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Efficient Gene Delivery into Dendritic Cells by Fiber-Mutant Adenovirus Vectors

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Recent studies have demonstrated the usefulness of dendritic cells (DCs) genetically modified by adenovirus vectors (Ad) to immunotherapy, while sufficient gene transduction into DCs is required for high doses of Ad. The RT-PCR analysis revealed that the relative resistance of DCs to Ad-mediated gene transfer is due to the absence of Coxsackie-adenovirus receptor expression, and that DCs expressed adequate α_v-integrins. Therefore, we investigated whether fiber-mutant Ad containing the Arg-Gly-Asp (RGD) sequence in the fiber knob can efficiently transduce and express high levels of the LacZ gene into DCs. The gene delivery by fiber-mutant Ad was more efficient than that by conventional Ad in both murine DC lines and normal human DCs (NHDC). Furthermore, NHDC transduced with fiber-mutant Ad and conventional Ad at 8000-vector particles/cell resulted in a 70-fold difference in β-galactosidase activity. We propose that α_v-integrin-targeted Ad is a very powerful tool with which to implement DC-based vaccination strategies. © 2001 Academic Press

Key Words: **adenovirus vector; dendritic cell; Coxsackie-adenovirus receptor; α_v-integrin; fiber-mutant; Arg-Gly-Asp sequence; gene-immunotherapy.**

Antigen-presenting cells are key vehicles for delivering antigens in tumor immunotherapy, and the most potent of them are dendritic cells (DCs). These bone marrow-derived leukocytes express on their surface high levels of major histocompatibility complex (MHC) class I and class II molecules, adhesion and costimulatory molecules, all of which assist in T cell activation (1). After antigen acquisition and processing, DCs migrate via lymph vessels or blood to the T-cell areas of regional lymphoid tissues, where they present MHC class I- and II-restricted peptides to naïve T cells (2). Because of these properties, DCs have recently constituted a very active area of tumor immunotherapy research. A number of groups have reported that DCs pulsed with peptides from tumor-associated antigens (TAA) can induce antigen-specific antitumor responses *in vivo* in a variety of murine tumor models (3–5). These results have supported the use of autologous, peptide-pulsed DCs in recent clinical trials (6, 7).

In developing strategies to optimize the use of DCs in tumor immunotherapy, viral transduction of DCs with TAA genes may offer important advantages over peptide-pulsed DCs. The vaccine efficacy of TAA peptide-pulsed DCs might be limited *in vivo,* because peptides pulsed onto DCs only transiently bind to MHC molecules due to variations in peptide binding affinities, peptide-MHC complex dissociation, and MHC turnover (8). Additionally, the use of peptide-pulsed DCs is greatly dependent on identification of the TAA peptide epitopes corresponding to the MHC haplotype of the patient. On the other hand, viral transduction of DCs with TAA genes may allow constitutive expression of the full-length protein, leading to prolonged antigen presentation *in vivo,* as well as presentation of multiple or unidentified antigen epitopes appropriate to MHC class I, and possibly class II molecules.

Adenovirus vectors (Ad) can efficiently transduce foreign genes into a wide variety of cell types and tissues of various species (9, 10). Entry of Ad into target cells involves the interaction of virus particles with at least two distinct cell receptors. The Ad-fiber knob mediates

Abbreviations used: 2-ME, 2-mercaptoethanol; Ad, adenovirus vectors; β-gal, β-galactosidase; CAR, Coxsackie-adenovirus receptor; DC, dendritic cell; FBS, fetal bovine serum; MHC, major histocompatibility complex; NHDC, normal human dendritic cell; RGD, Arg-Gly-Asp; RT-PCR, reverse transcription-polymerase chain reaction; TAA, tumor-associated antigens; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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TABLE 1

Primer Sequences Used for PCR Amplification

Species	Gene		Sequence (5′ to 3′)	Denaturation	Annealing	Extension	Cycle no.
Mouse	CAR	Forward	TGATCATTTTGTATTCTGGA	for 45 s at 94°C	for 60 s at 50°C	for 90 s at 72°C	40
		Reverse	TTAACAAGAACGGTCAGCAG				
	α_v-integrin	Forward	CCAGCCTGGGATTGTAGAAG	for 45 s at 94°C	for 60 s at 53°C	for 90 s at 72°C	40
		Reverse	ACTCCAGTGGGTCATCTTTG				
	β_3-integrin	Forward	TCTGGCTGTGAGTCCTGTGT	for 45 s at 94°C	for 60 s at 55°C	for 90 s at 72°C	40
		Reverse	GCCTCACTGACTGGGAACTC				
	β_5-integrin	Forward	TCGTGTGAAGAATGCCTGTT	for 45 s at 94°C	for 60 s at 53°C	for 90 s at 72°C	40
		Reverse	GCTGGACTCTCAATCTCACC				
	β-actin	Forward	TGTGATGGTGGGAATGGGTCAG	for 45 s at 94°C	for 45 s at 60°C	for 120 s at 72°C	20
		Reverse	TTTGATGTCACGCACGATTTCC				
Human	CAR	Forward	AGCCTTCAGGTGCGAGATGTTACG	for 30 s at 94°C	for 60 s at 52°C	for 120 s at 72°C	35
		Reverse	TACGACAGCAAAAGATGATAAGAC				
	α_v-integrin	Forward	GAGCAGCAAGGACTTTGGG	for 60 s at 94°C	for 60 s at 60°C	for 60 s at 72°C	30
		Reverse	GGGTACACTTCAAGACCAGC				
	β_3-integrin	Forward	GAGGATGACTGTGTCGTCAG	for 30 s at 94°C	for 60 s at 58°C	for 120 s at 72°C	35
		Reverse	CTGGCGCGTTCTTCCTCAAA				
	β_5-integrin	Forward	CAGGATGGGGAGAACCAGAGC	for 60 s at 94°C	for 90 s at 55°C	for 90 s at 72°C	30
		Reverse	CTGGTCATCTTTCACGATGGT				
	β-actin	Forward	CCTTCCTGGGCATGGAGTCCTG	for 60 s at 94°C	for 90 s at 55°C	for 90 s at 72°C	20
		Reverse	GGAGCAATGATCTTGATCTTC				

binding to the Coxsackie-adenovirus receptor (CAR) on the cell surface (11), followed by internalization of the virion facilitated by the interaction of Arg-Gly-Asp (RGD) motifs located in Ad-penton base, with $\alpha_v\beta_3$- or $\alpha_v\beta_5$-integrins (12). Although the Ad system should theoretically be applicable to the delivery of TAA genes into DCs, the relative resistance of DCs to Ad causes difficulties in the realization of tumor immunotherapy using DCs genetically modified by Ad (13). Therefore, the manipulation of Ad, which leads to the highly efficient delivery of foreign genes into DCs, can greatly advance DC-based gene immunotherapy. We demonstrated that the fiber-mutant Ad harboring the RGD sequence in the HI loop of the fiber knob could more efficiently transduce marker genes into human aortic smooth muscle cells that expressed little or no CAR on their surface compared with conventional Ad. We reasoned that this fiber-mutant Ad system might target α_v-integrins during the first attachment to human aortic smooth muscle cells (14).

The present study demonstrated using reverse transcription-polymerase chain reaction (RT-PCR), that the relative resistance of DCs to Ad-mediated gene transfer is due to the absence of CAR expression, and that DCs expressed adequate levels of α_v-integrins. Based on these findings, we investigated whether or not the α_v-integrin-targeted Ad could efficiently transduce and express high levels of foreign genes in DCs.

MATERIALS AND METHODS

Cells. The immortalized murine DC lines, DC2.4 (H-2^b) (15), FSDC (H-$2^{b/d}$) (16), and XS52 (H-2^d) (17), were provided by Dr. K. L. Rock (Department of Pathology, University of Massachusetts Medical School, Worcester, MA), Dr. P. Ricciardi-Castagnoli (Department of Biotechnology and Bioscience, University of Milano-Bicocca, Milan, Italy), and Dr. A. Takashima (Department of Dermatology, University of Texas Southwestern Medical Center, Dallas, TX), respectively. Each cell line was grown in complete RPMI 1640 medium (10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin) supplemented with 100 μM nonessential amino acid and 50 μM 2-mercaptoethanol (2-ME) (for DC2.4 cells), Iscove's modified Dulbecco's medium supplemented with 5% FBS, 50 μM 2-ME, and antibiotics (for FSDC cells), or complete RPMI 1640 medium supplemented with 1 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor (Pepro Tech EC LTD., London, England), 10% culture supernatant collected from the NS47 stromal cell line, and 50 μM 2-ME (for XS52 cells), respectively. Normal human DCs (NHDC) obtained from Clonetics Cell Systems (Walkersville, MD) were cultured according to the manufacturer's instructions. B16 BL6 cells and HeLa cells were cultured in minimum essential medium supplemented with 10% FBS and antibiotics. EL4 cells were cultured in complete RPMI 1640 medium supplemented with 50 μM 2-ME. Colon 26 cells were cultured in complete RPMI 1640 medium. HEK293 cells, A549 cells and Caco-2 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS and antibiotics. Normal human aortal endothelial cells (HAEC) obtained from KURABO Industries, Ltd. (Osaka, Japan) were cultured according to the manufacturer's instructions.

RT-PCR analysis. Total RNA was isolated from culture cells using TRIZOL reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription proceeded for 60 min at 42°C in a 100 μl reaction mixture containing 10 μg total RNA, 10 μl 10 $\times$ PCR buffer, 20 μl 25 mM $MgCl_2$, 40 μl 2.5 mM dNTP mix, 1 μM random hexamer, 1 μM oligo(dT), and 200 U ReverTra Ace (TOYOBO Co., Ltd., Osaka, Japan).

PCR amplification of the CAR and integrins transcripts proceeded in 50 μl of a reaction mixture containing 5 μl of RT-material, 1.25U Taq DNA polymerase (TOYOBO Co., Ltd.), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.5 μM primers. The sequences of the specific primers used for PCR amplification and PCR conditions and cycle number are defined in Table 1. The PCR product was resolved by electrophoresis in 3% agarose gel, stained with ethidium bromide, and visualized

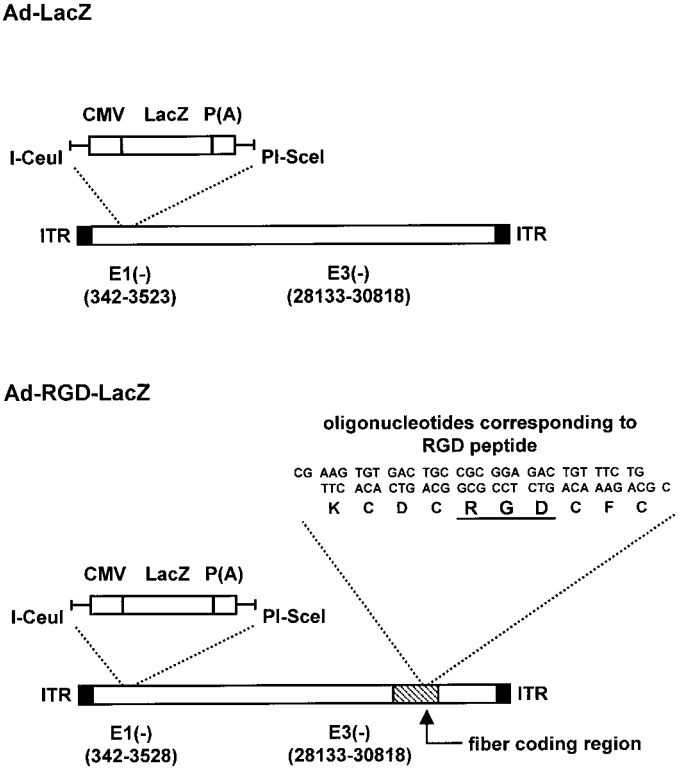

FIG. 1. Schematic representation of Ad. Construction procedure of Ad-LacZ and Ad-RGD-LacZ has been reported (14, 18, 19). ITR, inverted terminal repeat; CMV, cytomegalovirus promoter; P(A), bovine growth hormone polyadenylation signal.

under ultraviolet light. EZ Load (BIO-RAD, Tokyo, Japan) was used as 100 bp molecular rulers. The predicted PCR product sizes were: murine CAR, 211 bp; murine α_v-integrin, 105 bp; murine β_3-integrin, 115 bp; murine β_5-integrin, 126 bp; human CAR, 366 bp; human α_v-integrin, 619 bp; human β_3-integrin, 232 bp; and human β_5-integrin, 510 bp. To control the quality of the procedure, RT-PCR was performed on the samples using specific primers for β-actin.

Preparation of recombinant Ad. The replication-deficient Ad used in this study are based on adenovirus serotype 5 with deletion in early regions 1 and 3. Both Ad-LacZ and Ad-RGD-LacZ, which carried the LacZ gene under the control of the cytomegalovirus promoter, were constructed by improved *in vitro* ligation as described (18, 19). The RGD sequence was introduced into the HI loop of the fiber knob in Ad-RGD-LacZ using the two-step method developed by Mizuguchi *et al.* (14). The Ad constructs are shown schematically in Fig. 1. Both types of Ad were propagated in HEK293 cells, purified by two rounds of CsCl density centrifugation, dialyzed and stored at −80°C. Virus particle titer was spectrophotometrically determined by the method of Maizel *et al.* (20).

Viral transduction and evaluation of β-galactosidase (β-gal) expression. Cells cultured in 24-well plates were infected with Ad-LacZ or Ad-RGD-LacZ for 1.5 h at various numbers of vector particles/cell in 400 μl of FBS-free medium, and then 1 ml of culture medium was added to each well. Cell viability was not affected by at least 8000 vector particles/cell of both types of Ad (data not shown). Two days later, the cells were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to assess the ability of Ad to transfer and express LacZ genes in DCs. At the same time, β-gal activity and the protein level in lysates of Ad-infected cells were quantified by using the Luminescent β-gal Detection kit II (CLONTECH, Tokyo, Japan) and the DC Protein Assay kit (BIO-RAD), respectively.

RESULTS AND DISCUSSION

Among various methods of gene transduction, Ad provide high-level transduction efficacy to a broad range of cell type, regardless of the mitotic status of the cell, and cannot transform cells by insertional mutagenesis (9, 10). Vaccination with DCs transduced with the TAA (21, 22) or cytokine genes (23, 24) using Ad can induce potent tumor immunity in murine tumor models. However, extremely high doses of Ad were required for sufficient gene transduction into DCs. The internalization of Ad into target cells is mediated by two steps: the fiber knob of Ad particles initially attaches to CAR on the cell surface (11), then $\alpha_v\beta_3$- or $\alpha_v\beta_5$-integrins subsequently interact with the RGD motif in the Ad-penton base and facilitate internalization of the virion (12). Based on understanding of this Ad-entry pathway, the relative resistance of DCs to Ad-mediated gene transfer was thought to be due to a lack or low levels of CAR expression on DCs.

We firstly investigated the mRNA levels of CAR, α_v-integrin, β_3-integrin and β_5-integrin on murine DC and other cell lines by RT-PCR analysis (Fig. 2). This analysis revealed the absence of CAR expression and adequate expression of α_v-integrin on all murine DC lines. In contrast, PCR products of CAR and α_v-integrin were detected using RT materials from B16 BL6, EL4, and Colon 26 cells. Moreover, the expression of β_5-integrin but not β_3-integrin on murine DC lines suggested that these cells have $\alpha_v\beta_5$-integrins as heterodimers on their surface. These findings support the notion that the absence of CAR expression is the major cause of the relative resistance of DCs to Ad-mediated gene transfer, and that the modification of Ad-fiber knob to target α_v-integrins during cell attachment is an

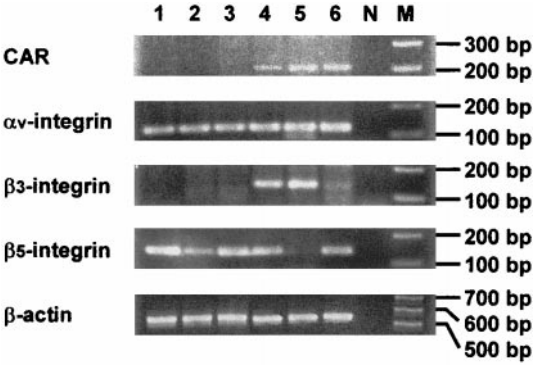

FIG. 2. RT-PCR analysis of CAR, α_v-integrin, β_3-integrin, and β_5-integrin in murine cell lines. Total RNA was prepared from three DC lines and three tumor cell lines, and then RT-PCR was performed as described under Materials and Methods. Lane 1, RT-PCR from DC2.4 cells; lane 2, RT-PCR from FSDC cells; lane 3, RT-PCR from XS52 cells; lane 4, RT-PCR from B16 BL6 cells; lanes 5, RT-PCR from EL4 cells; lane 6, RT-PCR from Colon26 cells; lane N, PCR with water as template; lane M, 100 bp molecular rulers.

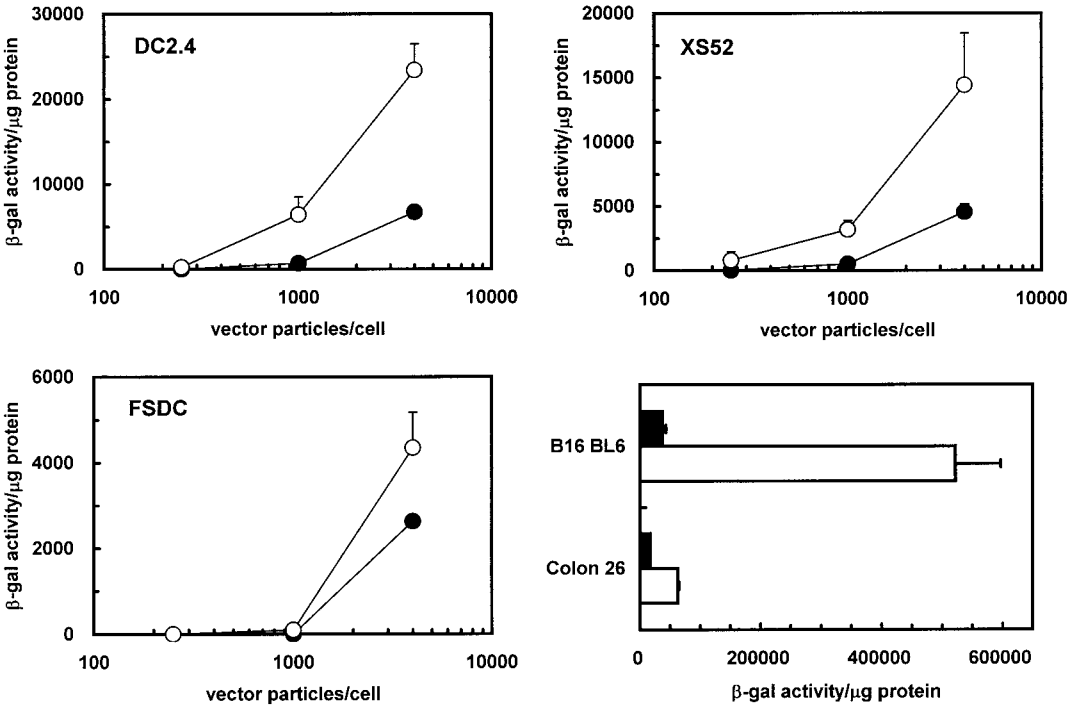

FIG. 3. Comparison of β-gal activity in murine cell lines transduced with Ad-LacZ and Ad-RGD-LacZ. DC lines were transduced with 250, 1000, or 4000 vector particles/cell of Ad-LacZ (●) or Ad-RGD-LacZ (○) for 1.5 h. Similarly, B16 BL6 cells and Colon 26 cells were infected with Ad-LacZ (closed column) or Ad-RGD-LacZ (open column) at 1000 vector particles/cell. Two days later β-gal activity was measured using a chemiluminescent β-gal assay. All data are presented as means ± SE of three experiments.

attractive strategy for altering the Ad tropism for efficient gene transduction into DCs.

We therefore compared the transfection efficiency on murine DC lines between Ad-RGD-LacZ, which was inserted with an oligonucleotide corresponding to RGD peptide in the fiber coding region, and conventional Ad (Ad-LacZ). Figure 3 shows quantifiable amounts of β-gal activity, as an indicator of the expression level of LacZ gene, on three DC lines infected with Ad-RGD-LacZ or Ad-LacZ. The β-gal activity of Ad-RGD-LacZ-infected DC2.4 and XS52 cells was three to fourfold higher at 4000 vector particles/cell, six to ninefold higher at 1000 vector particles/cell than that of Ad-LacZ-infected cells. In addition, 1000 vector particles/cell of Ad-RGD-LacZ were sufficient to attain the level of β-gal expression obtained by infection with 4000 vector particles/cell of Ad-LacZ (Fig. 3, upper). However, the enhancement of β-gal expression using Ad-RGD-LacZ was only slight on FSDC cells (Fig. 3, lower left). The difference in the enhancement effect using Ad-RGD-LacZ between the two DC lines and the FSDC cells appeared to be due to the variation in mRNA level of β_5-integrin (Fig. 2, fourth panel), namely less expression of $\alpha_v\beta_5$-integrin on the surface of FSDC cells compared with that on the surface of DC2.4 and XS52 cells. Taken together, these results were interpreted as follow. The Ad-RGD-LacZ attached to DCs through the interaction between the RGD sequence in the fiber knob and $\alpha_v\beta_5$-integrin in a CAR-independent manner, and then was internalized by interaction between the penton bases with $\alpha_v\beta_5$-integrins. Furthermore, B16 BL6 and Colon 26 cells that express CAR, when transduced with Ad-RGD-LacZ and Ad-LacZ at 1000 vector particles/cell resulted in 14- and fourfold differences in β-gal activity, respectively (Fig. 3, lower right). This finding demonstrated that the Ad-RGD-LacZ can target both α_v-integrins and CAR during cell attachment, and thus increase the transduction and expression ef-

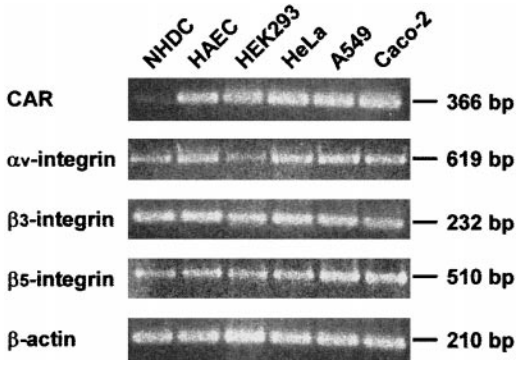

FIG. 4. RT-PCR analysis of CAR, α_v-integrin, β_3-integrin, and β_5-integrin in normal human DCs and other cell types. Total RNA was prepared from NHDC, HAEC, and four cell lines, and then RT-PCR was performed as described under Materials and Methods.

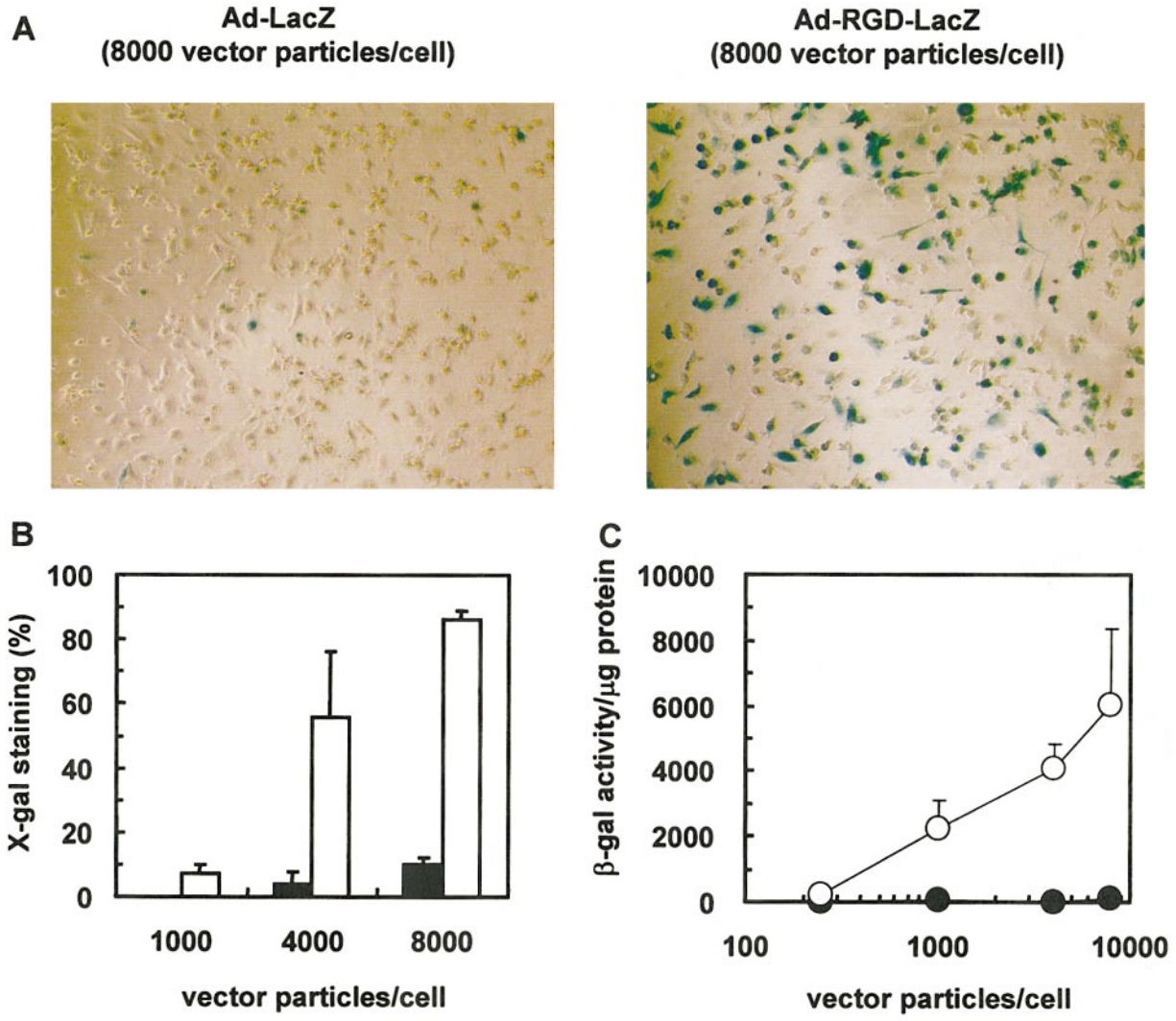

FIG. 5. The ability of Ad-RGD-LacZ to transfer and express LacZ genes into NHDC. NHDC were infected with Ad-LacZ or Ad-RGD-LacZ for 1.5 h. After 2 days of culture at 37°C, the cells were washed, fixed, and stained with X-gal to detect LacZ expression, and cells were observed under phase-contrast microscopy. (A) X-gal staining of NHDC infected with 8000 vector particles/cells of Ad-LacZ (left) or Ad-RGD-LacZ (right). (B) LacZ gene-transduction of NHDC infected with indicated vector particles/cell of Ad-LacZ (closed column) or Ad-RGD-LacZ (open column) was expressed as X-gal staining (%) calculated using the following formula: X-gal staining (%) = (number of X-gal staining cells per field)/(number of total cells per field) × 100. Data are presented as means ± SD of five fields. (C): NHDC were infected with indicated vector particles/cell of Ad-LacZ (●) or Ad-RGD-LacZ (○) for 1.5 h. Two days later β-gal activity was measured by chemiluminescent β-gal assay. Data are presented as means ± SE of three experiments.

ficiency of foreign genes irrespective of CAR expression on the surface of target cells.

We also investigated the mRNA levels of CAR by RT-PCR analysis on NHDC. PCR products of CAR were scarcely detected using RT materials from NHDC, whereas HAEC and four cell lines (HEK293, HeLa, A549, and Caco-2) adequately expressed CAR mRNA (Fig. 4). In contrast, PCR products of α_v-, β_3-, and β_5-integrin were found in both NHDC and other cell types in similar amounts. These findings suggested that α_v-integrin-targeted Ad was an effective method for gene transduction to human DCs as well as murine DC line. Therefore, we compared gene transduction and expression on NHDC by Ad-RGD-LacZ and by Ad-LacZ. X-gal staining revealed that 8000 particles/cell of Ad-RGD-LacZ transfected LacZ genes to more than 85% of the NHDC, while Ad-LacZ-infected NHDC were scarcely stained (Figs. 5A and 5B). Moreover, β-gal activity of NHDC was dose-dependently increased by transduction with Ad-RGD-LacZ, and NHDC infected with Ad-RGD-LacZ and Ad-LacZ at 8000 vector particles/cell resulted in 70-fold difference in β-gal activity (Fig. 5C). These results demonstrated that the gene delivery and expression efficiency of transgenes by α_v-integrin-targeted Ad was more efficient than by conventional Ad on human DCs.

Because Ad is cytopathic at high doses, less Ad is preferred in DC-based gene immunotherapy to prevent adenoviral toxicity from damaging DCs (23, 25–27). Several approaches, such as using bispecific antibodies (28), or cationic liposomes (29), have been developed to improve Ad-mediated gene transfer into DCs. However, the extensive application of these methods is limited due to difficulties associated with constructing new vectors. In contrast, the fiber-mutant Ad targeting α_v-integrins not only significantly reduced the amount of Ad needed for efficient transduction to DCs, but also required only a simple *in vitro* ligation using standard molecular biology reagents for the vector construction without generating wild-type or null vectors (14, 18, 19). We believe that this fiber-mutant Ad system will contribute considerably to advance in DC-based gene immunotherapy.

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