

Maturation and Lineage-Specific Expression of the Coxsackie and Adenovirus Receptor in Hematopoietic Cells

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ABSTRACT

Adenovirus vectors have been used to transfer genes into both hematopoietic progenitor cells and tumor cells, including carcinoma cells that have metastasized to bone marrow (BM). However, the relative susceptibility of different subsets of hematopoietic cells is unknown. In permissive cells adenoviral-mediated gene transfer is mediated by the coxsackievirus and adenovirus receptor (CAR) protein and α_v integrins expressed on the cell surface of the target cells. This prompted us to investigate the expression of CAR on subpopulations of hematopoietic cells, determine whether this protein played a role in adenovirus-mediated gene transfer of hematopoietic cells and whether we could modulate CAR to enhance

gene transfer efficiency. In this report we show that CAR is expressed on approximately 40% of all human BM cells, including erythroid and myeloid cells, but not lymphoid cells. Of the CD34⁺ cells, 10%-15% expressed CAR, but this did not include most colony-forming progenitor cells, nor the most primitive CD38⁻ subpopulation. The presence of CAR correlated well with gene transfer efficiency, but we were unable to induce CAR expression on immature, noncommitted progenitor cells. In conclusion, our results show that primitive hematopoietic progenitor cells lack CAR expression, but that expression is acquired during erythroid and myeloid differentiation. *Stem Cells* 2000;18:176-182

INTRODUCTION

The easy accessibility of hematopoietic progenitor cells and their ability to generate long-term progeny in vivo are two characteristics that make these cells important targets for gene therapy. For this purpose, a wide variety of viruses have been used including retro-, adeno-, adeno-associated, and lentiviruses [1-6]. Adenoviruses are able to infect noncycling cells and can be concentrated to extremely high titers; however, gene expression is transient. Thus, for gene therapy applications in which transient gene expression is desired, adenovirus may be the preferred vector for gene delivery into quiescent hematopoietic progenitor cells. Examples are the delivery of the amphotropic retroviral receptor or a mitogen to increase the sensitivity of cells to subsequent retroviral infection or improve the success rate of integration of a retrovirus-encoded transgene into the genome, respectively

[7, 8]. The susceptibility of CD34⁺ hematopoietic progenitor cells to adenovirus is somewhat controversial. Recent studies suggest that adenovirus vectors carrying a "suicide" gene may be suitable for bone marrow (BM) purging of cancer cells; in these experiments the breast carcinoma cells tested were much more easily transduced than freshly isolated BM cells, which were relatively resistant [9, 10]. Because of interest in using adenovirus as a purging vector, it is extremely important to establish the susceptibility of primitive BM cells to infection and the different mechanisms by which virus may enter the cell.

Cellular infection by adenovirus is a multistep process that involves the interaction of the trimeric fiber protein and the pentameric penton base protein of the virus with specific receptors on the target cells. First, the virus attaches to the cell, a process mediated by the fiber protein. The cellular

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receptor for the fiber protein was recently identified with the isolation of the common coxsackie and adenovirus receptor (CAR) protein [11]. After attachment, virus internalization and membrane permeabilization occur through the interaction of the penton base protein with α_v integrins on the target cells [12]. Although adenovirus infection is most efficient when both CAR and α_v integrins are present on the target cells, there is increasing evidence for successful adenovirus-mediated gene transfer using alternative pathways that circumvent the lack of either type of receptor [13, 14].

Adenovirus infection of human CD34⁺ hematopoietic progenitor cells, a population that includes long-term repopulating stem cells, requires certain culture conditions and a high multiplicity of infectious (MOI) particles per cell [2]. Compared to certain primary cells or tumor cell lines, the relatively inefficient adenovirus-mediated gene transfer of hematopoietic progenitor cells may be in part due to the lack of expression of α_v integrins on their cell surface [15, 16]. Little is known about the expression of CAR on hematopoietic cells, although mRNA for the CAR protein has been demonstrated in CD34⁺ cells isolated from leukopheresis products [17]. In light of these findings, we wanted to investigate whether the CAR protein is expressed on the cell surface of subpopulations of hematopoietic cells. If so, we were interested in answering the following questions: A) does the expression of the CAR protein correlate with susceptibility to adenoviral gene transfer in hematopoietic cells and B) can we identify cytokines that modulate the expression of CAR and therefore the gene transfer efficiency? We show that CAR expression on freshly isolated BM cells is mainly found on differentiated erythroid and myeloid cells, on a small proportion of CD34⁺ progenitor cells, but not on lymphoid cells. Gene delivery into freshly isolated CD34⁺ cells correlates well with the level of CAR expression, but still requires large amounts of virus.

MATERIALS AND METHODS

Preparation of Human BM Cells

Discarded bags and attached filters from BM harvests of normal donors were rinsed with Iscove's modified Dulbecco's medium (IMDM) (Life Technologies; Grand Island, NY; <http://www.lifetech.com>) containing 2% fetal bovine serum (FBS) (Sigma Chemical Co.; St. Louis, MO; <http://www.sigma-aldrich.com>) to obtain the remaining BM cells. The cells were then centrifuged over a layer of Histopaque®-1077 (Sigma) to deplete erythroid and granulocytic cells. The cells were frozen (in IMDM, 50% FBS, 10% dimethylsulfoxide [Sigma]) and further separated on the day of the experiment. CD34⁺ progenitor cells were enriched using a positive selection method as recommended by the

manufacturer (Ceprate LC separation system, CellPro; Bothell, WA).

Cell Staining and Sorting

Flow cytometric detection of CAR on the cell surface was performed using the monoclonal antibody (mAb) RmcB [18], which was either directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin. To define the different hematopoietic subpopulations and their expression of CAR, BM cells were simultaneously stained with anti-CD34-cyanine 5 (Becton Dickinson; San Jose, CA; <http://www.bd.com>), RmcB-FITC, and a mAb directed against one of the following lineage markers: CD33, CD14, or CD38 (Becton Dickinson), glycophorin-A, CD4 together with CD8, or CD19 (PharMingen; San Diego, CA; <http://www.pharMingen.com>). In every experiment irrelevant isotype controlled mAbs were used to determine background staining. All staining procedures were done in phosphate buffered saline (PBS) (Life Technologies) that contained 2% FBS. The cell labeling was performed on ice (35 min) after which the cells were washed twice. Propidium iodide (PI) (Sigma) (2 μ g/ml) was added during the second wash prior to resuspension in PBS, 2% FBS. Three-color flow cytometric analysis and cell sorting were performed on a Coulter Epics® Elite ESP (Coulter; Hialeah, FL; <http://beckmancoulter.com>).

To analyze individual colonies for CAR expression, colonies were plucked from methylcellulose (MC), incubated for 1 h in PBS containing 2% FCS, to allow the MC to dissolve, spun down once and subsequently stained with the appropriate mAbs. Two-color fluorescence-activated cell sorter (FACS) analysis of the MC colonies and the suspension cultures (see later) were analyzed on a single laser FACScan (Becton Dickinson; Mountain View, CA).

Colony-Forming Cell (CFC) Assay

To determine the CFC content of the sorted CD34⁺ BM cells, cells were plated in IMDM/0.9% MC media (Methocel MC, Fluka; Buchs, Switzerland; <http://www.sigma-aldrich.com>) containing 30% defined FBS (HyClone Laboratories Inc.; Logan, UT; <http://www.hyclone.com>) and the following human recombinant cytokines: *Steel* factor ([SF] 50 ng/ml), interleukin 3 ([IL-3] 20 ng/ml), GM-CSF (20 ng/ml), and erythropoietin ([Epo] 3 U/ml). IL-3, IL-6, and GM-CSF were generous gifts from Genetics Institute (Cambridge, MA; <http://www.genetics.com>). SF and Epo were purchased from R&D Systems (Minneapolis, MN; <http://www.rndsystems.com>). Duplicates of 1,000 cells (or as otherwise indicated) per 35 mm dish were plated. Colonies were scored in situ after 14-20 days of incubation at 37°C in a humidified atmosphere of 5% CO₂ in air using well-established criteria [19].

Serum-Free Suspension Cultures

Enriched CD34⁺ BM cells were cultured in serum-free medium prepared as described previously [20]. Cells were initially cultured in 1 ml volumes in 24-well culture plates and kept at a density below 1×10^6 cells/ml. The medium was supplemented with various combinations of the following cytokines: SF (50 ng/ml), Flt-3 ligand ([FL] 100 ng/ml), IL-6 (10 ng/ml), IL-3 (20 ng/ml), IL-11 (25 ng/ml), GM-CSF (20 ng/ml), and Epo (2 U/ml). FL was kindly provided by Immunex (Seattle, WA; <http://www.immunex.com>) and IL-11 by Genetics Institute. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. At subsequent days, the cultures were harvested, viable cells (excluding trypan blue) were counted using a hemocytometer and phenotypic analysis was performed as described above.

Adenovirus Construction and Preparation

The adenovirus vector that contains the green fluorescent protein gene (AdGFP) was kindly provided by *Bob Carter* and *Richard Mulligan* (Howard Hughes Medical Institution, Children's Hospital, Boston, MA; <http://www.hhmi.org>), and was constructed by first subcloning the GFP cDNA into pAdlox, a shuttle vector that contains a single loxP site. This expression cassette was linearized and cotransfected into CRE8 cells with the ψ 5 helper virus, which is an E1- and E3-deleted version of Ad5 that contains loxP sites flanking the packaging site. Recombination occurs between the two linear molecules at the loxP sites [21]. We then plaque-purified the virus and expanded it on 293 cells using standard techniques. Each virus inoculum was purified by a CsCl step gradient followed by a CsCl equilibrium gradient, dialyzed against a glycerol buffer and stored at -20°C.

Adenoviral Infection Protocol and Analysis

After a short culture period (~4 h) CD34⁺-enriched cells were incubated with AdGFP for 20 h at 37°C, at a MOI of 500, or otherwise indicated. The incubations were done in serum-free medium, supplemented with SF, FL, IL-6, and Epo in 100-200 μ l volumes in 96-well plates when $<10^5$ cells were to be infected, or in 1 μ l cultures in 24-well plates when the cell number was between 10^5 - 10^6 . The analysis by FACS for green fluorescence intensity as a measure for gene transfer was performed immediately after the 24 h of culture.

RESULTS

Expression of CAR on the Cell Surface of Subpopulations of Hematopoietic Cells

BM cell suspensions were stained with mAbs directed against CAR and various lineage markers representative for

erythroid (glycophorin-A), myeloid (CD33 and CD14), and lymphoid (CD19 and CD4/CD8) cells. Figure 1 shows representative FACS profiles: CAR is expressed on ~40% of total BM cells, including glycophorin-A⁺ cells, CD14⁺, and CD33⁺ cells. In contrast, very few CAR⁺ cells can be demonstrated among the lymphoid CD19⁺ or CD4/8⁺ cells. To identify CAR expression on more primitive hematopoietic progenitor cells, BM cells were stained with a cocktail of mAbs identifying CD34, CD38, and CAR. From Figure 2A it is immediately clear that the level of CAR expression on CD34⁺ cells is considerably lower than that on mature myeloid or erythroid cells (Fig. 1). Only 10%-15% of CD34⁺ cells express CAR at a level comparable to that of, e.g., CAR⁺CD33⁺ cells (box 2, Fig. 2A) and 1%-2% express high levels of CAR (box 1, Fig. 2A). Counterstaining CD34⁺CAR⁺ cells with a combination of lineage markers (glycophorin-A, CD14, CD33, CD38, CD19, CD4, and CD8) revealed that these cells expressed one or more of these markers (data not shown). This finding suggests that CAR expression on hematopoietic BM cells may be limited mostly to mature erythroid and

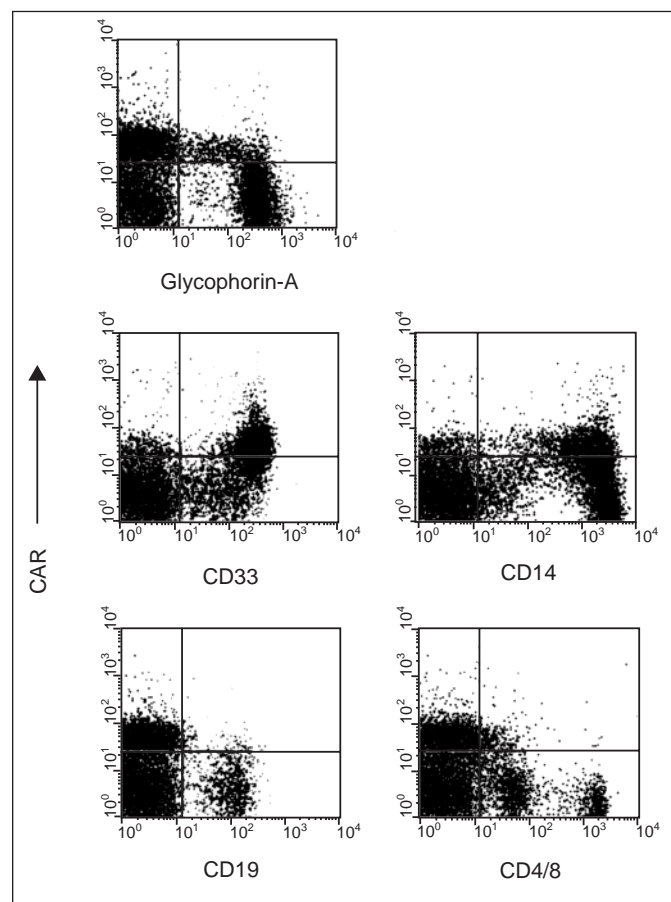


Figure 1. CAR is expressed on the cell surface of erythroid and myeloid, but not lymphoid cells. Depicted are representative profiles of live (PF) unseparated BM mononuclear cells.

myeloid cells and a small proportion of committed progenitor cells. Indeed, when CAR expression was determined on CD34⁺CD38⁻ cells, a population of cells that contains primitive nonobese diabetic/severe combined immunodeficiency (NOD/SCID) repopulating cells [22, 23], CAR expression was not detectable (Figs. 2B and 2C).

CAR Expression on CFC

The phenotypic analysis suggested that the majority of the CD34⁺ progenitor cells does not express CAR. We wanted to investigate whether functional analysis could validate this result. CD34⁺ BM cells were separated on the basis of CAR expression

as indicated in Figure 2A (box 1-3) and the different subsets were then analyzed for their ability to form colonies in MC. One such analysis is shown in Table 1. Most of the colonies are recovered in the CAR⁻ fraction, a distribution that is in accordance with the relative CAR expression on CD34⁺ cells.

Since the progeny of CFC are more differentiated cells, we were interested to determine the CAR expression on these cells. Individual colonies from the CAR⁻ fraction were therefore isolated and the cells stained with an anti-CAR mAb together with the appropriate lineage marker to confirm the morphological appearance of the colony. Colonies scored as BFU-E were counterstained with glycophorin-A and colonies

Figure 2. CD34⁺CD38⁻ progenitor cells do not express CAR. CD34⁺-selected cells were stained with mAb directed against the indicated cell surface antigens. Shown are representative FACS profiles from live (PI⁻) cells. A. In this experiment 11.4% of all CD34⁺ cells expressed intermediate levels of CAR (box 2) and 1.2% high levels (box 1). B. Simultaneous staining with antiCD34 and antiCD38 mAbs revealed that the CD34⁺CD38⁻ cells (indicated by the cells in box 4) do not express CAR (C).

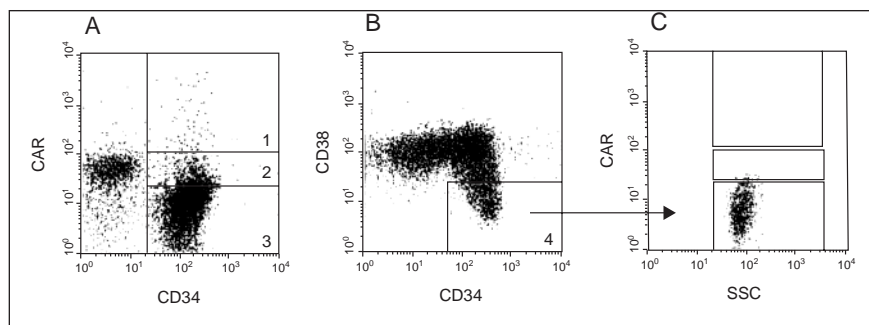


Table 1. The majority of CFC does not express CAR

aCD34 ⁺ subset	Fraction of all CD34 ⁺ cells (%)	Number of Colonies (per 10 ⁴ cells)			bRecovery of Colonies (%)		
		BFU-E	G/M/GM	GEMM	BFU-E	G/M/GM	GEMM
CAR ⁻	85.1	600	450	110	83	78	93
CAR ^{im}	11.4	660	750	50	12	18	6
CAR ⁺⁺	3.5	767	600	33	4	4	1

Data represent one of two experiments; the other experiment showed very similar results.
 aCD34⁺ cells were sorted as indicated in Figure 2A (boxes 1-3). Duplicates of 1,000 cells per dish were plated.
 bThe recovery was calculated as follows: for each subset and type of colony, the observed number per 10⁴ cells was multiplied by the respective fraction that the subset represented of the total CD34⁺ cells. This corrected number was then divided by the total number of colonies recovered (i.e., the sum of the corrected colony numbers of the three subsets) and multiplied by 100.

Figure 3. CAR⁺ cells can be found among the progeny of all types of CFC. Shown are representative FACS profiles of live (PI⁻) cells obtained by plucking MC colonies 14 days after the cells had been plated. Erythroid colonies (A) mostly showed a profile as presented here, but occasionally a level of CAR that was comparable to that of cells obtained from CFU-G/M/GM colonies (B) was detected. (C) Mixed lineage colonies contained cells from the erythroid, myeloid and megakaryocytic lineage.

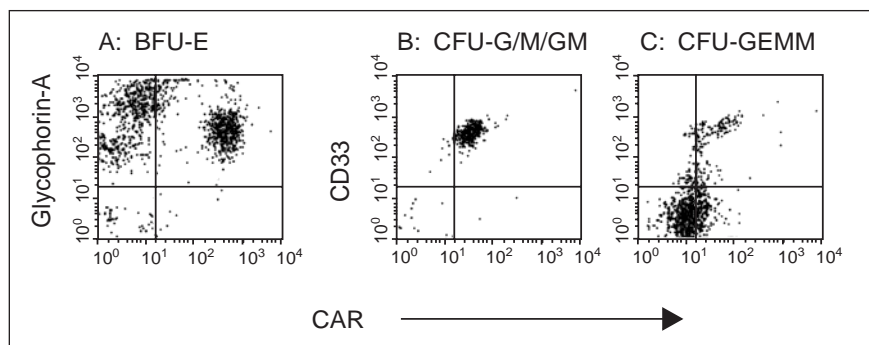
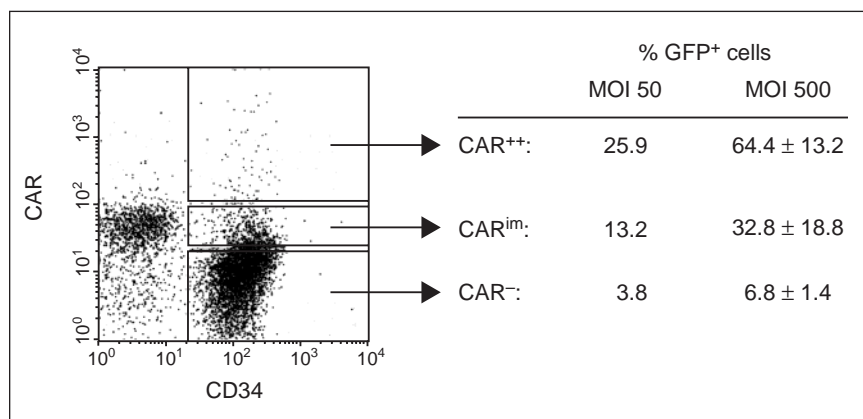


Figure 4. Expression of CAR correlates with adenovirus-mediated gene transfer efficiency. Pre-enriched CD34⁺ cells were simultaneously stained with anti-CAR and anti-CD34 mAbs. CD34⁺ cells were sorted on the basis of CAR as indicated by the boxes. The results of 20 h exposure to AdGFP during a 24-h culture period for each fraction are shown in the figure. Each data point with MOI 500 consists of two to three independent experiments. The data points obtained with MOI 50 represent a single experiment. CAR^{im} cells are CD34⁺ cells that express intermediate levels of CAR. im = intermediate.



scored as granulocytic and/or monocytic (CFU-G/M/GM) were counterstained with CD33. The multilineage colonies (CFU-GEMM) were also stained with CD33 to identify the myeloid component in an often dominant erythroid appearance. Figure 3 shows the various types of colonies that were identified by flow cytometry. A large proportion of cells isolated from erythroid colonies stained brightly positive for CAR (Fig. 3A). Cells from myeloid colonies all stained intermediate positive for CAR (Fig. 3B), as did the CFU-GEMM, but the level of CAR expression was on average lower than that of the myeloid colonies (Fig. 3C). Thus, while the majority of clonogenic progenitors is CAR⁻ (Table 1), their progeny show an increase in the level of CAR expression (Fig. 3).

Adenoviral Gene Transfer Efficiency in Relation to CAR Expression

To determine whether there was a correlation between CAR expression and efficiency of adenoviral gene transfer in CD34⁺ cells, CD34⁺ cells were separated on the basis of CAR expression, and the different fractions were cultured for 24 h. During the last 20 h of culture, cells were exposed to an adenovirus construct that contained the gene for the AdGFP. After 24 h, the culture was then analyzed by FACS for GFP expression (i.e., green fluorescence intensity). Figure 4 shows the combined results of three such experiments. The best gene transfer efficiency was indeed obtained with cells that expressed the highest level of CAR (CAR⁺⁺ cells); 64.4 ± 13.2%, compared to 6.8 ± 1.4% in cells that did not express CAR (CAR⁻ cells). The cells that expressed intermediate levels of CAR (CAR^{im} cells) showed intermediate levels of gene transfer: 32.8 ± 18.8%. This effect was dose-dependent; decreasing the MOI 10-fold reduced the gene transfer rate considerably. Thus, the level of CAR expression on freshly isolated CD34⁺ BM cells correlates well with the proportion of GFP⁺ cells after a 24-h exposure to AdGFP.

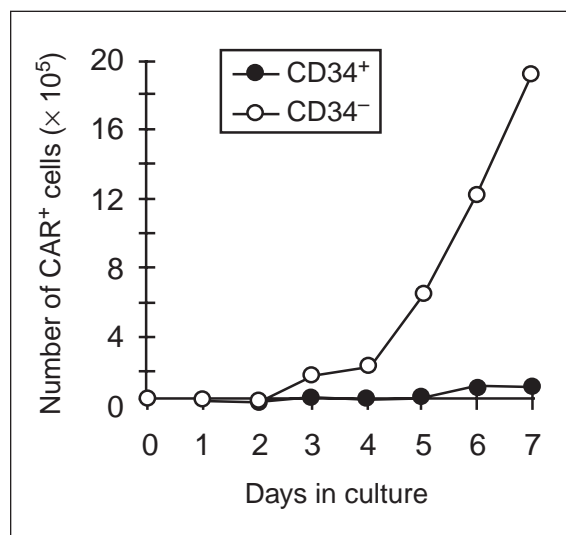


Figure 5. CAR⁺ cells produced in culture are mostly CD34⁻. Presented is one of three experiments, showing the number of CAR⁺ cells (CD34⁺ and CD34⁻) that initiated the culture at day 0 and the production of CAR⁺ cells (CD34⁺ and CD34⁻) at subsequent days. The number of cells was calculated by multiplying the total cell number by the fraction of cells of a particular phenotype obtained by FACS analysis.

Cytokines Do Not Induce CAR Expression

BM cells enriched for CD34⁺ progenitor cells were cultured under serum-free conditions to determine whether one or a combination of cytokines could induce CAR expression on such cells. The following cytokines were tested in one, two, and four-day cultures, either alone or in combination: SF, FL, IL-6, IL-3, IL-11, GM-CSF, and Epo. No cytokine or combination thereof—33 conditions were tested—could be identified that showed a superior effect on CAR expression (data not shown). All subsequent cell cultures were therefore performed in serum-free medium, supplemented with SF, FL, IL-6, and Epo, a culture condition demonstrated to maintain the most

primitive hematopoietic cells [1, 24, 25]. Over a seven-day culture period, the number of CD34⁺ cells that express CAR stays nearly constant (Fig. 5). However, the number of CD34⁺ cells that express CAR increases dramatically with time. Thus, as CD34⁺ cells lose CD34 expression, they acquire CAR. This result, together with the phenotypic analysis and functional CFC data, suggest that expression with CAR in hematopoietic cells is related to myeloid and erythroid differentiation.

DISCUSSION

Adenovirus-mediated gene transfer is highly efficient in permissive cells, such as HeLa cells, or nonpermissive cells stably infected with the gene encoding CAR [11, 14]. In contrast, we show here that the transduction of genes into primitive CD34⁺ hematopoietic cells by an adenovirus construct is not very effective. In our hands, only 15%-20% of CD34⁺ cells exposed for 20 h to adenovirus were transduced, a result that directly correlated with the level of CAR expressed on the cell surface. However, CAR expression was found to be associated with cellular differentiation. These results predict very low adenovirus-mediated gene transfer into immature long-term repopulating hematopoietic stem cells (HSCs). Indeed, in one experiment in which purified CD34⁺CD38⁻ cells were exposed to AdGFP for 20 h of the 24 h in culture, only 2% gene transfer efficiency could be demonstrated (data not shown). These results appear to contrast with a previous report showing that quiescent CD34⁺CD38⁻ cells were GFP⁺ after exposure to an adenovirus GFP construct [2]. Several reasons may account for this difference: first, the post-infection time allowing for gene expression (24 h in our experiment versus 48 h), and second, the starting population that was infected. We infected purified CD34⁺CD38⁻ cells, whereas *Neering et al.* used total CD34⁺ cells and analyzed the proportion of transfected CD34⁺CD38⁻ cells by FACS. It is possible that there are accessory cells present in the CD34⁺ cell population that facilitate gene transfer into other cells. The mechanism by which this occurs is unclear, but it is tempting to speculate that these cells produce certain cytokines that upregulate cell surface molecules, as yet unidentified, that are important for adenoviral infections in primitive hematopoietic cells. Interestingly, although adenoviral gene transfer into human primitive cells is inefficient at best, murine long-term repopulating HSCs are quite efficiently transducible with the same construct (unpublished data, 1999).

Several approaches have been taken to improve adenovirus infection of otherwise nonpermissive cells, such as modulating the viral surface structures with which the virus may attach to the target cells and the use of agents to facilitate the virus-target cell binding [26-28]. Our approach, i.e., trying to induce CAR expression on hematopoietic progenitor cells, has so far been unsuccessful. The cytokines we tested were chosen based on previous studies describing their (relative) beneficial effect on CD34⁺ cells in maintaining NOD/SCID mouse repopulating ability [1, 29, 30]. Because our goal was not only to induce CAR, but also to maintain phenotype/function, the cytokines that were tested were limited to SF, FL, IL-6, IL-3, IL-11, GM-CSF, and Epo. However, studies with other cell types may point us towards examining other cytokines, not usually thought of in relation to culturing CD34⁺ cells. In this regard it is of interest that IL-2 was found to induce the expression of the fiber receptor on the cell surface of lymphocytes, whereas this receptor was undetectable in noncultured, freshly isolated lymphocytes [31]. Whether this receptor was indeed CAR needs to be verified, or, for example the $\alpha_M\beta_2$ integrin, as others have shown to be involved in adenovirus binding to human mononuclear cells [32]. Interestingly, a small subset of CD34⁺ cells do express this integrin [33] and may provide a tool to improve adenovirus-mediated gene transfer into hematopoietic progenitor cells.

In conclusion, our results clearly demonstrate that CAR is expressed on hematopoietic cells and that its expression is directly related to the susceptibility of these cells to adenoviral gene transfer. We also demonstrate that the majority of CAR⁺ hematopoietic cells are lineage-committed cells and not the more primitive CD34⁺ progenitor cells. Our findings, along with those of others [2], that high MOIs are needed to successfully infect hematopoietic cells with adenovirus, suggest that at low MOI, adenovirus may provide a good vehicle for oncolytic therapy in cancer.

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