High-Efficiency Gene Transfer Into Ex Vivo Expanded Human Hematopoietic Progenitors and Precursor Cells by Adenovirus Vectors

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Replication-deficient adenoviral vectors (AdVec), which infect cycling and noncycling cells with high efficiency, low toxicity, and ease of delivery, provide ideal vehicles to study the expression of regulatory genes controlling different stages of hematopoiesis. To examine the infection efficiency of AdVec in hematopoietic precursor and progenitor cells, we used a replication-deficient adenovector expressing the humanized form of the cDNA for green fluorescent protein (AdGFP), permitting assessment of infection efficiency and kinetics of transgene expression in viable hematopoietic cells using flow cytometry and fluorescence microscopy. Flow-cytometric analysis of ex vivo expanded hematopoietic precursor cells infected with a multiplicity of infection (MOI) of 100 of AdGFP show that 78% of megakaryocytic (CD41a+) and CD42b+ cells, 82% of dendritic (CD1a+) cells, 41% of RBC precursors (glycophorin A+), and 32% of monocytic (CD14+) cells expressed GFP. Nineteen percent ± 1% of freshly isolated CD34+ cells from peripheral blood leukapheresis products infected under the same conditions expressed GFP. Morphologic evaluation of ex vivo expanded, AdGFP-infected CD34+ cells showed normal maturation. The functional capacity of AdGFP-infected CD34+ cells was analyzed by quantifying clonogenic efficiency and proliferative capacity. Infection of CD34+ progenitor cells with MOIs of 1 to 100 did not impair clonogenic efficiency of CD34+ cells. However, MOI greater than 100 resulted in a significant inhibition of colony-forming unit-granulocyte/granulocyte-macrophage (CFU-G/GM) formation. In sequential dilution expansion over 3 weeks (Delta assay), the cytokine-driven proliferative potential of CD34+ cells was not impaired following exposure to AdGFP at MOIs of 1 to 1,000. The GFP+ population expanded 10- to 15-fold at high MOIs (500 to 1,000), indicating multiple copies of the transgene in the initially infected CD34+ cells, which were expressed in subsequent progenies. These data show that AdVec deliver transgenes with high efficiency and low toxicity to hematopoietic progenitor and precursor cells. Introduction of marker genes such as GFP into hematopoietic cells by AdVec will provide a valuable system for study of development, homing, and trafficking of hematopoietic precursor and progenitor cells in vitro and in vivo. Furthermore, these results provide insights into the design of gene therapy strategies for treatment of hematologic disorders by AdVec.

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negatively influenced by the presence of the transgene. Our experimental design provides a new model to study the physiology of hematopoietic progenitor and precursor cells in vitro and in vivo. In addition, this model provides new insights into the design of gene therapy protocols for the treatment of hematologic disorders using transient gene expression mediated by AdVec.

MATERIALS AND METHODS

**Purification of Human CD34+ Cells**

Human CD34+ cells were purified from frozen leukapheresis products collected from patients with solid tumors after stem-cell mobilization with granulocyte colony-stimulating factor (G-CSF)/cyclophosphamide. Permission to obtain leukapheresed samples was obtained from the Sloan-Kettering Institute and Cornell University Medical College institutional review boards. Briefly, the units were thawed at 37°C and washed several times with phosphate-buffered saline (PBS)/0.1% fetal calf serum (FCS) at room temperature (23°C). The cells were then concentrated to 5 x 10^10 to 1 x 10^11 cells/mL in PBS/0.1% FCS and incubated at 4°C for 30 minutes with monoclonal mouse antihuman CD34 antibodies, IgG1, clone 11.1.6 (developed by M.A.S. Moore, licensed to Organon Teknika, Cambridge, MA) at a concentration of 50 µg antibodies/10^8 cells. Thereafter, the cells were washed twice with PBS/0.1% FCS and incubated with magnetic immunobeads (30 µL/10^8 cells; Dynal beads, Dynal Inc, Oslo, Norway) at 4°C for 30 minutes. Then, the CD34+ cells, now rosetted by immunobeads, were collected using a magnetic particle concentrator (MPC; Dynal) and placed in Iscove’s modified Dulbecco’s medium (IMDM)/20% FCS at 37°C overnight. After the CD34+ cells were detached from the beads, the cells were washed and collected in fresh IMDM/20% FCS. The purity of the CD34+ cell fraction ranged routinely between 85% to 95% as assessed by flow cytometry.

**Ex vivo Expansion of CD34+ Cells**

CD34+ cells (4 x 10^6 to 1 x 10^9/mL) were expanded in 12-well polystyrene plates (Corning, New York, NY) using conditions and cytokine cocktails designed to generate progenitors of choice. Megakaryocytic (CD41a+) and erythroid precursors (glycophorin A+) were generated in serum-free medium X-Vivo 15 (Bio Whittaker, Walkersville, MD). For expansion of CD41a+ cells, thrombopoietin (TPO) and cytokines were added to the medium. To generate erythroid precursors, erythropoietin (EPO) and KL were used. Myelomonocytic cells (CD14+CD15+) were expanded in IMDM/20% FCS in the presence of G-CSF, KL, and IL-3 and dendritic cells were expanded in IMDM/20% FCS in the presence of KL, tumor necrosis factor-alpha (TNF-alpha), and granulocyte-macrophage colony-stimulating factor (GM-CSF). All expansion cultures were incubated at 37°C in 100% humidity, 5.2% CO2, and replenished with fresh medium containing the appropriate cytokines every 7 days.

**Antibodies and Cytokines**

**Monoclonal antibodies.** All of the monoclonal antibodies used for flow cytometry experiments were directly conjugated with phycoerythrin (PE). Dendritic cells were detected by CD1a-PE (SK9, IgG2b; Becton Dickinson, San Jose, CA), monocytic cells by CD14-PE (UCHM-1, IgG2a; Sigma, St. Louis, MO), myeloid cells by CD15-FITC (DU-HL60-3, IgG; Sigma), stem cells by CD34-PE (8G12, IgG1; Becton Dickinson), megakaryocytic progenitors and progenitor cells by CD41a-PE (GPIIbIIa, HPI8, IgG1; Pharmingen, San Diego, CA), mature megakaryocytes by CD42b-PE (GPIb S22, IgG1) and CD62-P (GMP140, AC 1.2, IgG1; Becton Dickinson), and erythroid precursor cells by rhodamine-conjugated antibody to glycophorin (AD2, 10 Immunotech, Westbrook, ME).

**Cytokines.** The following cytokines were used: Kit ligand (20 ng/mL; Amgen, Thousand Oaks, CA); G-CSF (100 ng/mL; Amgen), GM-CSF (100 ng/mL; Sandoz, Basel, Switzerland), TPO (50 ng/mL; R&D Systems, Minneapolis, MN), IL-3 (50 ng/mL; Sandoz), IL-6 (20 ng/mL; Amgen); EPO (6 U/mL; Amgen), and TNF-alpha (100 ng/mL; Genentech, San Francisco CA).

**AdVec Construction and Preparation**

The humanized GFP cDNA12 was subcloned as a NotI fragment into the plasmid pCMVSV2+, which creates an expression cassette with the CMV immediate-early promoter and a synthetic splice site upstream of the gene and the SV40 early polyadenylation site downstream of the gene. In pCMVSV2+, the expression cassette lies between the left end of the adenovirus genome (nucleotides 1 to 355) and the truncated E1B, pIX region (nucleotides 3333 through 5790). Coinfection of human embryonic kidney (HEK) 293 cells with the pCMVSV2+ GFP plasmid and the adenoviral backbone prepared from delE3 adenovirus backbone plasmid produced a full-length, replication-incompetent, E1- and E3-deficient adenovector expressing GFP. AdGFP was prepared by expansion of a single plaque generated in HEK 293 cells, which gave fluorescence. Large-scale preparations were routinely tested for titer (plaque-forming units [PFU]) by plaquing on HEK 293 cells and replication-competent adenovirus (RCA) by plaquing on A549 cells.15,16 A PFU to RCA ratio of greater than 10^6 was considered acceptable. The absolute amount of RCA present in the preparations is less than 1 RCA in the total dose administered. This is the same criteria the Food and Drug Administration has established for clinical studies, and the method used to detect RCA is the same as we used for clinical preparations. There is no detectable RCA+ virus or E1+ GFP virus in the preparation, and assessment of the cultures demonstrated no RCA+ virus.

**Adenovector Infection of Ex Vivo Expanded Hematopoietic Precursor Cells**

On day 10 of expansion, the cells were incubated with AdGFP (12 hours at 37°C) at various MOIs in 200 to 300 µL of serum-free medium (X-Vivo 15; BioWhittaker, Walkersville, MD) in flat-bottom 12-well plates. Following incubation, the cells were resuspended in the original expansion medium, including cytokines and serum as appropriate, and expanded for 7 to 14 more days. Subsequently, aliquots of cells were stained with lineage-specific, fluorescein-conjugated antibodies and analyzed by two-color flow cytometry for GFP expression.

**Flow Cytometry**

Before flow cytometry, viability of the cells was routinely determined by trypan blue exclusion. On average, 80% to 95% of cells were viable. AdGFP-infected hematopoietic precursor cells were washed and stained (30 minutes at 4°C) with PE- or rhodamine 1 (RD1)-conjugated monoclonal antibodies. Noninfected ex vivo expanded hematopoietic cells were stained with PE- or fluorescein isothiocyanate (FITC) conjugated IgG control isotype. Immediately after staining, the cells were washed with PBS/0.1% bovine serum albumin (BSA) and analyzed by two-color flow cytometry using an Elite Profile II flow cytometer (Coulter, Hialeah, FL). For determination of cells coexpressing GFP and the lineage-specific marker, a fluorescence intensity 1/fluorescence intensity 2 (FL1/FL2) dot-plot display was used. Log FL1 (x-axis) represents the fluorescence intensity of GFP+ cells. Log FL2 (y-axis) represents the fluorescence intensity of ex vivo expanded hematopoietic precursor cells labeled with the PE- or RD1-conjugated monoclonal antibody. GFP+ cells labeled with lineage-specific antibody are designated as FL1(GFP+) · FL2+. GFP+ cells labeled with lineage-specific antibody are designated as FL1(GFP+) · FL2-. The absolute amount of RCA present in the preparations is less than 1 RCA in the total dose administered. This is the same criteria the Food and Drug Administration has established for clinical studies, and the method used to detect RCA is the same as we used for clinical preparations. There is no detectable RCA+ virus or E1+ GFP virus in the preparation, and assessment of the cultures demonstrated no RCA+ virus.
ing formula:
\[
\% \text{ labeled precursors} = \frac{\text{FL1(GFP\textsuperscript{+})} \cdot \text{FL2\textsuperscript{+} cells}}{\text{FL1(GFP\textsuperscript{+})} \cdot \text{FL2\textsuperscript{+} cells} + \text{FL1(GFP\textsuperscript{+})} \cdot \text{FL2\textsuperscript{+} cells}} \times 100.
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**Cell Sorting of GFP-Expressing CD34\textsuperscript{+} Cells**

Pure human CD34\textsuperscript{+} cells (purity, 99.6\%) were infected with AdGFP at MOI of 100 as described earlier. Twenty-four hours after infection, 11\% of the CD34\textsuperscript{+} cells expressed GFP. Subsequently, the cells were stained and propagated in the presence of propidium iodide and the viable cells (>90\%) were sorted into GFP\textsuperscript{+} and GFP\textsuperscript{−} fractions using fluorescence-activated cell sorting (FACS Star Plus cell sorter and LYSIS Program; Becton Dickinson). Analysis after sorting showed a recovery of 70\% of input CD34\textsuperscript{+} cells with viability of greater than 90\% for both fractions.

**Blockage of Vimentin Receptor Before AdGFP Infection**

CD41\textsuperscript{a} cells expanded with TPO/KL/IL-6 were incubated with 0.1 mM of monoclonal antibody to vimentin receptor (VnR) (α,β3 integrin, L203, a gift from T.J. Wickham, GenVec, Rockville, MD) for 2 hours after AdGFP infection. After washing, the cells were infected with AdGFP (100 MOI, 12 hours, 37°C). Twelve hours and 36 hours after infection, the expression of GFP by CD41\textsuperscript{a} cells was analyzed by flow cytometry and compared with AdGFP-infected CD41\textsuperscript{a} cells that were not preincubated with anti-VnR antibodies.

**Morphology and Cell Counts**

Liquid cultures and cytospin preparations were assayed by UV microscopy for intracellular GFP expression. Phase-contrast microscopy of liquid culture and light microscopy (Nikon, UFX-IIA) of Wright-Giemsa–stained cytospin preparations were used to document cell morphology and maturation features (camera, Nikon FX-35A; color print film, Kodak ASA 200–400). Cell numbers and viability of expanded cells were routinely determined by trypsin blue exclusion using Neubauer hematocytometer chambers.

**Southern Blot Analysis for Adenoviral DNA**

Total DNA was extracted using protease K digestion and phenol-chloroform extraction. The DNA was digested over night with HindIII at 37°C. Ten micrograms of digested DNA were electrophoresed on a 3% agarose gel and subsequently transferred to nitrocellulose membranes. Hybridization (30 minutes at 65°C) was performed with a 32P-labeled adenovirus-specific probe detecting the 2.9-kb digestion product of the E4 gene.

**Semiquantitative Reverse-Transcriptase Polymerase Chain Reaction for the Common Coxsackie and Adenovirus Receptor (CAR)**

Total RNA extracts were prepared from CD34\textsuperscript{+} cells and expanded CD41\textsuperscript{a} cells, which were enriched for megakaryocytic cells to 98\% purity using immunoaffinity columns. 15G RNA was extracted using the Ultraspec RNA isolation system (Biotec Laboratories, Houston, TX). Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed for simultaneous amplification of the transcription products of the recently described common receptor for coxsackie B and adenovirus (CAR)\textsuperscript{18} and the housekeeping gene for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers for CAR were designed using the cDNA sequence obtained from the National Center for Biotechnology Information gene bank (accession no. Y07593) to provide an amplification product of 432 bp (5′-primer: TCTCATCTGTCCTTCCTG; 3′-primer: TAAATCTTGGG-GAGACTGTC). The primers for GAPDH were designed using the gene sequence published at NCBI gene bank (accession no. M33197) to provide an amplification product of 627 bp (5′-primer: GGAAGGTGAAGGTCG-GAGTC; 3′-primer: AACATCATCCTCGCCTCTAG). Briefly, primers were synthesized using 392 DNA/RNA Synthesizer (Applied Biosystem, Perkin-Elmer Co, Foster City, CA) oligo synthesizer and purified with NAP-25 columns (Pharmacia LKB Biotechnology, Arlington, IL) according to the manufacturer’s instructions. Then, 1 µg of RNA was mixed with 5 U AMV RT (Promega, Madison, WI) and 5 U TFI DNA polymerase (Promega) in the presence of dNTP mix and of 5× reaction buffer (Tris base 242 mM, glacial acetic acid 57.1 µL/mL, 50 mmol/L EDTA, pH 8.0, Promega). For the RT reaction, the mixture was incubated at 48°C for 45 minutes followed by a denaturation step (94°C, 2 minutes) using a Perkin Elmer Thermocycler. Subsequently, 35 cycles of PCR reaction (94°C for 30 seconds, 56°C for 1 minute, and 68°C for 2 minutes) plus one cycle of extension (68°C for 7 minutes) were performed. The RT-PCR product was then electrophoresed on a 2% TBE-agarose gel and stained with ethidium bromide. RNA from CD34\textsuperscript{+} and CD41a\textsuperscript{+} cells was compared with RNA isolated from human umbilical cord vein endothelial cells (HUVEC; positive control) and the KG1a leukemia cell line (negative control). For control of the RT-reaction, RNA from the first sample of CD34\textsuperscript{+} cells was treated with RNase H (Boehringer Mannheim, Indianapolis, IN) 1 U/µL at 37°C for 15 minutes before RT-PCR.

**Clonogenic Assay**

CD34\textsuperscript{+} cells (10\(^3\) cells/mL) were plated in IMDM/20% FCS and 0.36% agarose in the presence of KL, EPO, IL-3, IL-6, and G-CSF (Table 1) and incubated at 37°C in 100% humidity and 5.2\% CO\textsubscript{2} for 14 days. Colonies that consisted of more than 50 cells were quantified using an inverted microscope (×40).

**Sequential Dilution Expansion (Delta Assay)**

AdGFP-infected CD34\textsuperscript{+} cells (4 × 10\(^4\) cells/mL) were expanded for 7 days in IMDM/20% FCS in the presence of KL, IL-3, IL-6, G-CSF, and EPO. After 7 days, the cells were washed with IMDM/20% FCS and the viable cells were quantified using trypsin blue exclusion. An aliquot of 4 × 10\(^4\) cells was expanded again in the same conditions for another 7 days. This was repeated for three rounds and the cumulative number of cells produced over 21 days of expansion was calculated. Additionaly, every week, the number of GFP\textsuperscript{+} cells was determined by flow cytometry and the cumulative number of GFP\textsuperscript{+} progeny generated during the expansion period was quantified.

**Statistics**

Results are shown as the mean ± SD of at least three experiments. For statistical comparison Student’s t test for nonparametric data was used. For multivariable analysis, two-way analysis of variance (ANOVA) was performed.

**RESULTS**

**Adenovirus Infection Efficiency of Hematopoietic Precursor Cells**

Human CD34\textsuperscript{+} cells purified from leukapheresed peripheral blood were expanded ex vivo for 10 days with various cytokine cocktails to generate cell populations enriched for lineage-committed precursor cells. Ex vivo expanded cells were then infected with different MOIs of AdGFP and analyzed for GFP expression by lineage-committed precursor cells using two-color flow cytometry 3 days after infection. A typical profile of GFP expression in different ex vivo expanded precursor cells with a MOI of 100 is shown in Fig 1A. Using PE-conjugated monoclonal antibody to different lineages, GFP expression (FITC expression) could be detected in 78\% of megakaryocytic (CD41\textsuperscript{a} and CD42b\textsuperscript{−}) cells, 82\% of dendritic (CD1a\textsuperscript{+}) cells,
41% of RBC precursors (glycophorin A⁺), and 32% of monocytic (CD14⁺) cells. As summarized in Fig 1B, on average (n = 3 to 4), infection with 50 MOI of AdGFP resulted in GFP expression of 61.9% ± 8.2% of dendritic cells (CD1α⁺) generated with KL, GM-CSF, and TNF-α; 29.7% ± 4.1% of megakaryocytic cells (CD41α⁺) generated with TPO, KL, and IL-6; 15.9% ± 6.2% of monocytic cells (CD14⁺) obtained with GM-CSF, KL, and IL-3; and 26.6% ± 4.0% of glycophorin A⁺ RBC precursors obtained with KL and EPO.
RBC precursors obtained with KL and EPO. Higher MOIs (100 to 500) of AdGFP resulted in a higher proportion of cells expressing GFP.

Phenotypic and functional maturation of hematopoietic precursor cells is controlled in part by the cytokine milieu. The influence of cytokines on infectibility of hematopoietic precursor cells was studied on megakaryocytes (CD41a⁺). For all vector MOIs used (10 to 500), CD41a⁺ cells generated in the presence of IL-6/TPO/KL showed the most intense fluorescence as compared with CD41a⁺ cells grown in the presence of IL-3/TPO/KL or TPO/KL (data not shown). This suggests that either the vector load per cell or transgene expression or both were enhanced at a given MOI in the presence of IL-6 as compared with other cytokine combinations.

Seven days after infection of CD41a⁺ cells with AdGFP, the viability as assessed by trypan blue exclusion and propidium iodide staining was between 80% and 90% over a range of MOIs of 10 to 500. There was no difference in cell viability in three different cytokine combinations used for expansion of the megakaryocytes. Southern blot analysis was performed on total DNA extracts from megakaryocytes 7 days after infection; the amount of adenoviral DNA recovered was proportional to the initial MOIs.

Kinetics of AdGFP Expression in Megakaryocytic and Erythroid Progenitor Cells

The expression of the transgene over time was investigated in megakaryocytic (CD41a⁺) and erythrocytic (glycophorin A⁺) cells. After a lag phase of 2 to 4 hours following infection, the number of GFP⁺ CD41a⁺ cells, as well as the intensity of GFP expression (mean fluorescence intensity), increased as a function of time after infection and of the MOI used for infection (Fig 2). Seventy-two to 96 hours after infection, the number of GFP-expressing cells and their fluorescence intensity plateaued. Subsequently, the number of GFP-expressing megakaryocytes decreased, particularly if the cells were exposed to high MOI levels (100 to 500) (Fig 2A). The number of GFP-expressing glycophorin A⁺ cells increased with similar kinetics, reaching a maximum 120 to 144 hours after infection and remaining unchanged thereafter (Fig 2B).

Blockage of Adenovector Infection by Anti-VnR Antibodies

AdVec use a variety of integrins expressed on hematopoietic cells for attachment and cell entry. Megakaryocytes express αvβ3 integrins (VnR), which function as adenoviral vector coreceptors. CD41a⁺ cells were infected with AdGFP (MOI, 100) after preincubation with blocking monoclonal antibody (L203) to VnR. Subsequently, infection efficiency was assessed by flow cytometry. Compared with CD41a⁺ cells that had not been preincubated with VnR monoclonal antibody, blockage of αvβ3 integrins partially inhibited the infection of CD41a⁺ cells. As shown in Fig 3, 36 hours after AdGFP infection, the frequency of GFP-expressing CD41a⁺ cells was reduced from 50% (no preincubation with antibodies) to 15% (preincubation with 0.1 µg/mL of VnR monoclonal antibody).

Infection Efficiency of Purified CD34⁺ Cells

A maximal expression of GFP in 19% ± 1% of CD34⁺ cells was achieved with an MOI of 50, 72 hours after infection (Fig 1B). Twenty-four hours after infection (MOI, 100), CD34⁺ cells expressing GFP were sorted by FACS. As shown in Fig 4A, 11% of the CD34⁺ cells expressed GFP. Analysis after sorting showed 98% of positively sorted cells expressing GFP (Fig 4B). The assessment of viability of GFP⁺ and GFP⁻ fraction by trypan blue exclusion showed greater than 90% viable cells in both populations.
Proliferative Potential and Plating Efficiency of AdGFP-Infected CD34\(^{+}\) Cells

The progenitor content of AdGFP-infected CD34\(^{+}\) cells was determined by clonogenic assay in 0.36% agarose. The plating efficiency of unsorted, adenovector-exposed CD34\(^{+}\) cells remained unchanged over a range of 0 to 100 MOI (Fig 5). At an MOI of 1,000, there was a significant decrease in clonogenic capacity to 20% to 30% of noninfected CD34\(^{+}\) cells. FACSorted 98% pure CD34\(^{+}\)/GFP\(^{+}\) cells that were previously exposed to AdGFP with an MOI of 100 maintained normal plating efficiency (Fig 5).

The proliferative capacity of AdGFP-infected CD34\(^{+}\) cells was analyzed in a cytokine-driven sequential dilution expansion assay over 3 weeks (Delta assay\(^{19}\)). Before expansion, CD34\(^{+}\) cells were exposed to AdGFP using MOIs of 0 to 1,000. The proliferative capacity of CD34\(^{+}\) cells remained unchanged independent of the dose of AdGFP used for infection and showed a cumulative expansion of 2,000- to 3,000-fold over 21 days (Fig 6). Only CD34\(^{+}\) cells that were exposed to a high MOI (>100) cumulatively generated increasing numbers of GFP\(^{+}\) progeny (expansion, 10- to 15-fold). If CD34\(^{+}\) cells were exposed to lower MOIs (<100), there was no detectable expansion of GFP\(^{+}\) cells or they disappeared completely during cell expansion.

Morphologic Assessment of AdGFP-Infected Precursor Cells

Labeling of hematopoietic cells with GFP facilitates morphologic evaluation of stability of transgene expression and the fate of infected precursor cells over time during ex vivo expansion.

Fig 3. Suppression of AdGFP infection by blocking of VnR (\(\alpha_v\beta_3\) integrin). VnR expressed on megakaryocytic precursor cells was blocked with a saturable dose of monoclonal antibody to VnR (L203) before incubation of the cells with AdGFP (500 MOI). As assessed by flow cytometry 36 hours after infection, the number of GFP expressing megakaryocytic cells (CD41a\(^{+}\)) was reduced from 50% in control culture to 18% in culture that was preincubated with VnR antibodies. \(\square\) Preincubation with anti-VnR antibody (0.1 \(\mu\)g/mL). \(\square\) No anti-VnR antibody.

Fig 4. Cell sorting of GFP-expressing CD34\(^{+}\) cells. Purified CD34\(^{+}\) cells were infected with 100 MOI of AdGFP and analyzed 24 hours after infection for GFP expression. (A) Eleven percent of CD34\(^{+}\) cells expressed GFP, allowing sorting of the cells into GFP\(^{+}\) and GFP\(^{-}\) fractions. (B) Reanalysis after cell sorting showed 98% purity of the GFP-expressing population of CD34\(^{+}\) cells. (C) The GFP\(^{-}\) fraction was 97% pure.
Ninety-six hours after infection of CD41a<sup>+</sup> megakaryocytes, the majority of the cells showed intensive GFP expression (Fig 7A). Cytospin preparations of the same expansion culture confirmed the presence of normal, polyploid megakaryocytes (Fig 7B) that elicited bright green fluorescence upon excitation with UV light (Fig 7C). Morphologic details, such as cytoplasm pseudopods, blebs, and platelet-like particles, were recognizable. Dendritic cells in liquid culture generated from CD34<sup>+</sup> cells with GM-CSF, KL, and TNF-α showed typical maturation features, such as long cytoplasmic veils (Fig 7D), and displayed striking cytoplasmic GFP expression in UV light (Fig 7E). In expansion cultures of myelomonocytic cells (Fig 7F), mainly monocyctic cells showed bright GFP expression (Fig 7G).

CD34<sup>+</sup> Cells Express CAR

CD34<sup>+</sup> cells do not express α<sub>v</sub>β<sub>3</sub> or α<sub>v</sub>β<sub>5</sub> integrins.<sup>22,23</sup> Therefore, we speculated that the CD34<sup>+</sup> population may express the recently described common receptor for coxsackie B virus and adenovirus (CAR).<sup>18</sup> In this regard, we performed RT-PCR on RNA derived from purified populations of CD34<sup>+</sup> cells. As controls, RNA derived from HUVEC (positive control) and RNA from the leukemic cell line KG1α, which is not infectable with AdV ec vectors even at MOIs greater than 500 (negative control), were used. RT-PCR performed with RNA extracts from two different samples of CD34<sup>+</sup> cells was positive for the expected amplification product for CAR of 432 bp (Fig 8).

DISCUSSION

Noncycling hematopoietic cells are resistant to stable transgene expression using currently available gene-transfer techniques. Introduction of transgenes with standard techniques, including calcium phosphate transfection, lipofection, or electro-
samples of peripheral blood CD34 cells treated with RNAse H before RT-PCR. Two different HUVEC (lane 4) demonstrated a strong signal of expected size (432 bp) for CAR; as well as RT control was performed with RNA from CD34 cells (lanes 1 and 2), as well as KG1a (negative control) included in this experiment. RT control was performed with RNA from CD34 cells that were treated with RNase H before RT-PCR. Two different samples of peripheral blood CD34+ cells (lanes 1 and 2), as well as HUVEC (lane 4), demonstrated a strong signal of expected size (432 bp) for CAR. The presence of GAPDH (627 bp) in all samples demonstrates equal representation of mRNA from each sample.

There are several lines of evidence indicating the susceptibility of hematopoietic cells to AdVec infection. First, adenovirus binds to integrins expressed on certain hematopoietic precursor cells and uses these adhesion molecules for cell entry. Second, cells of hematopoietic origin play an important role as a reservoir for adenoviruses in immunocompromised individuals. Third, in this report, we show that various molecules, known to function as AdVec receptors, are expressed on CD34+ progenitors, as well as on differentiated progeny.

To assess infection efficiency and subsequent transgene expression kinetics in human hematopoietic progenitor and precursor cells, we used a replication-deficient adenovector expressing the humanized GFP gene derived from the jellyfish *Aequorea victoria* that is driven by CMV promoter. The intracellular expression of GFP facilitates real-time analysis and quantification of infected cells by fluorescence microscopy, flow cytometry, and functional analysis of the infected cells following FACS sorting.

To evaluate infection and expression efficiency of adenovirally delivered transgenes in progenitor and lineage-committed hematopoietic cells, we infected ex vivo expanded hematopoietic precursors of various lineages with AdGFP. Purified human peripheral blood CD34+ cells were expanded in the presence of cytokine combinations designed to promote lineage-specific differentiation. On day 10 of expansion, the cells were infected with AdGFP at MOIs of 10 to 500. Relative to erythroid and myeloid lineages, AdGFP showed higher affinity for megakaryocytic and dendritic cells. At MOIs greater than 100, more than 80% of dendritic cells and 75% of megakaryocytes expressed GFP. Myeloid and monocytic precursors showed the lowest infection efficiency, while glycophorin A+ RBC precursor cells showed intermediate susceptibility of infection to AdVec.

Time-course studies performed with megakaryocytic and glycophorin A+ RBC precursors showed maximal transgene expression occurring 72 to 96 hours after infection, resulting in 10% to 60% transduced cells depending on the MOI of AdGFP used. Due to maturation and disintegration of megakaryocytes into proplatelets, the relative number of GFP expressing CD41a+ cells decreased later on. In contrast, the proportion of transduced RBC precursors remained unchanged upon reaching maximal expression over at least 6 days.

Dendritic cells represent one of the most versatile types of antigen-presenting cells (APC) that reside in bone marrow, skin, parenchymatous organs, and lymphoid tissue. By circulation in parenchymatous organs, and lymphoid tissue. By circulation in the bloodstream, dendritic cells and their precursor cells orchestrate cellular immunity through interaction with T cells. Dendritic cells are characterized by a unique morphology and repertoire of cell-surface molecules, including major histocompatibility antigens (MHC) class I and II, adhesion, and costimulatory molecules. Ex vivo expansion of large numbers of functionally active dendritic cells has sparked interest in the potential of genetically modified dendritic cells for the treatment of cancer, autoimmune diseases, and human immunodeficiency virus. AdVec deliver and express transgenes with...
high efficiency into dendritic cells.\textsuperscript{54,55} Forced expression of target antigens in dendritic cells using AdVec provides a novel strategy to engineer dendritic cells for these therapies.

Infection of hematopoietic precursor cells with AdVec does not impair the final differentiation of the targeted cells. Despite the presence and expression of the GFP gene, precursor cells underwent normal differentiation into megakaryocytes, neutrophils, monocytes, nucleated RBCs, and dendritic cells. The amount of transgene recovered from megakaryocytes 7 days after infection correlated with the vector dose used for infection, suggesting stable coexistence of the transgene in maturing precursor cells. Based on these findings, adenovirally mediated transgene expression in hematopoietic cells can lead to measurable amount of transgene product in postmitotic, mature hematopoietic cells and their progeny. This may have therapeutic implications for the treatment of inherited hematologic disorders, such as sickle cell disease and hemophilia, where transient and high level expression of normal hemoglobin in RBCs or clotting factors in platelets may alleviate complications of the underlying genetic disorders.

Our results show that purified CD34\textsuperscript{+} cells can be reproducibly infected with AdGFP. Overall, we found a saturable infection efficiency of CD34\textsuperscript{+} cells of 20% to 25%, which was accomplished by using adenovirus doses of 50 to 100 MOI. Plating efficiency of AdGFP-infected CD34\textsuperscript{+} cells is not diminished by virus load corresponding to an MOI of 1 to 100. However, a higher MOI (>500) leads to significantly reduced colony formation. These results are in agreement with Watanabe et al.\textsuperscript{a} and Neering et al.\textsuperscript{b} who have shown, that 6% to 45% of CD34\textsuperscript{+} cells obtained from bone marrow or peripheral blood can be infected with AdVec expressing \textit{Escherichia coli} β-galactosidase using high MOIs (>500) of AdVec.

However, the fate of adenoviral transgene in transduced CD34\textsuperscript{+} progenitor cells during the process of proliferation and differentiation has not well studied. Adenoviral transgenes delivered to CD34\textsuperscript{+} progenitor cells remain epichromosomal and are not duplicated during cell division. Since it is difficult to study the fate of adenoviral transferred gene to the CD34 progeny by β-galactosidase, we took advantage of GFP expression in viable replicating progenitor cells to evaluate the adenoviral gene expression in proliferating CD34\textsuperscript{+} progenitor cells. We show in Delta expansion cultures of infected CD34\textsuperscript{+} cells that progeny derived from GFP\textsuperscript{+} CD34\textsuperscript{+} cells express GFP if high MOIs (>100) are used for infection of the original CD34\textsuperscript{+} cells, suggesting that the introduced transgenes were transferred from CD34\textsuperscript{+} cells to the daughter cells. The expansion capacity of the whole population of CD34\textsuperscript{+} cells that were exposed to AdGFP was 2,000- to 3,000-fold and independent of the MOI used for infection of the CD34\textsuperscript{+} cells (MOI, 1 to 1,000). In summary, this implies that adenovirally infected CD34\textsuperscript{+} cells disappear during cell expansion, either by loss of the transgene during cell division or by dilution and overgrowth of the GFP\textsuperscript{+} cells with noninfected cells. Therefore, adenoviral gene transfer for therapeutic purposes may achieve optimal results if mature postmitotic precursor cells are targeted. On the other hand, AdVec used for tumor-purging purposes should be approached with caution, since uncommitted CD34\textsuperscript{+} cells can demonstrate significant susceptibility to AdVec infection and toxicity upon exposure to higher MOIs (>100).

Infection efficiency of hematopoietic cells varies considerably in the different lineages. This may be explained by the differential expression of integrins on the cell surface of various hematopoietic cell populations.\textsuperscript{35-40} Expression of α\textsubscript{v} and β\textsubscript{3}/β\textsubscript{1} integrins by hematopoietic cells may play a critical role in adenovirus entry into precursor cells.\textsuperscript{42,20,24} During differentiation of uncommitted hematopoietic progenitors into lineage-restricted precursors and mature hematopoietic cells, the expression of integrins is controlled in part by cytokines released by the stromal cells in the bone marrow microenvironment.\textsuperscript{49,51} Megakaryocytic and dendritic precursor cells express integrins\textsuperscript{35,36,39,43,46} and were found to be most susceptible to AdVec infection. Our finding that uptake of AdGFP by megakaryocytes is only partially inhibited by α\textsubscript{v}β\textsubscript{3} antibodies imply that adenovirus internalization by megakaryocytes may partially be mediated through integrins.

We found that mRNA of the recently described CAR\textsuperscript{18} is expressed by peripheral blood CD34\textsuperscript{+} cells. Transfection with CAR has been shown to enhance the susceptibility of rodent cells to transduction by AdVec. Expression of CAR protein may thus be important for gene delivery to CD34\textsuperscript{+} cells.\textsuperscript{18,53} Differential expression of CAR and integrins may account for the wide range of susceptibility for adenovector infection observed in the different stages of differentiation and maturation of hematopoietic precursor cells. Identification of factors that modulate CAR and integrin expression may allow augmentation of infectibility of hematopoietic precursor and progenitor cells with AdVec.

GFP-cDNA delivered by AdVec provides an excellent tool for quantifying infection and expression efficiency of these vectors over time. Labeling of hematopoietic cells, particularly megakaryocytic progenitor and precursor cells, with GFP in vitro and in vivo will allow studies that will lead to new insights into proliferation, maturation, trafficking, and intercellular interactions of various hematopoietic subpopulations. Such studies will be facilitated by the fact that GFP-expressing hematopoietic cells maintain their functional capacity, can be FACS sorted, and can ultimately be used for proliferation and differentiation assays. Furthermore, based on the presented data, we speculate that transient expression of therapeutic transgenes in terminally differentiated hematopoietic cells using an adenoviral strategy may be used for the treatment of various inherited and acquired disorders. High-level expression of therapeutic proteins such as cytokines, growth factors, hemoglobin, clotting factors, receptor molecules, and tumor antigens in terminally differentiated hematopoietic cells may become applicable for the treatment of acute and chronic hematologic and infectious disorders, as well as lymphoproliferative and myeloproliferative diseases.

\textbf{REFERENCES}

ADENOVIRAL GENE TRANSFER INTO PROGENITORS


High-Efficiency Gene Transfer Into Ex Vivo Expanded Human Hematopoietic Progenitors and Precursor Cells by Adenovirus Vectors

Beat M. Frey, Neil R. Hackett, Jeffrey M. Bergelson, Robert Finberg, Ronald G. Crystal, Malcolm A.S. Moore and Shahin Rafii