Coxsackievirus Adenovirus Receptor Expression Predicts the Efficiency of Adenoviral Gene Transfer into Non-Small Cell Lung Cancer Xenografts

Min Qin, Shuang Chen, Tina Yu, Brian Escuadro, Sherven Sharma, and Raj K. Batra[1]

Department of Medicine and The Lung Cancer Research Program [M. Q., S. C., T. Y., B. E., S. S., R. K. B.], Jonsson Comprehensive Cancer Center [R. K. B.], David Geffen School of Medicine at University of California at Los Angeles and Greater Los Angeles Veterans Administration Healthcare System, Los Angeles, California 90073

ABSTRACT

Purpose: **Current paradigms postulate that inefficient adenoviral (Ad) gene transfer is a consequence of poor Coxsackievirus adenovirus receptor (CAR) expression in tumors *in vivo*. To test whether exuberant CAR expression alone is sufficient to mediate efficient Ad gene transfer, we compared Ad gene transfer efficiency in a panel of non-small cell lung cancer (NSCLC) cell model systems in which we systematically measured CAR expression *in vitro* and *in vivo*.**

Experimental Design: **NSCLC cells were selected for study on the basis of (*a*) differences in Ad transduction, (*b*) identical requirements for growth *in vitro*, (*c*) capacity to grow as xenografts in immunocompromised mice, and (*d*) similar amounts of α_v integrin expression as measured by flow cytometry. CAR expression and Ad transduction profiles of these NSCLC cells were generated *in vitro* and *in vivo*.**

Results: **Ad transduction efficiency of NSCLC cells *in vitro* can be directly related to CAR expression at both the mRNA and protein level. CAR expression *in vitro* favorably predicts a comparable pattern of expression in transplanted NSCLC xenografts *in vivo*. Xenografts generated from NSCLC cells exhibiting increased CAR expression showed evidence of higher Ad gene transfer, although the efficiency of transduction was reduced compared with *in vitro* measurements. Thus, in NSCLC cells with high basal expression of CAR, Ad vector doses that enabled uniform transduction *in vitro* achieve a gene transfer efficiency ranging from 10% to 70% after a single intratumoral injection in the xenografts.**

Conclusions: **These studies indicate CAR expression is predictive for more efficient gene transfer into NSCLC cells *in vitro* and *in vivo* but is not sufficient to achieve uniform transduction by Group C Ad vectors *in vivo*.**

INTRODUCTION

Gene delivery to target cells is required for effective gene therapy. Investigations over the last several years have elucidated mechanisms by which Ad[2] vectors mediate gene transfer into cells *in vitro*. In cell culture systems, studies indicate that adenovirus initially attaches to a specific high-affinity cell surface receptor [CAR (1, 2)]. This attachment apparently triggers a clustering of cell surface integrins, viral endocytosis using coated pit mechanisms, and cellular actin stabilization, (3–5). The virus begins to disassemble at the cell surface and, after internalization, disrupts endosomal vesicles to escape into the cytoplasm (6–8). Endosomal escape, which also uses integrin-mediated processes (9, 10), is followed by microtubule-mediated transport of the residual Ad nucleocapsid to the nucleus (11–13) and episomal expression of the vector DNA. Despite the elucidation of cellular mechanisms underlying Ad gene transfer, however, successful translation in the clinic is yet to be realized, in part because of inefficient gene delivery. For example, in the experimental treatment of advanced human lung cancer with the use of adenovirus-mediated gene delivery, investigators instilled high doses of adenovirus (up to 7.5×10^{12} particles) directly into lung tumors by bronchoscopy or transthoracic computed tomography-guided injection (14–16). Using intratumoral transgene mRNA (by RT-PCR of vector-specific sequences) as a marker of transduction efficiency, Swisher *et al.* (14) and Nemunaitis *et al.* (15) reported less than 40% to 50% expression, and Schuler *et al.* (16) reported transgene expression in 68% of subjects with repeated testing. Unfortunately, it is currently not possible to extrapolate clinical benefits derived from molecular evidence of *in situ* transgene expression. Collectively, these results suggest that efficient *in vivo* gene delivery to target cells remains a major limitation for the successful gene therapy of NSCLC (17–19).

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[1] To whom requests for reprints should be addressed, at David Geffen School of Medicine at University of California at Los Angeles and Division of Pulmonary and Critical Care Medicine, Veterans Administration Greater Los Angeles Health Care System, 111Q, 11301 Wilshire Boulevard, Los Angeles, CA 90073. Phone: (310) 268-3021; Fax: (310) 268-4712; E-mail: rbatra@ucla.edu.

[2] The abbreviations used are: Ad, adenoviral; CAR, Coxsackievirus adenovirus receptor; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-PCR; MOI, multiplicity of infection; PBST, PBS + 0.1% Tween 20; IHC, immunohistochemistry; SCID, severe combined immunodeficient.

The studies detailed here have focused on the expression and importance of CAR in mediating gene transfer into NSCLC cells *in vivo*. CAR is a M_r 46,000 transmembrane protein that interacts with the Group C Ad fiber protein with high affinity [with an estimated dissociation rate constant (K_d) of ~0.5 to 1 × 10^{-8} M (20, 21)]. The cellular function of CAR, aside from serving as the attachment receptor for viruses, is largely unknown. Recent reports suggest that the protein serves to mediate homotypic cell-cell adhesion as a component of the tight junction (22). *In vitro* analyses suggest an absence or paucity of CAR expression in tumor cells (23–27), leading to the supposition that the usefulness of Ad vectors for cancer gene therapy is likely to be limited *in vivo* (28). It is unknown, however, whether CAR expression *in vitro* is a reliable predictor of expression *in vivo*, and the data are conflicting with respect to whether CAR expression *in vivo* is sufficient to mediate efficient gene transfer into target cells (29, 30). Moreover, even if CAR expression is a correlate of adenovirus-mediated gene transfer efficiency *in vivo*, it is not clear whether specific and/or physical barriers *in vivo* negate the efficiency of Ad transduction that is conferred by CAR expression (30–36).

Investigating gene transfer to lung cancer, our previous studies provided conclusive evidence that gene transfer into NSCLC cells is heterogeneous (18). Whereas transduction-sensitive cells bind Ad vector with markedly greater efficiency and specificity for the Ad fiber knob, transduction-refractory cells bind and internalize vector by less efficient pathways (18). Thus, these pilot studies suggested a primary difference in CAR expression between transduction-sensitive and transduction-refractory NSCLC cells. Using these data as a platform for further investigation, we report (*a*) that differences in CAR expression between transduction-sensitive and transduction-refractory NSCLC cells are present *in vitro*, (*b*) that these differences in CAR expression persist in xenograft models, and (*c*) that controlling for adequate vector internalization mechanisms (3), CAR expression is predictive for more efficient gene transfer but insufficient to achieve uniform transduction by Group C Ad vectors *in vivo*.

MATERIALS AND METHODS

Cell Lines and Viral Vectors. NCI-H226, NCI-H1703, NCI-H1437, and NCI-H2122 cells (a gift of Dr. Herbert Oie, National Cancer Institute) were maintained in RPMI 1640 (Irvine Scientific, Santa Ana, CA) with 10% fetal bovine serum (Gemini, Woodland, CA) and penicillin (100 units/ml)/streptomycin (100 μg/ml) [complete growth medium (18)]. Ad vectors were constructed in the Vector Core at the Gene Therapy Center of the University of North Carolina School of Medicine and amplified in the University of California at Los Angeles-Jonsson Comprehensive Cancer Center. Ad5LacZ is E1a/E1b and partially E3-deleted and expresses the reporter LacZ gene under the control of the cytomegalovirus promoter region. Ad vectors were typically purified and concentrated with double CsCl ultracentrifugation and stored at −20°C in a nonfreezing solution containing 25% glycerol, 0.05% BSA, 4 M CsCl, 50 mM NaCl, 0.5 mM $MgCl_2$, and 5 mM Tris buffer. Immediately before use, vectors were gel filtered (G-50 Sephadex; Boehringer Mannheim, Indianapolis, IN) and eluted into growth medium for transduction studies as described previously (18).

RT-PCR and Quantitative Real-Time RT-PCR Protocols. Total RNA was extracted from transduction-sensitive and -resistant cells using TRIzol reagent (Life Technologies, Inc., Grand Island, NY). RT-PCR was performed using the SuperScript One-Step RT-PCR system (Life Technologies, Inc.). Primers were as follows: for CAR expression, 5′-AAACCGCCTACCTGCAGCCG-3′ (sense) and 5′-GAGCTTTATTTGAAGGAGGGACAACG-3′ (antisense) (product size, 747 bp); and for β-actin (control) expression, 5′-CTCGTCGTCGACAACGGCTC-3′ (sense) and 5′-AAACATGATCTGGGTCATCTTCTC-3′ (antisense) (product size, 353 bp). The RT-PCR protocol was as follows: cDNA synthesis (1 cycle: 54°C for 30 min) followed by 94°C for 2 min. DNA amplification followed (38 cycles: 94°C for 15 s; annealing at 55°C for 30 s; and extension at 72°C for 1 min) with a final extension at 72°C for 10 min. A plasmid [pcDNA3 containing the CAR cDNA insert; a kind gift from Dr. David Curiel (University of Alabama at Birmingham Gene Therapy Center)] encoding for CAR was used as positive control for cDNA amplification and for confirmation of the amplicon as CAR by Southern hybridization.

For quantitative real-time RT-PCR, total RNA was extracted from cells in log-phase growth according to the manufacturer's protocol using the TRI Reagent (Molecular Research Center, Cincinnati, OH). The extracted total RNA was solubilized (in diethyl pyrocarbonate-H_2O) at a concentration of 100 ng/μl, and 500 ng were used for amplification in an iCycler (Bio-Rad, Hercules, CA). Reaction components and cycling conditions were modified from manufacturer's protocol in the QuantiTech SYBR Green RT-PCR Kit (Qiagen, Valencia, CA). Briefly, a 124-bp CAR amplicon was reverse transcribed and amplified using the following primers: sense, 5′-AGGGACCGCTGGACATCGAG-3′; and antisense, 5′-ACTCGGCCTTTCAGATCTGG-3′. Similarly, a 115-bp amplicon of the control β-actin gene was reverse transcribed and amplified using the following primers: sense, 5′-TGTGGGATCAGCAGAGGAG-3′; and antisense, 5′-CTCGTTTGGGAGCTGTTCA-3′. After reverse transcription (50°C, 20 min), Taq DNA polymerase was activated (95°C, 15 min, 1 cycle), and DNA PCR amplification was performed (45 cycles: denaturation at 94°C for 15 s; annealing at 55°C for 30 s; extension for 15 s). The threshold cycle for CAR expression in each cell line was normalized to β-actin gene expression and standardized to positive control amplification of the CAR cDNA insert in pcDNA3.

***In Vivo* Transduction Protocols and Reporter Gene Expression Analysis.** Four-week-old female *SCID/SCID* received s.c. injection in the flanks with 8 × 10^6 cells/mouse. When tumors reached a cross-sectional area between 0.5–1 × 0.5–1 cm^2 (2–4 weeks, depending on the cell line), they were directly injected with Ad5LacZ at a MOI of 100 in a volume of 50 μl of PBS. The number of cells in the tumor (for determination of MOI) were estimated based on the following measurements and assumptions: (*a*) bisecting diameters of the tumors were measured, and the tumor volume was approximated using the formula 0.4 (ab^2), where *a* is the long measured axis of the tumor, and *b* is the short measured axis (37); (*b*) the cells within the tumors were assumed to be spherical (volume = 4/3 πr^3) with a radius of 10 μm; and (*c*) the entirety of the tumor mass

was assumed to be comprised of tumor cells. Thirty h after vector injections, the tumors were extirpated, minced, and suspended in 4 IU/ml collagenase (type II collagenase; Sigma, St. Louis, MO) in RPMI 1640 at 4°C overnight. The following morning, cells were washed twice in PBS (Irvine Scientific), fixed (0.5% gluteraldehyde, 10 min, 4°C), washed twice in PBS containing 100 mM $MgCl_2$, and evaluated for LacZ expression by histochemistry (for intracellular 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside hydrolysis; Life Technologies, Inc., Rockville, MD). Tumor cells were identified morphologically, and the percentage of positive (blue) cells was determined by counting a total of at least 400 cells from each group under a hemacytometer as described previously (18).

CAR Immunolocalization and Flow Cytometry. Soluble recombinant CAR and rabbit antiserum to this protein (anti-recombinant CAR antiserum; Cocalico Biologicals, Reamstown, PA) were gifts from Dr. J. Bergelson [Children's Hospital of Pennsylvania (35)]. For Western immunoblots, total protein was extracted from cells during log-phase growth using Mammalian Cell-PE LB buffer (Genotechnology, Inc., St. Louis, MO) at 4°C and quantified using the Bio-Rad Assay Kit (Bio-Rad). Fifty μg of protein were suspended in 1× volume of Laemmli sample buffer (Bio-Rad), and the volume of the samples was brought to 50 μl with PE LB buffer for loading. The samples were heated at 95°C for 5 min, resolved by 10% SDS-PAGE, and transferred to immunoblot polyvinylidene difluoride membrane (Bio-Rad) on ice. After blocking the membrane with NAP-Sure Blocker (Genotechnology, Inc.), the membrane was washed three times in PBST over 30 min and probed with rabbit anti-CAR antiserum (1:2500 dilution in PBS, 1 h, room temperature). After three washes in PBST, the primary was labeled with antirabbit-alkaline phosphatase conjugate (1:3000 dilution, 2 h, room temperature; Promega, Madison, WI), and after repeat washes in PBST, specific bands were detected by chemiluminescence (ProtoBlot II AP System; Promega).

RmcB, a murine monoclonal antibody that recognizes the extracellular NH_2 terminus region of CAR (38), was purified from hybridoma (CRL-2379; American Type Culture Collection, Manassas, VA) SCID mouse ascites using protein G affinity chromatography (Amersham Pharmacia Biotech) and used to specifically detect cellular CAR expression by flow cytometry (fluorescence-activated cell sorting), immunocytochemistry, and IHC. To detect CAR using fluorescence-activated cell sorting, 1×10^6 cells were preincubated in 100 μl of 0.1% BSA (in PBS, 20 min, room temperature) before primary antibody (RmcB; mouse IgG1, 1:100 dilution in PBS/0.1% BSA, 90 min, room temperature) was admixed on an orbital shaker. Surface expression for the α_v integrin was accomplished in a similar manner, except the mouse monoclonal MAB1960 (Chemicon, Temecula, CA) was used for primary labeling. Cells were then sedimented and washed three times with PBS/0.1% BSA, incubated with secondary antibody [FITC-conjugated sheep anti-mouse $(Fab')_2$ (Sigma); 1:200 dilution in PBS/0.1% BSA, 30 min, room temperature in the dark], washed three times, and resuspended in 500 μl of PBS for flow cytometry by FACScan using CellQuest software (Becton Dickinson, Mountain View, CA). For all data acquisitions and analyses, gates were based on the forward and side scatter profiles of unstained cells, and surface expression of target proteins was normalized to that of cells that had been incubated with secondary antibody alone. For IHC, xenografted tumors were dissected, fixed in Bouin's liquid fixative (Fisher Scientific), and embedded in paraffin, and 5-μm sections were sliced. After deparrafinization and serial rehydration, the sections were rinsed for 5 min in tap water. Endogenous peroxidase activity was quenched by incubating the tissue sections in 0.3% H_2O_2 for 30 min. After a PBS rinse, the sections were blocked with 2% goat serum in PBS (30 min, room temperature) and washed, and the primary antibody (RmcB; mouse IgG1) was applied at a 1:100 dilution at 4°C overnight. After three washes in PBST followed by PBS, RmcB was labeled with biotinylated goat antimouse antibody [1:200, 30 min at room temperature (Vector-ABC method for IHC staining; Vector Laboratories Inc., Burlingame, CA)]. After washing with PBS, the tissue was incubated for an hour in horseradish peroxidase-conjugated streptavidin, and the CAR-reactive products were visualized using 0.5% diaminobenzidine and H_2O_2. The tissue was then counterstained with hematoxylin, and the histology was photomicrographed by light microscopy. CAR labeling imparted a brown color.

Statistical Methods. All transduction and surface labeling experiments were performed using at least three specimens a minimum of two separate times. To determine the statistical significance for differences in transduction efficiency, results were indexed to Ad MOI, and comparisons were made using Kruskal-Wallis ANOVA on ranks, followed by Bonferroni group comparisons. A statistically significant difference was defined as $P < 0.05$ between the groups compared.

RESULTS

CAR Expression *in Vitro* Correlates with Susceptibility to Ad Transduction. Ad transduction of NSCLC cells is heterogeneous, and these results prompted us to begin evaluating the structural basis for the observed differences in transduction efficiencies. The heterogeneity of adenovirus-mediated gene transfer into NSCLC cells can be exemplified by the disparity in LacZ expression between adenovirus-susceptible [NCI-H226 (□) and NCI-H1703 (○)] and adenovirus-resistant [NCI-H1437 (■) and NCI-H2122 (●)] cell lines after infection at four different Ad5LacZ-MOIs (Fig. 1). To discern the cause of this heterogeneity and its applicability to gene transfer *in vivo*, we chose to study these particular cells as models because (*a*) they required identical conditions for growth *in vitro*, (*b*) we had empirically confirmed their capacity to grow as xenografts in immunocompromised mice, and (*c*) they exhibited similar amounts of α_v integrin expression as measured by flow cytometry (Table 1). The latter observation suggested a functional equivalence between these cells in the capacity to internalize Ad vector after surface binding. Accordingly, these cells enabled us to more precisely define the role of CAR expression in determining Ad transduction efficiency *in vitro* and *in vivo*.

To begin with, RT-PCR and quantitative real-time RT-PCR were used to examine differences in CAR expression between the adenovirus-sensitive and adenovirus-resistant NSCLC cells. Semiquantitative RT-PCR (Fig. 2*A*) suggested that compared with the constitutive expression of the β-actin gene, the steady-state expression of CAR was reduced in cells

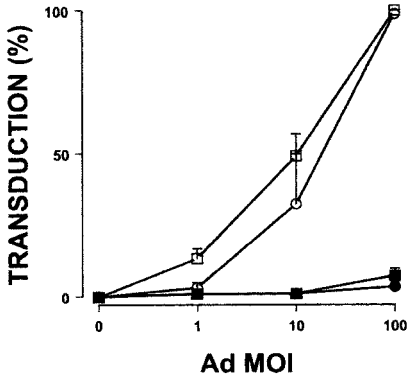

Fig. 1 NSCLC cells exhibit a heterogeneous Ad transduction profile. Comparison of Ad transduction of NSCLC subtypes as a function of MOI. Target cells were exposed to Ad LacZ vector at various MOIs for 60 min, and transduction efficiency (expression of β-galactosidase by histochemistry) was measured 24 h later using light microscopy. Depicted are the Ad transduction profiles (mean ± SE of four experiments) for the following NSCLC cells: NCI-H226, □; NCI-H1703, ○; NCI-H1437, ■; and NCI-H2122, ●.

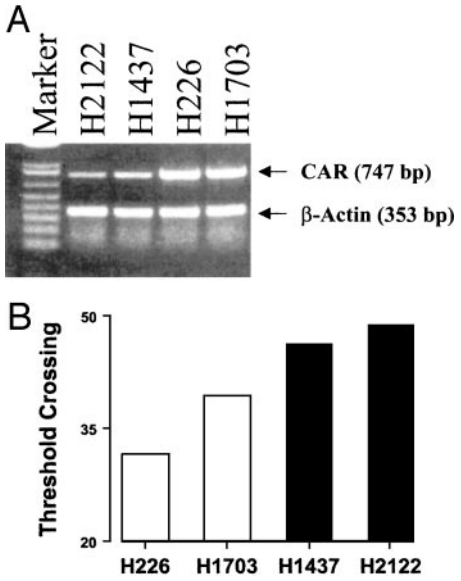

Fig. 2 Adenovirus-susceptible NSCLC cells (NCI-H226 and NCI-H1703) express increased amounts of CAR mRNA compared with adenovirus-resistant NSCLC cells (NCI-H1437 and NCI-H2122). *A*, RT-PCR analysis of steady-state CAR expression (RT-PCR product size, 747 bp) in Ad transduction-resistant (*Lanes 2* and *3*, NCI-H2122 and NCI-H1437, respectively) and Ad transduction-sensitive (*Lanes 4* and *5*, NCI-H226 and NCI-H1703, respectively) cells. *Lane 1* depicts relative molecular mass standards, and the higher mobility band represents the constitutive expression of β-actin (RT-PCR-product size, 353 bp). *B*, real-time RT-PCR analysis of steady-state CAR expression in transduction-sensitive (NCI-H226 and NCI-H1703; □) and transduction-resistant (NCI-H1437, NCI-H2122; ■) cells. Depicted is the normalized (to β-actin expression in the same cells) threshold detection (arbitrary fluorescence units − background) of the CAR-specific amplicon in the cell lines under identical growth conditions *in vitro*.

Table 1 Flow cytometric profiles of α_v integrin expression in transduction-sensitive (NCI H226 and NCI-H1703) and transduction-resistant (NCI H1437 and NCI-H2122) cells

Depicted are the percentage of gated cells that express the cell surface epitope and the fold increase in MCF over cells that were exposed to the FITC-labeled secondary antibody alone.

Cell line	Surface epitope	% gated cells	Fold MCF increase
H226	α_v	97.67	5.00
H1703	α_v	99.64	7.82
H1437	α_v	99.61	3.69
H2122	α_v	98.49	3.79

that exhibited relatively poor Ad transduction efficiency and was increased in adenovirus-susceptible cells. To confirm this observation, real-time RT-PCR was performed on these cells. The results were congruent (Fig. 2*B*), and the steady-state CAR expression in adenovirus-sensitive (NCI-H226 and NCI-H1703) cells was distinctly increased when compared with the transduction-resistant cells (NCI-H1437 and NCI-H2122). The CAR mRNA concentration in extracts of NCI-H226 cells was estimated to be 10 pg/μl, based on a standard curve generated using control amplification of the CAR cDNA insert in pcDNA3, although similar extrapolations could not be made from threshold values of the remaining cells because they fell outside the linear range of the standard curve.

To evaluate the surface expression of CAR in these cells, we performed a series of immunolocalization studies on cytological preparations and protein extracts. CAR-specific immunofluorescent tagging of cytological preparations demonstrated increased membrane labeling in adenovirus-susceptible cells (NCI-H226 and NCI-H1703) *versus* adenovirus-resistant cells [NCI-H1437 and NCI-H2122 (data not shown)]. Similarly, after surface labeling and evaluation by flow cytometry, CAR expression on NCI-H226 and NCI-H1703 cells was reliably 4-fold and 10-fold higher, respectively, when compared with NCI-H1437 and NCI-H2122 cells (Fig. 3*A*). Likewise, Western blots indicated increased CAR expression in NCI-H226 and NCI-H1703 cells as compared with the adenovirus-resistant cells (Fig. 3*B*). Thus, by all indications, the general patterns of CAR expression were consistent with the PCR data, and CAR expression was increased in adenovirus-susceptible cells (NCI-H226 and NCI-H1703) as compared with the adenovirus-resistant cells (NCI-H1437 and NCI-H2122). Collectively (Figs. 1–3), these data indicate that Ad transduction *in vitro* positively correlates with CAR expression, provided expression of integrins is a not limiting factor.

CAR Expression *in Vitro* Is Predictive of *in Vivo* CAR Expression in NSCLC Xenografts. The cellular function of CAR is unknown, although extracellular, transmembrane, and cytoplasmic domains have been identified (39, 40), and its putative role as an adhesion receptor that mediates homotypic intercellular contacts has been hypothesized (22). To date, however, it is not known whether CAR expression is affected by culture conditions, or how its expression as a component of tight junctions and/or adherens junctions is regulated. Accordingly, we believed that it was important to ascertain whether the observed differences in CAR expression *in vitro* between adenovirus-susceptible and adenovirus-resistant NSCLC cells persisted *in vivo*. To characterize this expression, we established

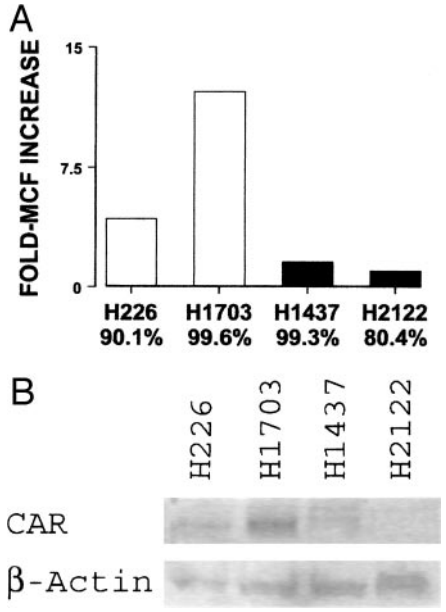

Fig. 3 Adenovirus-susceptible NSCLC cells express increased amounts of CAR compared with adenovirus-resistant NSCLC cells. *A*, flow cytometric assessment of surface CAR expression [primary labeling with RmcB, secondary labeling with FITC-conjugated sheep anti-mouse $(Fab')_2$] of transduction-sensitive (NCI-H226 and NCI-H1703; □) and transduction-resistant (NCI-H1437 and NCI-H2122; ■) cells. The percentage of gated cells that express the cell surface epitope is depicted on the *abscissa*, and the fold increase in mean channel fluorescence over cells that were exposed to the FITC-labeled secondary antibody alone is shown on the *ordinate*. *B*, CAR expression by Western immunoblots of total protein extracts (50 μg) from transduction-sensitive and transduction-resistant cells. Samples were resolved by 10% SDS-PAGE and transferred. The membrane was blocked, probed with rabbit anti-CAR antiserum, and labeled with antirabbit AP conjugate, and specific bands were detected by chemiluminescence. Depicted are the CAR- and β-actin (control for load)-specific bands from each cell line.

s.c. xenografts and examined CAR expression within these tumors by IHC (Fig. 4). These studies evidenced an expression pattern that was predicted by the *in vitro* analyses, with adenovirus-susceptible cells (NCI-H226 and NCI-H1703) exhibiting a demonstrably stronger staining pattern for CAR as compared with the adenovirus-resistant cells (NCI-H1437 and NCI-H2122; Fig. 4). Thus, these studies indicated that a pattern of CAR expression exhibited by NSCLC cells *in vitro* was qualitatively recapitulated in s.c. xenografts implanted in immunodeficient mice *in vivo*.

CAR Expression Correlates with Susceptibility to Ad Transduction, but the Efficiency of Gene Transfer *in Vivo* Is Reduced When Compared with Transduction Efficiency *in Vitro*. It is unknown whether CAR expression is mandatory and/or sufficient to mediate efficient Ad gene transfer *in vivo*. Indeed, when gene transfer efficiency has been directly examined after the controlled expression of CAR in polarized lung epithelial cells *in vivo*, the results have been conflicting (29, 30). Similarly, it remains to be determined whether exuberant CAR expression in tumors is a surrogate measure for efficient Ad transduction, and whether the extent of transduction efficiencies achievable *in vivo* are comparable with those exhibited by the same cells *in vitro*. To begin to answer this question, we injected AdLacZ, at a MOI of 100, into xenografts established using the adenovirus-susceptible (NCI-H226 and NCI-H1703) and adenovirus-resistant (NCI-H1437 and NCI-H2122) NSCLC cells. A MOI of 100 was chosen based on the observation that the adenovirus-susceptible (NCI-H226 and NCI-H1703) NSCLC cells exhibited uniform transduction at that dose *in vitro* (Fig. 1). Thirty h after single intratumoral vector injections, the tumors were extirpated, minced, and digested to yield tumor cell suspensions that were evaluated for LacZ expression by histochemistry. Our results, as shown in Fig. 5, indicate that although Ad gene transfer positively correlates with *in vivo* CAR expression, the efficiency and reliability of transduction of adenovirus-susceptible NSCLC cells may be variable. Thus, whereas NCI-H226 and NCI-H1703 cells exhibit uniform (100%) transduction *in vitro* at an Ad MOI of 100, the tumors generated using these cells displayed transduction efficiencies of 45.3 ± 26% and 24.1 ± 8.4%, respectively, after a single intratumoral injection *in vivo*. In contrast, gene transfer efficiency into CAR-deficient cells *in vivo* remains similar to that observed *in vitro* and peaks at approximately 5% of cells within the tumor mass. These results indicate that CAR expression is predictive for more efficient gene transfer but is insufficient to achieve uniform transduction by Group C Ad vectors *in vivo.*

DISCUSSION

A number of investigators have postulated that poor Ad transduction is caused by poor CAR expression in tumors *in vivo* (24, 25, 41). That postulate tacitly suggests that robust CAR expression will enable efficient Ad gene transfer and that enhancing (pharmacologically or otherwise) CAR expression will augment Ad transduction efficiency *in vivo*. To begin to understand the relevance and importance of CAR for mediating intratumoral gene transfer *in vivo*, we chose to directly investigate the relationship between CAR expression and Ad transduction in defined NSCLC cell model systems. First, we confirmed that the Ad transduction efficiency of NSCLC cells *in vitro* can be directly related to CAR expression (Figs. 1–3). Next, we demonstrated that CAR expression *in vitro* predicted a comparable pattern of CAR expression in transplanted NSCLC xenografts *in vivo* (Fig. 4). Finally, whereas increased CAR expression in the NSCLC xenografts may be a correlate, it is not a direct surrogate for predicting Ad transduction efficiency *in vivo* (Fig. 5). Accordingly, CAR expression alone may not be a guarantee for highly efficient or "therapeutic" gene delivery *in vivo*, and the efficiency and reliability of *in vivo* Ad transduction are likely also controlled by parameters other than the expression of attachment receptors for gene transfer vectors.

We have undertaken these studies while taking into account some of the variables that potentially limit an assessment of the relationship between CAR expression and Ad gene transfer efficiency. For example, we demonstrated differential CAR expression both *in vitro* and *in vivo* between the various NSCLC cells tested, and we made an effort to use cells that were controlled for the comparable expression of α_v integrin such that postadherence Ad internalization would not

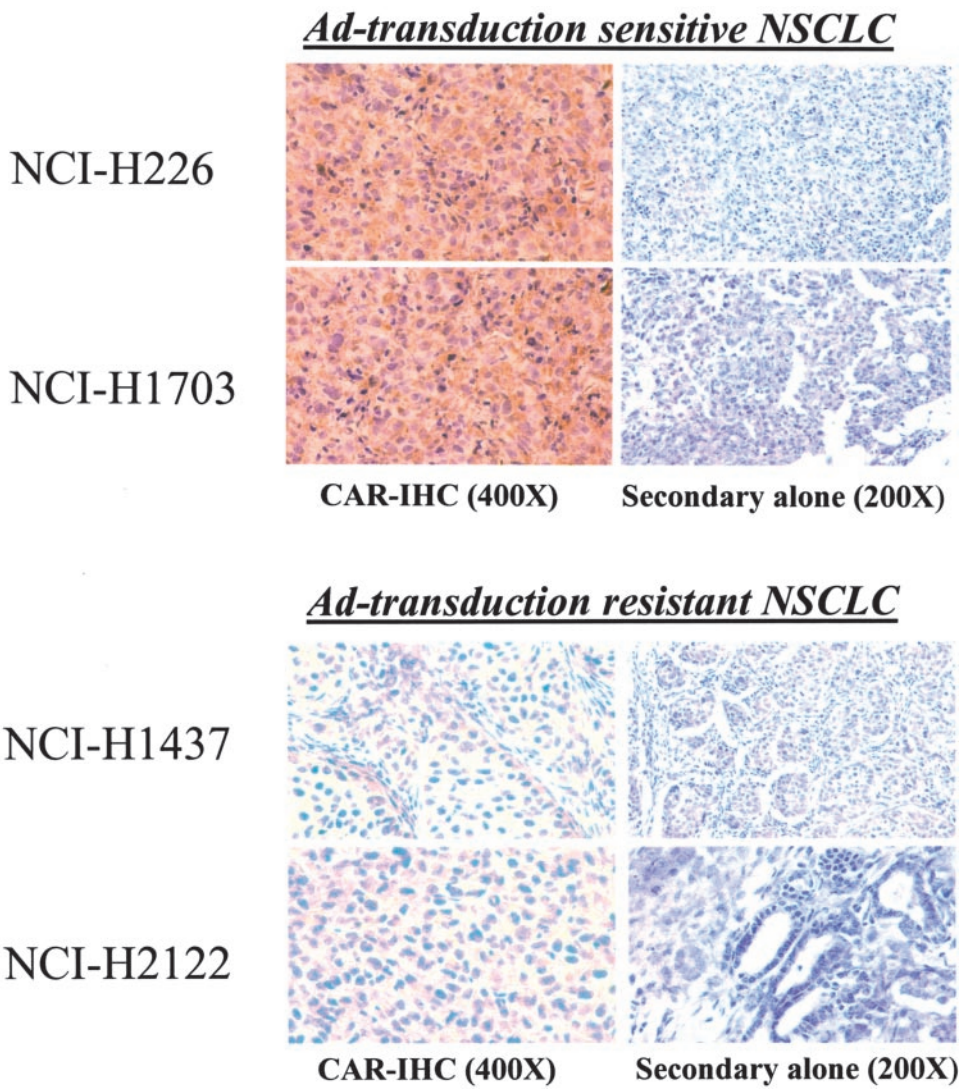

Fig. 4 Increased CAR expression *in vivo* in tumor xenografts generated using adenovirus-susceptible as opposed to adenovirus-resistant NSCLC cells. CAR expression in xenografted tumors was assessed by IHC. The primary RmcB (mouse IgG1) was labeled with biotinylated goat antimouse antibody and horseradish peroxidase-conjugated streptavidin, and the CAR-reactive products were visualized using 0.5% diaminobenzidine and H_2O_2. The tissue was then counterstained with hematoxylin, and the histology was photomicrographed by light microscopy. CAR labeling imparts a *brown color*. Depicted are representative photomicrographs of CAR IHC (×400) and control tissue labeling after incubation with secondary antibody alone (×200).

be limiting for transduction. For controlling study variables, we chose a methodology that called for a single viral dose administered with a single intratumoral injection. For analysis, we also deliberately chose a technically difficult but methodical approach to quantify transduction *in vivo*. In this respect, because we wanted to get a global assessment of intratumoral gene transfer after a single controlled vector injection, we systematically counted a sample of transduced cells digested out of extirpated primary tumors. In our view, this method allowed us to better control for the bias introduced by the heterogeneous distribution of transduced cells (*e.g.*, greatest density of transduced cells proximal to the injection site, and a lower proportion of transduced cells distant to the site of injection) within the tumor *in situ*. Despite these controls, we admit that the system for comparing the relative *in vitro versus in vivo* transduction efficiency of individual cell types may be somewhat artificial, with many residual confounding variables. For instance, our techniques did not take into account variations in the the intratumoral dispersion of vector, dwell time of vector within tumor bed, and/or clearance of vector by host circulation or reticuloendothelial cells *in vivo*. Similarly, the comparative doses of virus *in vitro versus in vivo* were estimates at best, using assumptions (see "Materials and Methods") that may be disputed. Nevertheless, these measurements do provide an awareness that the vector dose at which cells exhibit uniform transduction *in vitro* can be estimated to yield approximately 10% to 70% transduction *in vivo*, when using this intratumoral mode of vector delivery. Indeed, for many gene therapy paradigms with significant bystander effects and/or antitumor immune effects, this degree of gene transfer may be expected to yield clinically therapeutic effects. Thus, pharmacological manipulations designed to up-regulate CAR expression (42, 43) may some have efficacy in terms of improving Ad gene transfer *in vivo*. In conclusion, this study indicates that prediction of intratumoral Ad gene transfer efficiency needs to account for more than just an examination of Ad entry receptors and that alternative tumor- or host-

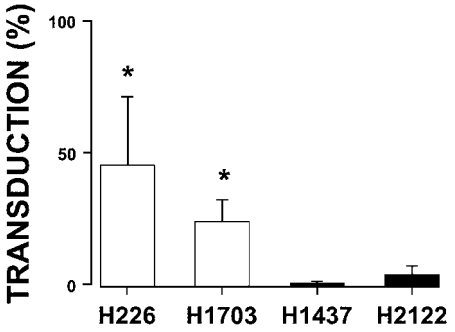

Fig. 5 Increased CAR expression *in vivo* predicts improved but not uniform Ad transduction of NSCLC cells in tumor xenografts. Four-week-old female *SCID/SCID* mice received s.c. injection in the flanks with 8×10^6 cells/mouse. Upon reaching a volume of ≥ 5 mm^3, tumors were injected with Ad5LacZ at a MOI of 100 in 50 μl of PBS. Thirty h after vector injections, the tumors were extirpated, minced, and suspended in collagenase, and cellular suspensions were fixed and evaluated for LacZ expression by histochemistry. The percentage of positive cells is presented as the mean ± SE of seven tumors for each cell type. *, represents a significant difference in the percentage of cells ($P < 0.05$) expressing β-galactosidase between the solid *versus* the empty bars.

specific variables may need to be defined for certain gene therapy strategies.

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