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RESEARCH ARTICLE

Efficient infection of primitive hematopoietic stem cells by modified adenovirus

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Almost all studies of adenoviral vector-mediated gene transfer have made use of the adenovirus type 5 (Ad5). Unfortunately, Ad5 has been ineffective at infecting hematopoietic progenitor cells (HPC). Chimeric Ad5/F35 vectors that have been engineered to substitute the shorter-shafted fiber protein from Ad35 can efficiently infect committed hematopoietic cells and we now show highly effective gene transfer to primitive progenitor subsets. An Ad5GFP and Ad5/F35GFP vector was added to CD34$^+$ and CD34$^-$lineage$^-$ (lin$^-$) HPC. Only 5–20% of CD34$^+$ and CD34$^-$lin$^-$ cells expressed GFP after Ad5 exposure. In contrast, with the Ad5/F35 vector, 30–70% of the CD34$^+$, 50–70% of the CD34$^-$lin$^-$ and up to 60% of the CD38$^-$ HPC expressed GFP and there was little evident cellular toxicity. Because of these improved results, we also analyzed the ability of Ad5/F35 virus to infect the Hoechst negative 'side population' (SP) of marrow cells, which appear to be among the very earliest multipotent HPC. Between 51% and 80% of marrow SP cells expressed GFP. The infected populations retained their ability to form colonies in two short-term culture systems, with no loss of viability. We also studied the transfer and expression of immunomodulatory genes, CD40L (cell surface expression) and interleukin-2 (secreted). Both were expressed at immunomodulatory levels for $>$5 days. The ability of Ad5/F35 to deliver transgenes to primitive HPC with high efficiency and low toxicity in the absence of growth factors provides an improved means of studying the consequences of transient gene expression in these cells. Gene Therapy (2001) **8,** 930–937.

Keywords: *chimeric adenovirus Ad5/F35; hematopoietic progenitor cells; CD34$^-$lineage$^-$ cells; SP cells; CD40 ligand; interleukin-2*

Introduction

Marrow-derived stem cells can differentiate to both hematopoietic and non-hematopoietic lineages, including bone, cartilage, muscle and perhaps even neurons. Hence, gene transfer to these cells may afford the opportunity to treat a number of diseases. However, the most primitive pluripotent stem cell subsets are infected poorly by most vectors currently available. For example, Moloney-derived retroviruses effectively infect only the small proportion of stem cells that are in or about to enter cell cycle.[1–3] Expression levels are often low and, even though these are integrating vectors, rapid silencing of the transgene expression is a frequent observation.[4] Lentiviral vectors have shown recent promise,[5] and may infect even quiescent cells, but their safety remains to be established, and there are still concerns that the level of gene expression may be inadequate. Adenoviral vectors infect many non-dividing cells with high efficiency and produce a high level of gene expression. Although they are non-integrating, even transient expression in stem cells could have many potential applications, for example for immunotherapeutic protocols, for efforts to enhance stem cell engraftment after transplantation, or for the expression of genes intended to promote differentiation down a particular lineage. Unfortunately, adenovirus serotype 5 (Ad5) infects human hematopoietic progenitor cells very poorly. Indeed this deficiency has been used as a means of purging human marrow of contaminating tumor cells[6,7] since these have a much higher susceptibility to infection by Ad5 vectors, which in this particular application encode a cytotoxic gene. It is possible to achieve stem cell infection with Ad5 serotype if the cells are first stimulated by a variety of hematopoietic growth factors. However, these may induce differentiation and loss of the most pluripotent population,[8] and a high proportion of cells die following infection. Low infectivity is in part due to the absence on most HSC of the Coxsackie-adenoviral receptor (CAR)[9] and of αV integrins.[10,11] Recently, a hybrid Ad5 vector was developed, in which the fiber protein from Ad35 has been substituted for the original fiber.[12] This vector binds in a CAR-independent manner,[13,14] and has successfully been used to infect committed hematopoietic cells. We now study the ability of this hybrid vector to infect the most primitive stem cell subsets, and to discern its effect on their viability and phenotype.

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Results

Comparison of Ad5 and Ad5/F35 infectivity of hematopoietic cell lines

Jurkat, HSB-2 (T cell leukemia), K562 (erythro–granulocytic malignant progenitor cell line) and EBV-LCL (EBV

immortalized mature B cells) were used for initial comparisons of the efficiency of Ad5 and Ad5/F35 infection. The cells were co-cultured for 6 h with 100, 500, 1000 or 2000 viral particles (VP) per cells and then washed to remove free viral particles. Twenty-four hours after infection the cells were analyzed by FACS for GFP expression. Figure 1 shows Ad5/F35 has a significantly higher infectivity than Ad5 for all cell lines. K562 and Jurkat cells (83% and 71% maximum expression respectively) both had a higher percentage of positive cells with Ad5/F35 infection than HSB-2 and EBV-LCL (34% and 38%, respectively). Ad5 had essentially identical and low infectivity for all cell lines, regardless of the dose of VP per cell (Figure 1a). In the successfully infected cells, the intensity of fluorescence is higher in all Ad5/F35 infected, compared with Ad5 infected cell lines (Figure 1a), while Figure 1b confirms the infectivity of the Ad5 particles on 293 control cells.

Kinetics of Ad5/F35 and Ad5 internalization in hematopoietic cells lines

To evaluate the time required by both viruses to infect cells efficiently, Jurkat, HSB-2, K562, and EBV-LCLs were co-cultured with 10^3 VP/cells of Ad5 and Ad5/F35 for 1 h, 2 h, 6 h and 24 h. At each time-point, the cells were washed twice in 4 ml of PBS to remove free viral particles and resuspended in new medium plus 10% serum and analyzed 24 h after infection. Figure 2a shows that more than 50% of the maximum infection efficiency was obtained with only 2 h co-incubation (6 h for HSB-2). The number of positive cells increases with the co-incubation time to a maximum at 24 h of infection. Intensity of fluorescence of positive cells follows a similar time-course, with 50% of maximum fluorescence expression obtained with 2 h exposure, with a peak at 24 h of co-culture. Figure 2b show that the two vectors are equally efficient on an Ad5 permissive cell line (293 cells).

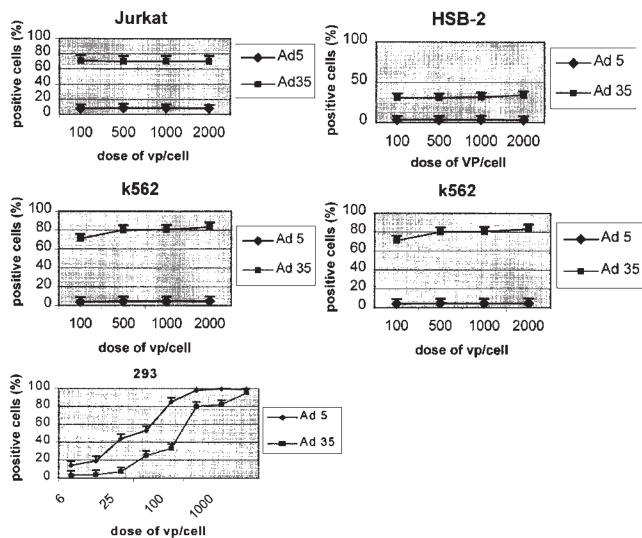

Figure 1 Comparison of Ad5 and Ad5/F35 infectivity in hematopoietic cell lines. K562, HSB-2, Jurkat, and EBC-LCL cells were co-cultured for 6 h with 100, 500, 1000, 2000 viral particles (VP) per cell, and then washed to remove free viral particles. At 24 h after infection, the cells were analyzed for GFP expression by FACS. Ad5-GFP is notated as Ad5 and Ad5/F35-GFP as Ad35. Results are expressed as the percentage of positive green cells and the median fluorescence intensity. The maximum efficacy is obtained with 500 VP and a higher concentration of virus does not increase the number of GFP-positive cells, implying that 600VP is the saturating dose. A dose–response curve is shown for the Ad5 permissive 293 cells.

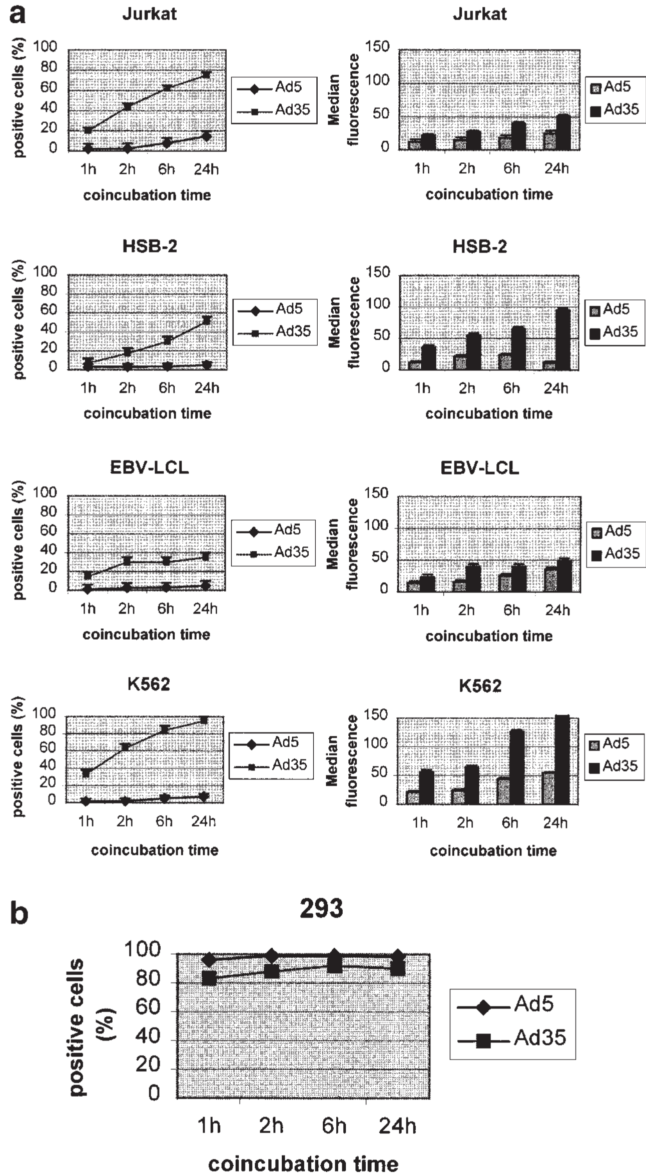

Figure 2 Kinetics of Ad5/F35 and Ad5 internalization in hematopoietic cell lines. K562, HSb-2, Jurkat and EBC-LCL cells were co-cultured for 1 h, 2 h, 6 h and 24 h with 10^3 VP. At each time-point, the cells were washed twice in 4 ml of PBS to remove free viral particles and resuspended in new medium plus 10% serum. Results are expressed as the percentage of GFP-expressing cells and the median of GFP fluorescence intensity. Ad5-GFP is notated as Ad5 and Ad5/F35-GFP as Ad35. The 50% maximum efficacy for the number of positive cells was reached 2 h after infection for most of the cell lines (at 6 h for HSB-2). The maximum GFP expression is observed after 24 h of co-incubation (a). 293 cells were highly permissive to both Ad5 and Ad5/F35 virus as soon as 1 h after infection (b).

CAR and integrin expression

To determine whether the low efficiency of Ad5 expression was associated with failure to express CAR and integrin molecules, the cell lines were stained with mAb to CAR, $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins. As controls we used the CAR-negative NIH3T3 and CAR-positive 293

and Y79 cells lines. Figure 3a and b show that K562 and LCL cell lines do not express CAR. Jurkat and HSB-2 cell lines expressed CAR but lacked expression of αVβ3 integrins.

Ad5/F35 is more infectious than Ad5 in normal human CD34⁺ and CD34⁻lin⁻ cells

The ability of Ad5/F35 to infect hematopoietic cells and cell lines led us to assess its activity in primitive stem cells. After immunoselection, CD34⁺ and CD34⁻lin⁻ cells were infected with 10^3 VP/cells of each virus for 6 h, washed and resuspended. Cells were analyzed by FACS 24 h after infection. Table 1 shows the percentage of GFP-expressing CD34⁺ cells at this time. The infection efficiency is 2.5- to 12-fold higher in Ad5/F35 infected CD34⁺ population compared with Ad5 infected cells. To discover if infectivity extends to the more primitive CD34⁺CD38⁻ subset of stem cells, Ad5/F35 infected CD34⁺ were double stained for CD34 and CD38 expression (Table 2). We next analyzed infection of the even more primitive CD34⁻ lin⁻ subset. infection was three to 74-fold higher in Ad5/F35-infected CD34⁻lin⁻ compared with the same cell populations infected with Ad5 (Table 1). Of note, in all subsets examined, there was wide inter-individual variation in the susceptibility to infection with these vectors, but with a consistent and significant advantage seen for the Ad5/F35 hybrid compared with the Ad5 vector. Regardless of the level of infection, the viability of cells measured by trypan blue staining 24 h after infection showed that Ad5/F35 had no adverse effect (>90% viability), whereas Ad5 significantly reduced viability (<50% viability).

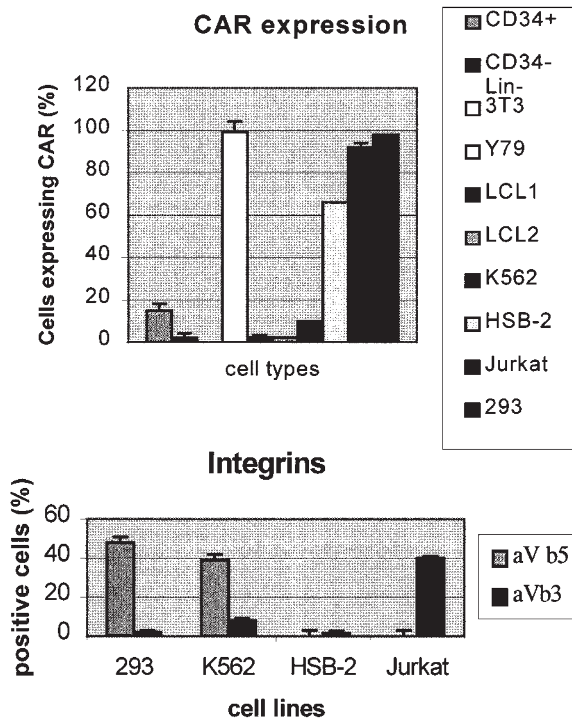

Figure 3 *CAR/integrins expression on cell lines. Cell lines were incubated with CAR, integrin and isotype control antibodies at 4°C in the dark. The cells were washed and incubated with a secondary antibody (rat anti-mouse-PE), and analyzed by FACS.*

Table 1 Ad5/F35 is more infectious thatn Ad5 in CD34⁺ and CD34⁻lin⁻ cells

Patient No.	*CD34⁺*		*CD34⁻lin⁻*	
	Ad5	*Ad5/F35*	*Ad5*	*Ad5/F35*
1	ND	42[a]	ND	57
2	ND	54	ND	77
3	ND	54	ND	68
4	5	37	4	35
5	15	37	1	74
6	4	22	1	10
8	30	72	5	40
9	3	34	0	20
10	13	20	10	30
11	2	25	1	22
12	7	40	ND	ND
13	11	57	15	67.3
14	2	46.7	1	68

After immunoselection, CD34⁺ and CD34⁻lin⁻ cells were infected with 10^3 VP/cells of both viruses for 6 h, washed, and resuspended in medium supplemented with 10% FCS. Cells were analyzed by FACS 24 h after infection.
ND not detemined.
[a]Data are expressed as the percentage of GFP-expressing cells at 24 h after infection.

Table 2 GFP expression by CD34⁺CD38⁺ and CD34⁺CD38⁻ subsets

Patient No.	*CD34⁺*		*CD38⁺*		*CD38⁻*	
	Total CD34⁺	*GFP² CD34⁺*	*Total CD34⁺ CD38⁺*	*GFP⁺ CD34⁺ CD38⁺*	*Total CD34⁺ CD38⁻*	*GFP⁺ CD34⁺ CD38⁻*
4	100[a]	33	70	17	29	17
7	100	47	22	8	79	38
8	100	52	100	36	0	0
9	100	43	90	22	8	2
3	100	72	70	41	30	30
6	100	25	70	10	29	14
10	100	57	95	54	5	3
3	100	20	94	14	4	4

Ad5/F35-infected CD34⁺ were double stained 24 h after infection with CD34-PercP and CD38-PE antibodies and analyzed by FACS. Data are expressed as the percentage of cells expressing CD38⁺ antigen and positive for GFP within the CD34⁺ population.

Side population (SP) progenitor cells are highly infectable

Because of the positive results obtained with the CD34⁺ and CD34⁻lin⁻ populations, we extended our Ad5/F35 infection study to 'side population' (SP) stem cells, a phenotype which defines one of the most primitive and most pluripotent subset of marrow cells.[15] Twenty-four hours after infection by Ad5/F35, the mononuclear bone marrow cells were stained using Hoechst dyes, and analyzed by FACS to verify that Ad5/F35 could infect SP cells and induce them to express GFP. Figure 4 confirms that Ad5/F35 infects 65% of the bone marrow cells determined by GFP expression 24 h after infection. In the SP population, 53% of cells have both the dye efflux characteristic of the SP population and GFP expression. Hence, the vector can infect the pluripotent marrow SP population. The toxicity of the Ad5/F35 is low, since the

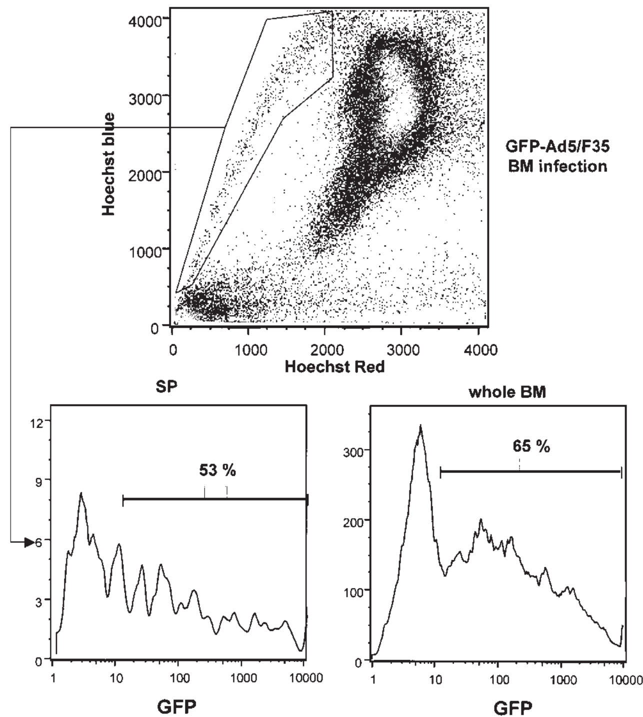

Figure 4 *SP stem cells are highly infectable. Twenty-four hours after infection by Ad5/F35 or Ad5, the mononuclear bone marrow cells were stained for 2 h and analyzed to verify the ability of Ad5/F35 to infect the SP stem cells and express the GFP protein. As assessed by FACS, 53% of SP cells had both efflux activity and expressed GFP protein. Less than 3% of cells were double positive using Ad5.*

viability of the SP cells (determined by PI incorporation) is essentially identical (>90%) in infected and non-infected samples.

Colony-forming ability of stem cells infected by Ad5/F35

To investigate the effect of adenovirus infection on stem cell progenitor function, infected cells were cultured for 2 weeks in methylcellulose. As shown in Table 3, $CD34^+$ cells exposed to Ad5/F35 formed nearly identical numbers of myeloid colonies as the non-infected control group. The same results were obtained for erythroid colony formation in both cases, demonstrating that Ad5/F35 does not preclude progenitor cell replication/maturation.

Expression of therapeutic genes using Ad5/F35

To assess the value of Ad5/F35 for gene therapy approaches, we expressed two genes that have been used in immunotherapy protocols. CD40L is a cell surface molecule involved in the initiation of the T cell immune response[16,17] in the induction of antigen-presenting cell maturation and in B cell activation,[16–19] while IL2 is a secreted molecule essential for T cell expansion during the immune response.[20,21] CD40L- and IL2-Ad5/F35 were used to infect both $CD34^+$ and $CD34^-lin^-$ as described above. The supernatant of IL2-Ad5/F35-infected cells was collected at 48 h after infection and analyzed by ELISA. Figure 5 shows that infected cells produced quantities of cytokine know to be immunomodulatory,[22–26] while cells infected with control Ad5/F35 GFP vector or with Ad5-IL2 produced only background levels of IL2. Seventy-two hours after infection CD40L-Ad5, CD40L-

Table 3 Colony-forming ability of Ad5/F35-exposed $CD34^+$ cells

Patient No.	*Infection efficiency*[a]	*$CD34^+$ population*	*Total colonies/10^4 input cells*[c]
1	27%	Mock[b]	490 ± 0.7
		Ad5/F35	510 ± 3.5
2	22%	Mock	280 ± 2.8
		Ad5/F35	340 ± 1.4
3	72%	Mock	640 ± 0.7
		Ad5/F35	450 ± 0
4	33.5%	Mock	1940 ± 4.2
		Ad5/F35	2270 ± 7.7
5	20%	Mock	1200 ± 2.8
		Ad5/F35	1180 ± 1.4
6	25%	Mock	1360 ± 21
		Ad5/F35	1310 ± 4.9

After immunoselection, $CD34^+$ cells were infected with 10^3 VP/cells of both Ad5 and Ad5/F35 viruses for 6 h, washed, and resuspended in medium supplemented with 10% FCS. Cells were infected with the virus and plated in duplicate at 10^3 to 10^4 cell/ml in 2.5 ml of complete methylcellulose medium with recombinant cytokines (rh-GM-CSF, rh-G-CSF, rh-IL3, rh-IL6, rh-erythropoietin, rh-stem cell factor). The cultures were incubated for 14 days in a 5% CO_2-humidified incubator. Colonies were scored using an inverted microscope. 10^3–10^4 control uninfected cells were plated under these conditions. Results showed that cells exposed to Ad5/F35 demonstrated normal plating efficacy, suggesting that the chimeric adenovirus did not impair $CD34^+$ proliferative function.
[a]Percentage in GFP-expressing cells in the infected population after 24 h.
[b]Uninfected cells.
[c]Represent the mean number of colonies from duplicate cultures.

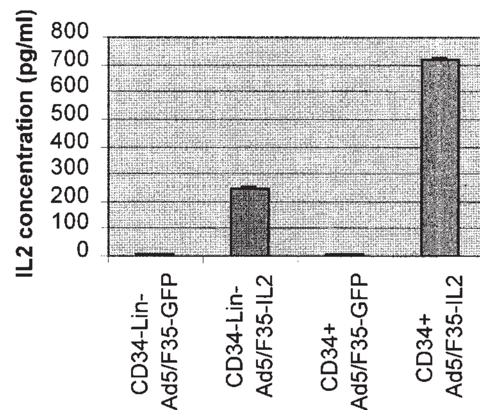

Figure 5 *IL2-Ad5/F35. $CD34^+$ and $CD34^-lin^-$ cells were infected for 6 h, with hIL2-Ad5/F35 and hIL2-Ad5 virus. The supernatant from the culture of non-infected, GFP-infected and infected cells was collected at 48 h after infection and analyzed by ELISA for the presence of hIL2.*

Ad5/F35-infected cells and control cells were stained with CD154 antibody to evaluate the expression of CD40L on the cell surface. The percentage of CD40L physiologically expressed at the cell surface of non-infected cells (consistently <5%) was subtracted from the percentage obtained with infected cells to measure expression due to the infection. As shown in Figure 6, 36% of $CD34^+$ and 69% of $CD34^-lin^-$ cells expressed the CD40L antigen after Ad5/F35-CD40L infection. In contrast, just 2.7% of $CD34^+$ and 6.8% of $CD34^-lin^-$ cells were positive following infection by Ad5-CD40L. The expression of CD40L persisted for 5 days at unchanged

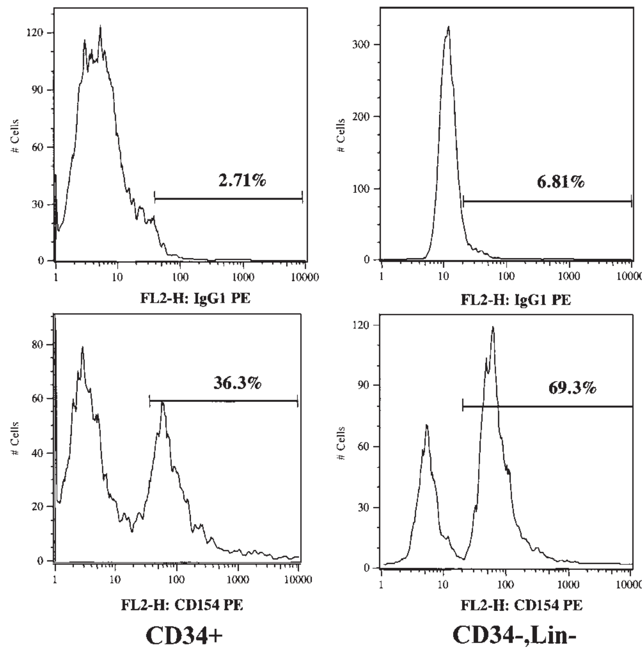

Figure 6 CD40L-Ad5/F35. CD34⁺ and CD34⁻lin⁻ cells were infected for 6 h with CD40L-Ad5 or CD40L-Ad5/F35 virus. The cells were analyzed by FACS 3 days after infection using PE-CD154 antibodies. The results are expressed as the percentage of cells expressing CD40L. The top panels are for CD40L-Ad5 infections and the bottom panels for CD40L-Ad5/F35 infections. The non-infected and mock-infected cells were stained with CD154 to evaluate the physiologic expression of CD40L on both populations. After CD40L-Ad5/F35 infection, 36.3% of CD34⁺ cells and 69.3% of CD34⁻lin⁻ cells expressed the transgene.

levels. No increase in the expression of CD40L was seen after infection with control GFP-Ad5/F35 vector. In these experiments too, we observed minimal toxicity when Ad5/F35 vectors were used, compared with >50% target cell death following exposure to Ad5 vectors.

Discussion

Ad5 is a valuable vector for transient gene expression in gene therapy protocols, since it can infect non-dividing cells and produce a high level of transgene expression. Unfortunately, this virus works poorly in hematopoietic cells, mainly because they lack the necessary receptor molecules for binding (CAR)[27] and for internalization (integrins).[28] Only 40% of the total bone marrow, 15% of CD34⁺ stem cells,[9] and a small proportion of T[29] and B lymphocytes express CAR, while integrins are generally absent on hematopoietic cells and cell lines. Mitogens and IL2 induce expression of integrins[30] and CAR, respectively, on T cells,[29] while CD40 cross-linking[31] and IL4 stimulation[32] up-regulate integrins on B cells and allow them to be infected by adenovirus. Similarly, CD34⁺ cells appear to be more permissive to Ad5 infection after culture with various cocktails of cytokines including SCF, IL3, G-CSF, Flt3 ligand, Tpo, and c-Kit ligand.[33] But even with such stimulation, the efficiency of infection remains poor. Moreover, the stimulation conditions themselves induce the cells to differentiate,[9,34] resulting in a loss of their pluripotency and often in increased toxicity.[8] Other approaches to infecting normal and malignant cells of the hematopoietic lineage have used lipofectamine,[35] bispecific antibodies that target both adenovirus epitopes and cell antigens, biotinylated adenoviruses[36] or adenoviruses with heparan sulfate binding domains.[37] These studies have generally required large amounts of vector and have not had proven success with highly primitive stem cell populations. Adenovirus subgenus B has been shown to be CAR independent and to infect hematopoietic cells.[14,38] Several different chimeric adenoviruses have been created from this subgenus, by a total or partial substitution of the fiber genes Ad5/F3,[39] Ad5/F7[40] and Ad5/F17.[41] These retargeted viruses differ from the original vector in their tropism and may be more infectious than Ad5 for hematopoietic cells.[42] Ad35 in particular has been described to have a broader cell tropism and to be CAR and integrin independent.[38] Hence, we used a chimeric Ad5/F35, in which the Ad5 fiber genes are substituted by the Ad35 fiber genes[12] and evaluated its capacity to infect hematopoietic cells without pre-activating them. Our initial results confirmed that Ad5/F35 efficiently transferred genes to T cells (Jurkat, HSB-2), B cells (EBV-LCL) and to multipotent cell lines (K562) that are resistant to Ad5, consistent with the use of distinct binding and internalization receptors. We obtained efficacy of infection of 30–70% even at low viral dose (100 VP per cell), with <6 h co-culture, thereby limiting toxicity due to prolonged serum starvation or directly to the vector.

We also found that Ad5/F35 infection was highly efficient in infecting progressively more primitive stem cell subsets. Ad5 has been shown to be non-infectious in primitive CD34⁺CD38⁻ cells due to their complete absence of appropriate receptors, but Ad5/F35 readily infected this population. To study the infectability of even more primitive stem cells,[43,44] we determined the permissivity of CD34⁻lin⁻ and SP cells. Essentially the same percentage of infected cells was found in all (CD34⁺ and CD34⁺CD38⁻ and SP) populations studied. Hence, Ad5/F35 receptors unlike CAR[33] are expressed on even the most primitive subsets of hematopoietic progenitor cells. There was, however, wide variability in infection efficiency between patient samples treated with a given lot of virus for both Ad5 and Ad5/F35 infection. We do not currently know the mechanism that underlies such variability, but it may arise from differences in Ad5/F35 receptors (at the nucleic acid sequence or protein expression level), differences in cell cycle status or differences in the cell activation status due to different cytokine concentrations in the CD34 cells' environment *in vivo*.

Trypan blue exclusion of infected cells showed minimal toxicity following Ad5/F35 infection, while Ad5 induced a significant loss of viability in the infected cells. These results were unexpected, because the same adenovirus backbone was used for both virus constructs. One explanation is that different CD34 subsets could have different sensitivity to Ad-mediated toxicity, with the most susceptible being those that can be infected by Ad5. However, there are no direct phenotypic data to support this.

Clonogenic assays further demonstrate that the Ad5/F35 was not toxic for the cells, since colonies were produced with the same distribution and at the same frequency as non-infected cells. Thus, Ad5/F35 does not affect the viability, the differentiation or the proliferation potential of committed progenitor cells. Of note, in the infected cultures only a few colonies were entirely green. While the signal may be lost due to silencing of the CMV

promoter, it is more likely to have occurred because the low number of viral particles used per cell meant only a few copies of the genome are internalized for each cell, and are progressively lost as the cells divide to form colonies.

We further investigated the infection efficacy of the chimeric vector using potentially therapeutic genes. IL2 is mainly produced by T cells during their activation and enhances their own expansion.[20,21] Production of IL2 is diminished in many patients with cancer due to T cell anergy[45–47] and several phase I studies[23–26,48] have shown that administration of Ad5-IL2 gene-modified tumor cells can induce specific antitumor responses mediated by T cells. Although successful in these studies in solid tumors, Ad5-IL2 is limited in gene therapy of hematopoietic malignancies by its low efficacy of infection and by a high toxicity. The Ad5/F35–IL2 used in this study may allow these problems to be overcome. Similarly, CD40 ligand (CD40L, also known as gp39 and CD154) is essential in T/B cell interaction[49,50] and for maturation of APC.[16,47] For example, the lack of CD40L on T cell leads to X-linked hyper-IgM syndrome,[51] and in many leukemias, T cell anergy has been attributed to defective CD40L expression by the malignant cells. CD40L gene therapy has been used to correct the expression level of this molecule on B-CLL cells and on other hematologic malignancies with encouraging results.[52–54] The chimeric hCD40L adenovirus vector we describe here may be useful in extending these studies. Although expression with any adenovirus vector is relatively transient, in this context such an effect may be of benefit since prolonged constitutive expression of CD40L may produce T cell lymphoproliferative disease.[55] It seems likely that other cell surface or secreted immunomodulatory molecules will be equally effectively infected by this chimeric vector, thereby broadening it applications. Together, these results show that chimeric Ad5/F35 vectors efficiently infected primitive hematopoietic stem cells, and may be used for the expression of a variety of genes of interest.

Materials and methods

Cells

HEK293 (human embryonic kidney cell line), A459 (human lung cancer cell line), M2–10B4 (murine fibroblast cell line), CHO (Chinese hamster ovary cell line), NIH-3T3 (Swiss mouse embryonic cell line), MRC-5 (human lung cell line), K562 (human erythro–granulocytic-malignant progenitor cell line), HSB-2, Jurkat (human T cell leukemia cell lines) were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). HEK293, CHO, A549 and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; BioWhitttaker, Walkersville, MD, USA). MRC5, M2–10B4, K562, HSB-2, Jurkat, and leukemia cells were cultured in RPMI-1640 (BioWhitttaker). Leukemic cells were obtained using IRB approved protocols. Mononuclear cells were isolated by Ficoll–Hypaque density separation (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and cryopreserved in 10% dimethyl-sulphoxide (DMSO; Igen International, Gaithersburg, MD, USA) plus 90% heat inactivated fetal calf serum (Hyclone, Logan, UT, USA) in liquid nitrogen, or used directly. EBV-LCL cells were obtained by Epstein–Barr virus (B95–8) immortalization of B cells isolated from the blood of a healthy donor[22] and cultured in RPMI-1640. All cells were grown in their specific medium supplemented with 10% FCS (Hyclone), 100 unit/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD, USA) at 37°C, 5% CO_2 in a humidified incubator.

Immunoselection of $CD34^+$ and $CD34^-lin^-$ cells

Leukapharesis products and bone marrow aspirates obtained using IRB approved protocols were our sources of stem cells. Mononuclear cells were isolated by Ficoll–Hypaque sedimentation. After mobilization, stem cells were purified using CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA) for positive selection. Cells were passed through these Minimacs separation columns according to the manufacturer's instructions. Two columns were used to increase the purity of this $CD34^+$ population. The $CD34^-$ fraction was depleted of lineage-committed cells using a cocktail of antibodies (StemSep antibody cocktail; StemCell Technologies, Vancouver, Canada). The negatively selected cells were used as a source of very early progenitor cells. Their purity was determined by fluorescence-activated cell sorting analysis (FACS) using direct immunolabeling. The purity of each immunoselected fraction was routinely >90% with a viability of greater than 90%.

SP cell characterization by Hoechst 33342 staining

Mononuclear cells were washed in cold Hanks' balanced salt solution (Life Technologies) containing 2% FCS and 5 mM Hepes (HBSS+; Life Technologies). Cells were resuspended at 37°C at 10^6 cells/ml in DMEM supplemented with 2% FCS, 5 mM Hepes buffer, 5 μg/ml Hoechst 33342 (Sigma Chemical Co, St Louis, MO, USA) and incubated at 37°C for 2 h. The cells were then pelleted and resuspended at 10^8 cells/ml in cold Hanks' balanced salt solution containing 2% FCS, 10 mM Hepes (HBSS+) and 2 μg/ml propidium iodide (PI) (Sigma Chemical Co). Flow cytometric analysis was performed on a triple-laser instrument (MoFlow; Cytomation, Fort Collins, CO, USA). An argon laser tuned to 350-nm emission was used to excite the Hoechst dye. Fluorescence emission was collected with a 405/30 BP filter (Hoechst blue) and 670/40 BP filter (Hoechst red).

Adenovirus vector

Cell lines and leukemic cells were infected using an Ad5 or a chimeric adenovirus Ad5/F35 described previously.[12] Both viruses have the identical E1/E3-deleted backbone structure. Ad5/F35-GFP is an Ad5 containing in its E3 region the green fluorescent protein gene (GFP) driven by the cytomegalovirus (CMV) promotor. This vector has the fiber gene substituted by the fiber gene of Ad35. Each virus type (Ad5 and Ad5/F35) was produced by calcium phosphate transfection of HEK293 cells. Ad5 and Ad5/F35 virus were prepared by expansion of a single plaque generated in transfected HEK293. At maximal virus cytopathic effect, the cells were harvested and pelleted. Virus was extracted from the HEK293 cells by three consecutive freeze/thaw cycles and amplified by infection of a larger culture of HEK293 cells. The virus was purified as previously described[56] with two series of cesium chloride gradient ultracentrifugation and desalted on an EconoPac 10DG exclusion column (Bio-Rad Laboratories, Hercules, CA, USA). The titer of the large-scale

virus preparation was established by plaque assay using HEK293 cells. Preparations were routinely tested for replication-competent adenovirus (RCA) by plaquing on A549 cells and all were >1 RCA/10^9 VP. Viral titers were also quantitated by OD_{260} and the particle to p.f.u. ratios were 100 and 400 for Ad5 and Ad5/F35, respectively. Except where stated, cells were infected at 37°C with 10^3 VP per cell in DMEM, 2% FCS. Six hours after infection, the cells were washed in 4 ml of PBS and resuspended in fresh medium supplemented with 10% FCS. Cells were analyzed at 24 h after infection for transgene expression and viability.

Ad5/F35-CD40 ligand and Ad5/F35-IL2 were constructed as follows. First, the GFP gene was removed from pE3/F35GFP described by Shayakhmetov *et al*.[12] This plasmid termed pE3/F35 was cleaved with *Msc*I and the DNA co-transfected with pAd HM4 cleaved with *Srf*I into the *E. coli* Bj5183 to allow recombination in this bacteria strain. The resulting plasmid was termed pAd5/F35. The cDNAs for CD40L and IL2 were cloned in the shuttle plasmid Pshutle X (Clontech, Palo Alto, CA, USA). The entire CMV promotor, either CD40L or IL2, followed by an SV40 polyadenylation site was then excised by I-*Ceu*I and pI-*Sce*I and this restriction fragment was transferred to pAd5/F35 cleaved with the same enzyme to form Ad5/F35-CD40L and Ad5/F35-IL2.

Flow cytometry

After sorting or adenovirus infection, cells were washed and stained with the appropriate fluorescein isocyanate (FITC), R-phycoerythrin (PE), or peridinin chlorophyll protein (PercP) conjugated antibodies for 20 min at 4°C, in the dark, in phosphate buffer saline (PBS) supplemented with 0.1% bovine serum albumin (BSA). Control cells were stained with PE or PercP-conjugated IgG control isotype. After incubation, the cells were washed twice and resuspended in PBS. Dead cells and debris were excluded from analysis by using PI. GFP expression was measured using a standard filter setup for fluorescein (525 nm, bandpass filter). Stem cells were stained by CD34-PercP (clone 8G12, IgG1; Becton Dickinson, San Jose, CA, USA), and CD38-PE (clone HB7, IgG1).

Cell viability

Cell viability was routinely determined by trypan blue exclusion or PI staining for FACS analysis.

Clonogenic assay

Cells were infected with the virus and plated in duplicate at 10^3 to 10^4 cell/ml in 2.5 ml of complete methylcellulose medium with recombinant cytokines (rh-GM-CSF, rh-G-CSF, rh-IL3, rh-IL6, rh-erythropoietin, rh-stem cell factor) (Methocult GF+ H4435; StemCell Technologies). Control uninfected cells 10^3–10^4 were also plated under these conditions. The cultures were incubated for 14 days in a 5% CO_2 humidified incubator. Colonies were scored using an inverted microscope.

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