**Introduction**

Subgroup C, replication-deficient recombinant adenoviruses (Ad) are used as vectors for gene transfer based on their ability to transduce many cell types efficiently (1–3). There is increasing evidence, however, that the efficiency of Ad vectors can be limited by a deficiency of the appropriate binding/entry mechanisms on the target cell (4–7). The binding/entry of subgroup C Ad is mediated through two specific cell-surface receptors. The knob of the Ad fiber first binds to the “Coxsackie virus and adenovirus receptor” (CAR), a 46-kDa transmembrane protein that functions as the high-affinity receptor for both subgroup C Ad and the Coxsackie B viruses (8–10). HLA class I molecules may also participate in Ad fiber binding to the cell surface (11). Ad internalization requires an interaction of an arginine-glycine-aspartate (RGD) sequence on the Ad penton base with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins on the cell surface (4, 6, 12–19).

Mesenchymal cells, such as primary human fibroblasts, are one example of cells that are not efficiently infected by subgroup C Ad. Although fibroblasts express $\alpha_v$ integrins (20–22), they are deficient in CAR. In the present study, we have used primary human dermal fibroblasts as a model to explore strategies by which Ad vectors can be designed to enter cells deficient in CAR. Using an Ad vector expressing the human CAR cDNA (AdCAR) at high multiplicity of infection, primary fibroblasts were converted from being CAR deficient to CAR sufficient. Efficiency of subsequent gene transfer by standard Ad5-based vectors and Ad5-based vectors with alterations in penton and fiber was evaluated. Marked enhancement of binding and transgene expression by standard Ad5 vectors was achieved in CAR-sufficient fibroblasts. Expression by Ad$\Delta$RGD$\beta$gal, an Ad5-based vector lacking the arginine-glycine-aspartate (RGD) $\alpha_v$ integrin recognition site from its penton base, was achieved in CAR-sufficient, but not CAR-deficient, cells. Fiber-altered Ad5-based vectors, including (a) AdF(pK7)$\beta$gal (bearing seven lysines on the end of fiber) (b) AdF(RGD)$\beta$gal (bearing a high-affinity RGD sequence on the end of fiber), and (c) AdF9sK$\beta$gal (bearing a short fiber and Ad9 knob), demonstrated enhanced gene transfer in CAR-deficient fibroblasts, with no further enhancement in CAR-sufficient fibroblasts. Together, these observations demonstrate that CAR deficiency on Ad targets can be circumvented either by supplying CAR or by modifying the Ad fiber to bind to other cell-surface receptors.

supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.1% Fungizone (all components from Gibco BRL, Gaithersburg, Maryland, USA).

Adenovirus vectors. The recombinant Ad vectors used in this study are E1Δ, partial E1Δ, and partial E3Δ, vectors based on the Ad5 genome. The expression cassette in the E1 position includes the cytomegalovirus (CMV) immediate/early enhancer/promoter (except AdΔRGD)gal, which uses the Rous sarcoma virus [RSV] long terminal repeat), an artificial splice signal, the transgene, and an SV40 poly[A]/stop signal (23, 24). The vectors include (a) AdCAR, containing the human CAR cDNA (8); (b) AdΔβgal, with the Echerichia coli β-galactosidase (βgal) gene (24); (c) AdGFP, with a humanized version of the jellyfish Aequorea victoria green fluorescent protein (GFP) gene (25, 26); (d) AdNull with no transgene (24); (e) AdΔ7CAT, a chimeric Ad5-based vector bearing the Ad7α fiber, and the chloramphenicol acetyl transferase (CAT) gene (27); (f) AdΔRGDβgal, with a deletion of the RGD sequence in the penton base; (g) AdF(pK7)βgal, with seven lysine residues at the COOH-terminal end of each fiber protein (28, 29); (h) AdF9sKβgal, with a shortened fiber, including the tail and first eight shaft repeats of Ad5 fiber attached to the last Ad9 fiber shaft repeat and Ad9 knob (30, 31).

For binding studies, AdGFP was labeled with carbocyanine dye, Cy3 (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA). The limit of detection was 10^3 relative light units/mg protein.

To quantify the efficiency of gene transfer by first generation Ad vectors in a population of cells that were either CAR-deficient or CAR-sufficient, human fibroblasts were first infected with AdCAR, AdNull (both 0–5 x 10^4 pfu/cell), or no virus (mock-infected), then infected a second time 24 h later with 300 pfu/cell of Adβgal or 200 pfu/cell of AdGFP. The AdGFP-infected fibroblasts were also labeled with RmC and Texas red secondary antibody. To demonstrate that the improved expression of transgene in CAR-sufficient fibroblasts resulted from a fiber–CAR interaction, fibroblasts infected with AdCAR, AdNull (both 10^4 pfu/cell), or no virus were incubated with increasing concentrations of purified Ad5 fiber (4) (0–1 μg/ml at 37°C) for 1 h before, and also during, infection with 300 pfu/cell Adβgal.

For all experiments using fiber- or penton-altered Ad vectors, fibroblasts were first infected with high moi AdCAR or AdNull (both 10^4 pfu/cell), or no virus followed 24 h later by a second infection with low moi (300 pu/cell) of altered vector or its control. Altered vectors included AdΔ7CAT, for which standard Ad5CAT served as control, and AdΔRGDβgal, AdF(pK7)βgal, AdF(RGD)βgal, or AdF9sKβgal, for which standard Adβgal served as control.

![Figure 1](image)

**Figure 1**

Evaluation of the ability of subgroup C, serotype 5 Ad vectors to transfer and express the βgal transgene in primary human fibroblasts compared with the Ad549 lung epithelial cell line. βgal activity was assessed 24 h after infection with 0–5 x 10^4 pfu/cell of Adβgal or 0–5 x 10^4 pu/cell AdNull. The dashed line represents the limit of detection of the assay (10^3 relative light units/mg protein). Each data point represents the mean ± SE of triplicate measurements. Ad, adenovirus.
Results

Gene transfer and expression of first generation Ad vectors in primary human dermal fibroblasts. A dose–response study comparing β-gal expression in human fibroblasts to A549 demonstrated that a much higher moi of Adβ-gal was required to generate comparable β-gal expression in the fibroblasts (Fig. 1). In fibroblasts infected with 100 pu/cell, the β-gal activity in these cells was approximately 100-fold less than in that of A549 cells infected with the same low moi (all P < 0.01, 10 – 5 × 10⁴ pu/cell; Fig. 1). Interestingly, the amount of β-gal expression in the A549 cells was maximal at >10⁵ pu/cell, whereas β-gal expression by the fibroblasts increased as a function of moi, so that with 5 × 10⁴ pu/cell, the expression of β-gal in the fibroblasts was only fourfold less than that of the A549 cells at the same moi. The control AdNull vector induced β-gal activity below the limit of detection in either A549 cells or fibroblasts.

Characterization of Ad cell-surface receptors. Primary human fibroblasts lack immunodetectable CAR (Fig. 2). A549 cells, which are easily infected with low moi of Ad (Fig. 1), demonstrated punctate cell-surface labeling when stained for CAR (Fig. 2a). In contrast, naïve fibroblasts stained for human CAR demonstrated no fluorescence over background (Fig. 2c). Although the fibroblasts did not exhibit detectable CAR, they did express αv integrins on the cell surface (Fig. 3a).

Enhanced Ad vector–mediated transfer and expression of the CAR cDNA in primary human fibroblasts resulting from prior infection with AdCAR

<table>
<thead>
<tr>
<th>Naive fibroblasts</th>
<th>CAR-positive (%)</th>
<th>GFP-positive (%)</th>
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<tbody>
<tr>
<td>+AdNull</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+AdCAR</td>
<td>57 ± 7%</td>
<td>31 ± 2%</td>
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Primary human fibroblasts were infected with AdCAR or AdNull (both 10⁴ pu/cell) or mock-infected (naive). After 24 h, the cells were exposed for 1 h to AdGFP (coding for green fluorescent protein, 200 pu/cell). The cells were then trypsinized, plated onto coverslip dishes, and, 48 h later, incubated with anti-CAR antibody or isotype control followed by a Texas red secondary antibody. The percentage of green (GFP⁺) and red fluorescent cells (CAR⁺) were quantified by evaluation of 200 cells by immunofluorescent microscopy. The data are presented as the mean ± SEM of triplicate measurements. Ad, adenovirus; CAR, Coxsackie virus and adenovirus receptor; GFP, green fluorescent protein.
comparing βgal expression in CAR-sufficient and CAR-deficient fibroblasts infected with either standard Adβgal or AdΔRGDβgal. Although βgal expression by AdΔRGDβgal in mock-infected fibroblasts was below the limit of detection, expression with the AdΔRGDβgal vector in CAR-sufficient fibroblasts was significant, approximately 10-fold above that of the background and fivefold greater than that of the AdNull control (P < 0.02; Fig. 9). This suggests that upregulation of CAR can improve Ad infection even in the absence of the penton–integrin interaction.

**Gene transfer and expression mediated by fiber-altered vectors.** Ad vectors bearing a variety of modifications in their fiber proteins bypass the usual fiber–CAR interaction and can be used to infect CAR-deficient cells (Fig. 10). Compared with the standard Ad5-based Adβgal vector, fiber-altered vectors demonstrated improved transgene expression in CAR-deficient fibroblasts. AdF(pK7)βgal was the most effective, inducing an approximately 200-fold increase in βgal expression in naive fibroblasts versus standard Adβgal (P < 0.01). An approximately 100-fold increase was observed with AdF(RGD)βgal (P < 0.01 compared to standard Adβgal), and an approximately eightfold increase was observed with AdF9sKβgal (P < 0.01 compared with standard Adβgal). Whereas CAR-sufficient fibroblasts demonstrate a 70-fold increase in βgal expression over that of AdNull-infected controls (P < 0.001) when infected with standard Adβgal, no significant difference was found between CAR-sufficient versus CAR-deficient cells infected with AdF(pK7)βgal (P > 0.8) or AdF9sKβgal (P > 0.1). These data suggest that the mechanism for binding and/or entry by these vectors is CAR-independent. With AdF(RGD)βgal, a small (about 1.5-fold) increase in βgal expression was noted in the AdCAR- versus the AdNull-infected cells (P < 0.03).

**Discussion**
Gene therapy by Ad-mediated gene transfer requires that the target cells bind and take up the Ad vector efficiently (15, 20–22). In this study, we have demonstrated that CAR is rate limiting for infection by standard Ad5 vectors in primary αv integrin–positive cells and that the upregulation of CAR in such cells leads to increased binding of Ad, as well as more efficient expression of Ad transgene, both of which depend on a subgroup-specific fiber–CAR interaction. Using a penton base RGD-deficient Ad vec-
tor, the data also demonstrate that the fiber–CAR interaction is sufficient for Ad entry and expression, although far less efficient than if the RGD–integrin interaction were available. Finally, by altering the Ad fiber to enable attachment to receptors other than CAR, it is possible to use Ad vectors effectively to transfer genes to CAR-deficient, αV integrin–positive cells.

Enhancement of Ad-mediated gene transfer in CAR-sufficient fibroblasts. Previous studies have demonstrated that CAR functions as the high-affinity cell-surface receptor for subgroup C Ad (8, 9). Chinese hamster ovary cells (CHO) permanently transfected with CAR are able to bind subgroup C Ad but not subgroup B Ad3 (9) and are able to express genes transferred with an Ad5 vector at least 100-fold more efficiently than nontransfected CHO cells (8). Roelvink et al. (31) have demonstrated that Ad subgroups A, C, D, E, and F, but not B, compete for CAR binding. The present study demonstrates that CAR is rate limiting for Ad infection of fibroblasts, and importantly, it demonstrates that CAR can improve Ad5 binding and gene transfer to normal, primary human cells (39).

Because Ad infection of primary human dermal fibroblasts is not efficient, the extent of CAR expression after infection with AdCAR is not uniform. This variation allowed us to demonstrate that the extent of Ad binding to a given cell correlates positively with the extent of CAR expression on that individual cell. Upregulation of CAR leads not only to increased Ad binding but enhanced Ad transgene expression. Importantly, this enhancement can be eliminated by the presence of excess recombinant Ad5 fiber. Consistent with the concept that CAR expression enables subgroup-specific Ad entry, a chimeric virus that is Ad5-based but bears Ad7a fiber does not infect CAR-sufficient cells any more efficiently than CAR-deficient cells. This is in agreement with previous findings that subgroup C Ad (serotypes 2 and 5) do not compete for the same receptor as subgroup B Ad (serotypes 3 and 7) (27, 31, 40–42), and with Tomko et al. (9), who

Figure 4
Binding of fluorescent Ad vectors by CAR-sufficient primary fibroblasts. AdCAR-infected fibroblasts were incubated with Cy3-labeled fluorescent AdGFP (10¹¹ pu/cell) for 10 min at 37°C. The cells were then fixed and stained for CAR as described for Fig. 2, but with FITC-labeled secondary antibody. Naive A549 cells served as positive control. (a) A549 cells, DIC. (b) A549 cells, Cy3-AdGFP binding. (c) A549 cells, RmcB. (d) AdCAR-infected fibroblasts, DIC. (e) AdCAR-infected fibroblasts, Cy3-Ad5GFP binding. (f) AdCAR-infected fibroblasts, RmcB. Scale bar, 10 μm. DIC, differential interference contrast.

Figure 5
Correlation of binding of fluorescent Ad vectors with CAR expression. Fibroblasts were infected and labeled with RmcB and Cy3-AdGFP as described in Fig. 4. Fields were selected in AdCAR-infected cultures that included intensely and minimally CAR-positive (green fluorescent) cells. Images were collected, and triplicate gray-scale measurements were made in arbitrary regions within the background (no cells) and within each cell in both green and red channels. CAR expression (green fluorescence) and virus binding (red fluorescence) in a total of 38 cells were expressed as a ratio of cell to background fluorescence. Spearman’s correlation analysis revealed a significant correlation, with ρ = 0.8 and P < 0.001.
observed that Ad5 bound five times more efficiently than Ad3 to CAR-transfected NIH 3T3 cells.

**CAR-independent mechanisms of Ad entry.** Whereas upregulation of CAR reflects one strategy for the improvement of Ad-mediated gene transfer to cells, an alternate strategy could be to bypass CAR by altering the fiber of the Ad vector to permit attachment to other receptors. The present study confirms that infection by such fiber-altered vectors do not rely on CAR. On a per particle basis, AdF(pK7)βgal and AdF(RGD)βgal were the most efficient at infecting CAR-deficient human fibroblasts, increasing transgene expression by about 100-fold over that of standard Adβgal. Expression in cells infected by AdF(pK7)βgal, which is designed to bind to cells via cell-surface heparan sulfate (28, 29), is not affected by the presence or absence of CAR on the cell surface. Although AdF(RGD)βgal is designed to use the RGD–αv integrin interaction for both attachment and internalization (29), the fiber modifications have left the CAR-binding domain available for interaction with CAR. Thus, a modest increase (1.5-fold) in transgene expression in CAR-sufficient vs. CAR-deficient cells is found. The main mechanism of entry, however, is most likely related to the RGD–αv integrin interaction, as the magnitude of the increased transgene expression of this vector over the standard Ad5 vector (70-fold) was much greater than that of AdF(RGD) in CAR-sufficient vs. CAR-deficient cells (1.5-fold).

The lack of significant improvement of infection by AdF9sKβgal, an Ad5-based vector with a short fiber and Ad9 knob, in CAR-sufficient versus CAR-deficient fibroblasts was somewhat surprising given that Ad9 has been demonstrated to compete for binding with CAR (30, 31).
This lack of improvement suggests that the Ad9 knob–CAR interaction is bypassed. The fiber of AdF9sKβgal only contains eight of the 22 shaft repeats usually present, and this shortening is designed to enhance entry via the RGD–αv integrin pathway. Expression from this vector in CAR-deficient fibroblasts is only about eightfold greater than that of standard Ad5 vector, whereas AdF(RGD)βgal could achieve an approximately 70-fold greater expression. This observation suggests that, although binding and entry of AdF(RGD)βgal and AdF9sKβgal may be through the same RGD-mediated mechanism, trafficking of these vectors may not be the same. Alternatively, replacing a subgroup C fiber with a subgroup D fiber could adversely effect particle assembly, resulting in fewer active particles in the vector preparation.

The chimeric vector, Ad5f7CAT, represents another alteration of fiber that enhanced transgene expression relative to standard Ad5 vector in CAR-deficient fibroblasts, albeit to a modest extent (threefold). As the receptor for subgroup B Ad has not been characterized, we cannot determine whether this increase is due to Ad7a fiber binding to its receptor. As with AdF9sKβgal, the Ad7 fiber is somewhat shorter than Ad5 fiber (30, 43), and the RGD–αv integrin mechanism may dominate here as well. This may be a more likely explanation, given that Ad7 has not been reported to have a tropism for skin (44).

The relative importance of cell-surface αv integrins versus CAR for the entry of Ad into target cells is not clear. The data of Wickham et al. (4, 14) demonstrate that Ad-mediated gene transfer is facilitated by an interaction between RGD on the Ad penton base and αv integrin on the cell surface. The present study suggests that even in the absence of RGD–αv integrin interaction, significant gene transfer and expression can be achieved in primary human fibroblasts if adequate fiber–CAR interaction is available, albeit much less (approximately 20-fold) efficiently than in the presence of RGD–integrin interaction. This concept is supported by data demonstrating that RGD-deleted Ad2 can infect fiber receptor–positive cells, but not fiber receptor–negative cells (7, 12). The lack of expression by AdΔRGDβgal in the naive, CAR-deficient cells supports the reciprocal concept that RGD–integrin interaction may be the primary mechanism of CAR-independent gene transfer.

The improved ability of the short fiber vectors over standard Ad5 controls to infect naive fibroblasts also point to the importance of the penton–integrin interaction in CAR-deficient fibroblasts. However, the exclusive use of the penton–integrin entry pathway by the short fiber vectors, including Ad5f7CAT and AdF9sKβgal, has not rigorously been proved. All the vectors we have used to demonstrate CAR-independent pathways of entry
have normal, RGD-bearing penton bases that probably bind to $\alpha_v$ integrin for cell entry. Although the AdF(pK7)$\beta$gal vector is able to infect resting T cells that are $\alpha_v$ integrin–negative (4, 5), future studies using fiber-altered vectors that are RGD negative will be necessary to discover whether the penton–integrin interaction is necessary in the absence of fiber–CAR interaction.

The data from our studies suggest that either fiber–CAR interaction or penton–integrin interaction is sufficient to permit Ad entry and gene transfer but that neither is as effective as the two-receptor system used together. Although the fiber-dependent, penton-independent pathway (AdARGD$\beta$gal in CAR-sufficient cells) and the fiber-independent, penton-dependent pathways (short fiber vectors in CAR-deficient cells) led to increases in transgene expression compared with their respective controls, these increases were modest. In contrast, when both mechanisms were available (e.g., standard Ad5 in CAR-sufficient, integrin-positive cells), the increase over that of the control was about 100-fold. Supporting this hypothesis is the observation that AdF(RGD)$\beta$gal demonstrates a much higher level of transgene expression than the short fiber viruses, although they all target the $\alpha_v$ integrin pathway of entry. Unlike the short fiber vectors, AdF(RGD)$\beta$gal has both fiber and penton available for binding to cell-surface integrin.

An interesting finding in the control groups was the increase in transgene expression in cells that had been infected with AdNull, an Ad vector with no transgene. The indirect immunofluorescence studies demonstrate that neither $\alpha_v$ integrin nor CAR is upregulated by AdNull infection of fibroblasts. That the presence of excess recombinant Ad5 fiber does not affect transgene expression in AdNull-infected cells also suggests that fiber–CAR interaction is not responsible for the increase in transgene expression. The observation of this Null vector effect with every vector tested, including the penton- and fiber-altered Ad vectors, suggests that the Null vector enhancement occurs at a step other than those related to binding or internalization and trafficking. It may be that Ad infection per se activates other biologic processes that, in turn, enhance Ad transgene expression. A recent study by R. Peila et al. (45) suggests that the CMV promoter and, to a lesser extent, the RSV promoter, can be activated by the internalization of Ad. Such activation may be occurring in the present study, because all of the vectors we used included the CMV or RSV promoters. Li et al. (46) have recently described the requirement of PI3-OH kinase activation for Ad internalization. Activation of downstream effectors and other related kinases, such as FAK and MAPK (including ERK1/ERK2), observed early during Ad internalization, may be able to activate the CMV promoter (46). Alternatively, the CMV promoter may be activated by Ad E4 products. Infection with E4-positive vectors has recently been demonstrated to be able to reactivate expression of transgene under either the CMV or RSV promoter that had declined after initial infection via an E4-negative Ad vector in immunodeficient mice (47). In the present study, only E4-positive vectors were used; thus, there could have been enhancement of the CMV-driven transgene by E4-products.

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