preclinical model demonstrated that the exposure to the suicide CD Ad followed by in vivo administration of the produg 5-FC significantly increased the survival of all of the purged mice. In addition to the improved overall survival, histological and molecular analysis results present evidence of the complete elimination of all contaminating tumor cells in vivo.

Our strategy has several important aspects. First, it combines the positive selection of human CD34+ cells with the capacity of the Ads to selectively infect the tumoral epithelial cells with minimum ex vivo graft manipulation. Second, we used a produg (5-FC) for which there is plenty of experience in the clinical setting. Third, we demonstrate that the strategy efficiently eradicates the tumor BCCs present in hematopoietic grafts while preserving the engraftment capacity of the hematopoietic cells. In summary, our protocol may have a positive impact on the subset of breast cancer patients with BCC contamination in their clinical graft products.
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