Soluble Coxsackievirus Adenovirus Receptor Is a Putative Inhibitor of Adenoviral Gene Transfer in the Tumor Milieu

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ABSTRACT

Purpose: Several barriers that collectively restrict gene delivery by viral vectors in vivo have been described. Previously, we identified soluble chondroitin sulfate-proteoglycans/glycosaminoglycans in malignant pleural effusions (MPEs) as inhibitors of retroviral vector transduction. Soluble components of MPE also inhibited adenoviral (Ad) gene transfer, and the factors were characteristically filterable, titrable, stable at 56°C, and blocked the binding of Ad to target cells. Depleting immunoglobulin from MPE, partially reversed the block to Ad transduction, instigating a search for additional factors that bound Ad in MPE.

Experimental design: Vector-protein interactions were identified after the resolution of MPE-components by SDS-PAGE. Viral overlays and immunoblots delineated significant interactions, and the potential relevance of those interactions was tested in transduction efficiency bioassays.

Results: Immunoglobulin is the predominant factor inhibiting Ad gene transfer in MPE. Albumin also interacted with Ad, although at predicted serum concentrations, it did not effect Ad transduction efficiency in vitro. Soluble coxsackievirus-Ad receptor (sCAR) was then identified in MPE. In a survey of 18 MPE, the mean concentration of sCAR was variable and estimated to be 3.51 ± 5.02 ng/ml by ELISA. The impact of sCAR on transduction efficiency in this milieu was next assessed. Whereas immunodepletion of sCAR from MPE by affinity chromatography resulted in enhanced gene transfer within MPE, the inhibition of adenoviral gene transfer was not evident when the predicted concentrations of recombinant sCAR were added into the transduction medium.

Conclusions: These studies indicate that, in addition to anti-Ad antibodies, other specific and nonspecific factors interact with viral vectors and may impair gene transfer in the tumor milieu. The presence of sCAR in MPE puts forward the notion that in certain contexts (e.g., within the extracellular matrix of solid tumors) the concentrations of secreted (or shed) CAR may be high enough to effectively compete with Ad gene delivery.

INTRODUCTION

There are ~180,000 new cases and >160,000 deaths annually attributable to lung cancer, making it the leading cause of cancer-related mortality in both men and women (1). Using conventional multimodality therapy, the overall cure rate for all patients with lung cancer is less than 15% and surgery for early-stage disease remains the only dependable curative option (2). Consequently, there is an obvious need for new and effective therapies. Treatment advances may originate from a variety of strategies that are currently under development, including gene therapy approaches (3, 4).

Investigations over the last several years have clarified mechanisms by which Ad vectors mediate gene transfer into cells in vitro. In model cell cultures, these studies indicate that Ad initially attaches to a specific high-affinity cell surface receptor, CAR (5). This attachment seems to trigger a clustering of cell surface integrins, cellular actin stabilization, and viral endocytosis using coated pit mechanisms (6–8). The virus begins to disassemble at the cell surface, and after internalization, disrupts endosomal vesicles to escape into the cytoplasm (9–11). Endosomal escape, which also uses integrin-mediated processes (12, 13), is followed by the transport of the Ad-nucleocapsid to the nucleus (14, 15) and epismal expression of the vector DNA.

For many gene therapy paradigms, efficient gene transfer...
into target cells in vivo is a prerequisite for successful translation into the clinical arena. However, despite the elucidation of cellular mechanisms underlying Ad gene transfer, successful translation to intratumoral and systemic Ad gene therapy remains enigmatic. Recently, investigators have instilled high doses of Ad (up to $7.5 \times 10^{12}$ particles) directly into the tumors by bronchoscopy or transthoracic computed tomography-guided injection (16–18). Even under these circumstances, using intratumoral transgene mRNA (by reverse transcription-PCR of vector-specific sequences) as a marker of transduction efficiency, Swisher et al. (16) and Nemunaitis et al. (17) both reported less than 40–50% expression, and Schuler et al. (18) reported transgene expression in 68% of subjects with repeated testing. These results indicate that a major limitation for the successful gene therapy of NSCLC is efficient in vivo gene delivery to target cells (19–21), which may be difficult to achieve for a number of reasons. To begin with, there are both soluble (19, 22–25) and structural barriers within the extracellular matrix that impede vector interaction with its target cell (26, 27). Secondly, either target cells may be deficient in the expression of attachment and internalization receptors for the vector (28, 29) or the expression of the receptors in polarized cells may not coincide with the mode of delivery (30). Accordingly, for the Ad vector system, gene delivery may be compromised by both Ad-specific (e.g., humoral immunity or lack of high-affinity Ad entry receptors) and nonspecific components (e.g., MUC-1, glycoalyx).

We have targeted intrapleural malignancy to model gene therapy for lung cancer, focusing on MPEs as the experimental and clinical correlate. In the process, we have studied gene transfer in the fluid component of MPEs derived from patients. MPEs arise in 10% of all cases of lung cancer, and mostly in patients with disseminated disease (31, 32). Because diminished lymphatic clearance underlies the pathogenesis of MPE, the effusion is a particularly rich and stagnant reservoir of soluble factors (plasma filtrate, matrix- and tumor-byproducts, and debris of dead cells) that can potentially interact with and impede viral gene transfer in this milieu. Previously, we recognized that chondroitin sulfate proteoglycans and glycosaminoglycans (CS-PG/GAG) inhibited gene transfer by retroviral vectors in MPE (19), and more recently, we observed that soluble factors in MPE inhibit gene transfer by Ad vectors as well (21). We determined that the inhibitory activity was filterable, sensitive to boiling, and could be titrated (21). In native effusions, using microporous filters, the inhibitory factors fractionated with molecular species that were greater than $M$, 100,000. However, unlike the inhibitors to retroviral transduction, complement proteins and chondroitin sulfate proteoglycans and glycosaminoglycans (CS-PG/GAG) were not implicated (21), and soluble fibronectin in the tumor milieu was excluded as a likely candidate. Importantly, specific binding of radiolabeled Ad by target cells was inhibited in the presence of effusions. Moreover, although preformed anti-Ad antibodies could account for a portion of the observed inhibition, significant inhibition to gene transfer persisted after the effusions were exhaustively depleted of immunoglobulin (21). Together, these studies strongly suggested the presence of other unidentified inhibitory elements in MPEs.

To identify other significant inhibitors to Ad gene transfer in this milieu, we began by screening for Ad-binding to factors in MPE that had been resolved by SDS-PAGE. Concurrently, given that our preliminary studies indicated that Ad binding was inhibited to target cells, we specifically probed the MPEs for inhibitory factors that inhibit cellular attachment, including antifiber antibody (33) and sCAR (the high-affinity cellular receptor that binds Ad; Ref. 5). Our results indicate that, in addition to the humoral response, sCAR is indeed present in MPEs, and its presence may further contribute to inefficient gene transfer by Ad vectors in some settings.

**MATERIALS AND METHODS**

**Cell Lines.** NCI-H226 NSCLC cells (a gift of Dr. Herbert Oie, National Cancer Institute, Bethesda, MD) were maintained in RPMI 1640 with 10% fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 µg/ml; growth medium; Irvine Scientific, Irvine, CA). These cells are efficiently bound by Ad-vectors by a fiber-knob dependent receptor, and they exhibit an efficient Ad transduction profile in vitro (20). Consequently, these cells are used as the target cells in Ad transduction bioassays, and also as a source of membrane-extracted CAR (see below: “Immunoprecipitation and Detection of sCAR.”).

**Viral Vectors.** Ad vectors were constructed in the Vector Core at the Gene Therapy Center of the University of North Carolina School of Medicine. Ad5LacZ is E1a/E1b- and partial E3-deleted, and expresses the reporter LacZ gene under the control of the cytomegalovirus-promoter region. Ad vectors were purified and concentrated with double CsCl ultracentrifugation, and stored at $-70\text{°C}$ in a buffer containing 10% glycerol, 0.05% BSA, 0.5 mM MgCl₂, and 5 mM Tris. Immediately before use, vectors were desalted by gel filtration (G-50 Sephadex; Boehringer Mannheim, Indianapolis, IN) and eluted into growth medium for transduction studies as described previously (20).

**MPEs.** Under an institutionally approved protocol, pleural effusions were collected using aseptic technique from symptomatic patients with known intrapleural malignancy. All of the effusions studied were exudative and pathologically positive for tumor (21). The cells in MPEs were pelleted, and the supernatants were sterile-filtered (to remove microscopic cellular debris) through 0.45 µm filters (Whatman, Clifton, NJ). The fluid components of the effusions were then used immediately for biochemical and transduction bioassays, and aliquots were also stored at $-70\text{°C}$ for additional analyses.

**Transduction Protocols and Reporter Gene Expression Analysis.** Target cells in 6-well or 96-well tissue culture plates (Costar, Cambridge, MA) were exposed to Ad vectors at various MOI (infectious viral particles/cell) ranging from 0 to 1000. The vectors were admixed in test [whole or fractionated effusions (50% v/v), total volume 1 ml/well for 6-well plates or 100 µl/well for 96-well plates] or control medium (without effusions) in humidified chambers at 37°C for 2 h. Ad transduction medium was then aspirated, the cells were washed twice in PBS (Irvine Scientific), and cultured in growth medium until LacZ expression was detected histochemically for intracellular 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal; Life Technologies, Inc., Rockville, MD) hydrolysis in fixed (0.5% gluteraldehyde, 10 min, 4°C) target cells. The percentage of positive (blue) cells
were determined by counting a total of at least 300 cells from each group under a hemacytometer as described previously (20). All of the transduction bioassays were performed a minimum of three times, and integrated results are presented.

**Gel Electrophoresis and Viral Overlays.** Proteins from whole or fractionated (immunoglobulin-depleted) effusions were separated by 10% SDS-PAGE (Bio-Rad, Hercules, CA). Briefly, 40 μg of reduced and denatured (heated at 95°C for 8 min in Laemmli sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 0.01% bromphenol blue, and 100 mM DTT]) MPE-proteins were resolved by electrophoresis through a 10% acrylamide Tris-HCl gel [25 mM Tris, 192 mM glycine, 0.1% SDS (pH 8.3)]. The proteins were subsequently transferred to Hybond nitrocellulose (Amersham Pharmacia Biotech, Piscataway, NJ) in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol). For viral overlays, the membrane was blocked in 5% (w/v) nonfat dry milk in TTBS (20 mM Tris base, 137 mM NaCl, and 0.1% Tween 20), overlaid with 10^11 Ad particles suspended in 5 ml of 5% nonfat milk in RPMI medium and gently rocked (2 h, room temperature). After washing in TTBS (10 min for three times each, room temperature), Ad-bound elements were labeled using rabbit anti-Ad antisera (34; Cocalico Biologicals, Reamstown, PA, 1:1000 dilution in W/B buffer). After rinsing in TTBS, the labeled proteins were detected by incubating the membrane in ECL solution and exposing it to autoradiograph (ECL; Amersham Pharmacia Biotech). In a like manner, species-specific albumins were purchased (Sigma, St. Louis, MO) and solubilized in deionized water, and 40 μg were loaded per lane for the viral overlay.

For total protein analysis, gels were fixed and stained with Coomassie Blue (4 h, 0.05% R-250 Coomassie Blue in 50% methanol, 10% acetic acid by gentle shaking at room temperature) and then destained (5% methanol, 7% acetic acid) until blue bands with clear background were obtained.

**Immunoprecipitation and Detection of sCAR.** RmcB, a murine monoclonal antibody that recognizes the extracellular NH2 terminus region of CAR (35) was purified from hybridoma (CRL-2379; American Type Culture Collection, Manassas, VA) and then destained (5% methanol, 10% acetic acid by gentle shaking at room temperature) the beads were sedimented and immunoglobulin-depleted supernatants collected. RmcB (2 μg) or control murine IgG1 (Accurate Chemicals, Westbury, NY) was admixed into the immunoglobulin-depleted effusion samples (4°C, 16 h in a tube inverter) and the RmcB-bound complexes were immunoprecipitated using Gammabind G-Agarose [W/B buffer (40 μl, 50% v/v in 10 mM sodium phosphate (pH 7.0), 150 mM sodium chloride, and 10 mM EDTA); 6 h on an orbital shaker at room temperature; Amersham Pharmacia Biotech]. Immune complexes were pelleted and washed sequentially with W/B buffer, W/B buffer + 0.5 μl NaCl, W/B buffer + 1% v/v Triton X-100 (Sigma), W/B twice, and water. Next, the beads were resuspended and heated to 95°C for 8 min in 30 μl of Laemmli sample buffer (Bio-Rad), and the precipitated immune complexes were then resolved by 10% SDS-PAGE. The separated proteins were transferred to nitrocellulose as above.

rCAR and mCAR were used as controls for the immunoblot. To generate recombinant CAR, a DNA fragment encoding the extracellular domain of human CAR was amplified with PCR primers and inserted into the baculovirus transfer vector pVT Bac (36) in frame with the honeybee melitin leader sequence. During the amplification process, six COOH-terminal histidine residues as well as restriction sites to permit insertion into pVT Bac were incorporated into the human CAR sequence. Recombination with baculovirus DNA, isolation of recombinant virus, protein production in Sf9 cells, and purification of His-tagged protein by chromatography on Ni2+ -Agarose (Qiagen, Valencia, CA) was as described for herpesvirus glycoproteins (37). Rabbit antiserum to the baculovirus-produced extracellular CAR (anti-rCAR antiserum; Cocalico Biologicals, Reamstown, PA) was tested for specificity by flow cytometry and Western blot analysis of CAR- and mock-transfected Chinese hamster ovary cells. Full-length CAR-protein was extracted from the membranes of NCI-H226 cells (mCAR) as described previously (5). The blot of RmcB immunoprecipitants was blocked (5% w/v nonfat dry milk in PBS + 0.1% Tween 20, 1 h, room temperature), and probed using the rabbit anti-rCAR antiserum (1:3000 dilution). After three washes in PBS-T, specific bands were detected using goat antirabbit HRP-linked antibody that had been preadsorbed against murine and human immunoglobulins (Biosource, Camarillo, CA), repeat washes in PBS-T, and ECL (Amersham Pharmacia Biotech).

**CAR and Immunoglobulin Depletion for Transduction Bioassays.** Four mg of RmcB antibody (0.8 mg/ml in PBS) were bound to Gammabind G-Agarose (Amersham Pharmacia Biotech; 2 ml, 50% v/v W/B buffer, mixed for 1.5 h on tube rotator at room temperature). The beads were sedimented, washed twice with 10 ml of W/B buffer and once with 10 ml of 0.2 mM triethanolamine (pH 8.5) TEA (Sigma) to block unreacted sites. Agarose-bound immunoglobulin was cross-linked in TEA containing 10 mg/ml of dimethylpimelimidate (ICN Biomedicals, Aurora, OH) for 30 min at room temperature. The bead complex was washed with TEA (for 3 h at room temperature) followed by washes in W/B buffer (38) and five washes with 10 ml of PBS, eliminating as much PBS as possible during the last wash. Sterile-filtered effusion (2.5 ml) was added to the RmcB beads, and the sample was incubated at room temperature on a tube rotator for 2 h. The beads were then sedimented, and the CAR-depleted supernatant was collected into a new tube. This sample was divided into two aliquots, one to be used directly in transduction bioassays, and the other was depleted of immunoglobulin by Protein A-affinity chromatography. A 1-ml HiTrap rProtein A column (sufficient to extract ~50 mg of immuno-
globulin; Amersham Pharmacia Biotech) was equilibrated in PBS, and 1.25 ml of effusion sample was passed through the column (flow rate of 0.2 ml/min). CAR and antibody-depleted samples were sterile filtered and used in transduction bioassays.

**Soluble CAR Quantitation in MPEs by ELISA.** ELA/RIA high binding polystyrene plates (96-well; Corning, NY) were coated with 40 g/ml of purified Rmcb [4 g/ml in 0.1 M NaHCO3 (pH 8.2)] overnight at 4°C. The wells were washed three times with PBS-T and subsequently blocked with 100 g/L of 10% fetal bovine serum/PBS solution (1 h, room temperature). Serial dilutions of the standard rCAR in concentrations ranging from 0.15625 ng/ml to 10 ng/ml (50 ml in blocking buffer) or 50 ml of each sterile-filtered effusion were applied to the sample plate in duplicate (1 h, room temperature). The wells were then washed three times with PBS-T, and 50 ml of rabbit anti-rCAR antiserum, diluted 1:3000 in blocking buffer, were added to each well (1 h, room temperature). After washing three times with PBS-T, 50 ml of goat α-rabbit HRP (Biosource) was added (1 h, room temperature). Next, the wells were washed five times with PBS-T and exposed to 100 ml of o-phenylenediamine substrate (buffered in 0.1 M citric acid and 0.2 M Na2HPO4) for 15 min. The reaction was quenched with 100 ml of 2 N H2SO4 and the absorbance read at 490 nm with a Dynatech MR5000 spectrophotometer (Chantilly, VA). The resultant standard curve had a linear fit (y = mx + b, where m = 0.0566 and b = −0.0044) with a correlation coefficient of 0.999.

**Statistical Methods.** Transduction analyses were performed using at least three distinct specimens a minimum of two separate times. To determine statistical significance for differences in Ad transduction bioassays, Ad transduction efficiencies were indexed to Ad MOI and comparisons 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**

Ad Interacts with Albumin and Immunoglobulin Heavy Chain in MPEs Resolved by SDS-PAGE. The pilot studies that initiated our present line of inquiry are detailed in an earlier article (21) and summarized in the “Introduction.” Briefly, we determined that Ad gene transfer was inhibited in MPEs, and that all of the MPEs tested possessed some inhibitory activity. To screen for factors within the effusions that interacted with Ad particles, we first fractionated the constituents of the effusion by SDS-PAGE and overlaid the resolved elements with a suspension of Ad (Fig. 1A). These viral overlay assays suggested that Ad consistently bound to factor(s) with approximate molecular weights of 73,000 and 50,000 (Fig. 1A). Because the Mm 73,000 element ran parallel with BSA, and because albumin is a known component of malignant effusions, we considered whether the Mm 73,000 factor that bound Ad was, in fact, human serum albumin in the MPE. To test this suggestion, we first confirmed that albumin was a prominent component of MPE (by size fractionation and Coomassie Blue and silver staining of SDS-PAGE-separated MPE; Fig. 1B). Next, we separated a series of species-specific serum albumins by SDS-PAGE and assessed Ad binding by viral overlays. Ad was observed to bind ubiquitously to the albumins extracted from a range of species (Fig. 1C), which suggested that this interaction may have significance in vivo. However, when the functional consequences of this interaction on gene transfer were assessed in viral transduction bioassays, albumin did not impact Ad transduction efficiency, even when cells were exposed to AdLacZ-vector in a medium containing human albumin approximating serum concentrations (up to 5 g/dl; data not shown). Because of the apparent lack of impact on Ad gene transfer, the affinity or the specificity of the Ad-albumin interaction was not further studied in this context.

The consistent binding of Ad to a Mm 50,000-factor (Fig.
inferred that the ascites likely contained antifiber antibodies. Accordingly, we evaluated Ad infectivity by blocking endosomal escape post-entry. Only the antifiber antibodies disable binding to the target cell factors in these fluids inhibited Ad binding to target cells (21).

Humoral response to adenoviral vectors, a synergistic response directed against the penton base has also been reported (23). Our observation, in particular, led us to speculate that in addition to anti-Ad antibodies, there were other soluble inhibitors to host-humoral immune response to the Ad is composed of antibodies to the major Ad capsid proteins, with neutralizing immunoglobulin in immune sera directed chiefly to the hexon-capsid protein and the fiber (24, 33, 39–42). In assessing the humoral response to adenoviral vectors, a synergistic response directed against the penton base has also been reported (23). Our results, and those of investigators studying Ad gene transfer in malignant ascites (25), collectively suggested that the inhibitory factors in these fluids inhibited Ad binding to target cells (21).

1A) in MPEs was then investigated. It is appreciated that the host-humoral immune response to the Ad is composed of antibodies to the major Ad capsid proteins, with neutralizing immunoglobulin in immune sera directed chiefly to the hexon-capsid protein and the fiber (24, 33, 39–42). In assessing the humoral response to adenoviral vectors, a synergistic response directed against the penton base has also been reported (23). Our results, and those of investigators studying Ad gene transfer in malignant ascites (25), collectively suggested that the inhibitory factors in these fluids inhibited Ad binding to target cells (21). Only the antifiber antibodies disable binding to the target cell surface (whereas the antihexon and antipenton antibodies attenuate Ad infectivity by blocking endosomal escape post-entry (33, 39–41), which suggests that both MPE and malignant ascites likely contained antifiber antibodies. Accordingly, we inferred that the $M_1$ 50,000 factor binding Ad on the viral overlays was immunoglobulin heavy chains that were derived from anti-Ad antibodies. In fact, passing the effusions through Sepharose A columns led to the removal of the $M_1$ 50,000-band (Fig. 1A) on viral overlays, which suggests that specific or nonspecific binding of Ad by immunoglobulin heavy chains were responsible. However, exhaustingively depleting the MPEs of immunoglobulin only partially restored transduction efficiency, and significant inhibition to gene transfer persisted (21). This observation, in particular, led us to speculate that in addition to anti-Ad antibodies, there were other soluble inhibitors to Ad gene transfer in the fluid component of MPEs.

sCAR Is Present within the Tumor Milieu. High-affinity (serotype C) Ad binding to cells is mediated by an interaction between the Ad fiber knob protein and the NH2-terminal domain of a $M_2$ 46,000 transmembrane receptor (CAR) on the target cell surface (5). This extracellular domain specifically binds the Ad fiber knob, which suggests that a soluble form of CAR in MPE may hypothetically inhibit Ad binding. To test for the presence of sCAR, we first evaluated immunoblots of gel-separated MPEs using RmcB (35), a murine monoclonal antibody that detects an epitope in the NH2-terminal region of CAR. Although RmcB inconsistently detected denatured and reduced CAR on Western blots, it did detect a broad band when MPE were resolved on native (nondenaturing) gels (Fig. 2A). The pattern of migration of sCAR in MPE was dissimilar (slower) to that of recombinant CAR on these native gels, which suggested that the native protein may complex with other components within MPE. This was consistent with our earlier results that fractionated the inhibitory components in native MPE to elements that were greater than $M_1$ 100,000 (21). Moreover, the ability to detect sCAR on native gels suggested that RmcB might be used to immunoprecipitate the native sCAR from MPE-fluid. As demonstrated in Fig. 2B, RmcB immunoprecipitated a $M_3$ 31,000 component of MPEs that was recognized using anti-rCAR antiserum, specifically raised against a recombinant extracellular domain of CAR. Interestingly, this $M_3$ 31,000-factor migrated parallel to the recombinant CAR ectodomain by denaturing PAGE, and was distinct from the full-length CAR protein (including the cytoplasmic and transmembrane domains) extracted from the membrane of NCI-H226 cells (Fig. 2B, Lane 2). Components of RmcB (data not shown) or human IgG (Fig. 2B, Lane 3) are not detected in this immunoblot, and the anti-rCAR antiserum does not recognize similar elements immunoprecipitated with irrelevant murine IgG1 (Fig. 2C). These data indicate that a truncated form of the cellular adenoviral receptor, which includes its extracellular domain, is present in a soluble form in MPEs.

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**Fig. 2** A, RmcB, an anti-CAR monoclonal antibody, recognizes sCAR in MPE resolved by native 10% PAGE. Three separate effusions (E1, E2, E3) along with control rCAR (first lane on left) were electrophoresed on a nondenaturing 10% polyacrylamide gel, transferred to nitrocellulose, and probed with RmcB. Specific bands were detected by ECL using goat antimouse-HRP-linked antibody. B, immunoprecipitation of MPE constituents by RmcB indicates the presence of a truncated sCAR in this tumor milieu. Sterile filtered effusions were preadsorbed with Sepharose rProteinA beads, and RmcB was admixed with the antibody-depleted fraction. Gammabind G-agarose was used to adsorb RmcB-bound components of the solution. The agarose-bound complexes were then pelleted, washed, heated in Laemmli sample buffer, and dissociated by 10% SDS-PAGE. After transfer to nitrocellulose, the blots were blocked and probed using rabbit anti-CAR antiserum. Specific bands were detected by ECL using goat antimouse-HRP-linked antibody. C, immunoprecipitation of MPE-constituents by irrelevant murine IgG1 confirms the specificity of truncated sCAR detected in this tumor milieu. Sterile filtered effusions were prepared and analyzed exactly as in B, except that irrelevant murine IgG1 was admixed with the antibody-depleted fraction. After transfer to nitrocellulose, the blot was blocked and probed using rabbit anti-CAR antiserum. Specific bands were detected using goat antirabbit HRP-linked antibody.
The sCAR Concentration in MPE Is Highly Variable. To estimate the concentration of sCAR that is present in malignant effusions, we developed an ELISA using Rmcb as the capture antibody and the anti-rCAR antisera for detection. Eighteen MPE samples were tested, and the mean sCAR concentration was estimated at $10^{-10}$ M ($3.51 \pm 5.02$ ng/ml) in the 14 samples in which CAR was detected. Four of the 18 effusions did not have detectable sCAR. The estimated sCAR concentrations in MPEs were heterogeneous and ranged from 1 ng/ml to over 20 ng/ml. For a variety of reasons (see discussion), these values may reflect an underestimate of the concentrations in situ.

sCAR May Inhibit Gene Transfer by Ad-Vectors in Vivo. To test whether sCAR in MPEs was impacting Ad-mediated gene transfer, we performed bioassays comparing Ad transduction efficiency in the presence of MPE, MPE depleted of sCAR or immunoglobulin, and MPE depleted of both sCAR and immunoglobulin. We had previously surmised that immunoglobulin was the predominant inhibitor of Ad gene transfer in MPE; however, exhaustively depleting MPE of immunoglobulin (as gauged by the inability to detect Fc by immunoblot) did not completely restore transduction efficiency in this milieu (21). Accordingly, to ascertain the contribution to the inhibition by CAR in those MPEs, soluble CAR and immunoglobulin were removed from MPEs by sequential affinity chromatography using protein G-Rmcb complexes and Sepharose protein A respectively. These results suggested that depletion of either sCAR or immunoglobulin from MPE augmented gene transfer (Fig. 3A), although the results confirmed that immunoglobulin was the dominant inhibitor. Moreover, depletion of both sCAR and immunoglobulin significantly enhanced transduction ($P < 0.05$ at Ad MOIs of 10, 100, and 1000) of NCI-H226 cells than depletion of either component alone. It is also noteworthy that at low Ad MOIs (10 and 1), there still remained other unidentified endogenous factors that continued to inhibit gene transfer as compared with transduction in the absence of all MPE components (Fig. 3A).

To test whether the concentration of sCAR estimated by ELISA could produce the degree of inhibition observed in MPEs after immunoglobulin depletion, we performed transduction bioassays with the exogeneous addition of rCAR (ectodomain generated in baculovirus) at various concentrations in the transduction medium (Fig. 3B). In cells that efficiently bind the Ad vector and that, by inference, have high surface expression of CAR (NCI-H226 NSCLC cells), the concentrations of sCAR (e.g., 3 ng/ml) that were present in MPEs were not observed to inhibit Ad gene transfer (Fig. 3B). In fact, much higher concentrations (e.g., 3 μg/ml) of rCAR were required to evidence significant inhibition, muddying the role of this factor in blocking Ad gene transfer in MPE, but confirming that the presence of sCAR may be inhibitory to Ad gene transfer in tumor milieus in which the local concentrations are in the μg/ml range.

DISCUSSION

We examined the biological basis for the observed inhibition to Ad gene transfer in biological fluid specimens (MPE) acquired from patients with pleural malignancy. Generally, inhibition of Ad binding and transduction in sera or body fluids 

Fig. 3 A. transduction bioassays suggest that sCAR is also an inhibitor of Ad gene transfer in MPEs. Purified Rmcb antibody was immobilized on Gammabind G-agarose and after washing with physiological buffer. These beads were resuspended in effusion samples to remove sCAR, and/or MPEs were depleted of immunoglobulin by Protein A-affinity chromatography. Whole, or the variably fractionated, MPEs were sterile filtered and tested for inhibition to transduction of NCI-H226 cells in in vitro bioassays. Ordinate, transduction efficiency; abscissa, Ad MOI. □, transduction efficiency in the absence of effusion components; ▲, CAR-depleted MPE; ●, immunoglobulin-depleted MPE; ★, both CAR and immunoglobulin-depleted MPE; ○, transduction efficiency in the presence of unfractonated effusions; results are means ± SE of two independent measurements of three separate effusion specimens. Depletion of both immunoglobulin and CAR from MPE resulted in significant increases in transduction efficiency at a range of Ad MOIs (10, 100, and 1000) when compared with either component alone. ★, $P < 0.05$. B, exogenous addition of recombinant sCAR at the predicted concentrations in MPE does not inhibit gene transfer. Histidine-tagged ectodomain of CAR is produced in a baculoviral system and purified using Ni$^{2+}$ chromatography. Various concentrations of this recombinant sCAR are sterile filtered and tested for inhibition to transduction of NCI-H226 cells in in vitro bioassays. Ordinate, transduction efficiency; abscissa, concentration of sCAR in the transduction medium. ■, Ad MOI of 100; ●, Ad MOI of 10; ▲, Ad MOI of 1; results are means ± SE of three independent measurements. There is no apparent inhibition of Ad gene transfer when sCAR is present in the transduction medium at the concentrations predicted to be in MPEs (3 ng/ml), although inhibition to Ad gene transfer is emerging when concentrations are in the μg/ml range.
(e.g., ascites) has been exclusively attributed to the presence of neutralizing antibodies. However, our studies indicate that all of the MPEs tested inhibit Ad gene transfer (21) to varying extents, and that the entirety of the inhibition cannot be accounted for by the preexistent anti-Ad humoral immunity.

Because viral overlays of gel-fractionated biological specimens have been used (43, 44) to identify viral receptors and vector-specific protein-protein interactions, we applied this technique to study interactions of Ad with MPE constituents resolved by PAGE. Interactions with serum albumin and immunoglobulin heavy chain were implicated (Fig. 1), although the importance of these interactions in inhibiting Ad gene transfer was not borne out in transduction bioassays. sCAR was also identified (Fig. 2A) and a truncated form of the receptor was specifically immunoprecipitated from MPEs (Fig. 2, B and C). An ELISA assay predicted that the concentration of sCAR in MPEs was highly variable, averaging ~3 ng/ml in a panel of MPEs that was tested. Whether the measured sCAR concentration in processed (frozen, thawed, and filtered) MPEs is reflective of in situ concentrations needs to be confirmed.

The cellular function of CAR is being uncovered. Extracellular, transmembrane, and cytoplasmic domains have been identified (45, 46) and its putative role as an adhesion receptor hypothesized. A recent report suggests that the transmembrane receptor may serve as an integral component of epithelial cell tight junctions, with the extracellular domain mediating homotypic cell-cell contacts (47). However, this receptor has largely been studied in the context of Ad-mediated gene transfer. In this context, CAR functions as a cellular adherence protein for a variety of Ad subtypes (48). Recent studies have identified the requisite regions of the protein needed to enable Ad binding and gene transfer (45), as well as its intracellular sorting to specific membrane regions of polarized epithelial cells (49). In this report, we have made a novel observation that a truncated soluble form of the cellular adenoviral receptor is present in the tumor milieu (Fig. 2B). Importantly, emerging reports suggest that this observation is credible, and alternative splice products encoded by the human CAR gene have been identified (47, 50). Interestingly, mRNA splicing leads to products that lack the transmembrane domain and the cytoplasmic tail of the full-length CAR, and these isoforms are secreted as soluble forms of the receptor (50). Because RmcB, the murine monoclonal antibody used to immunoprecipitate CAR from MPEs, recognizes the extracellular NH2 terminus region of CAR (35), the truncated form of CAR identified in the tumor milieu may indeed have arisen as a product of alternative mRNA splicing, which suggests that alternative CAR isoforms may be present in situ.

The presence of sCAR in tumor milieus may have implications for achieving efficacious Ad gene transfer in this setting, and additionally, may have ramifications for the biology of CAR. The observation also instigates an inquiry into how sCAR came to be present in MPE, and whether it has a specific biological role in the extracellular matrix. Which cells are responsible for its synthesis, and whether the CAR-ectodomain is secreted, cleaved or “shed” into the tumor milieu by a specific signaling mechanism also needs to be determined. The discovery of sCAR in the tumor milieu also provides credence to the concept that, in certain conditions, the extracellular matrix may serve as a sink for the therapeutic use of gene therapy vectors. The extent to which a matrix-component inhibits gene transfer may be patient and/or disease-specific. The inhibition itself may be specific for the vector, or it may be generic (e.g., anatomical entrapment of the vector, or interactions that may promote nonspecific phagocytic uptake or proteolytic cleavage of the vector).

In this respect, our studies verified that the predominant inhibitor of Ad gene transfer in MPEs is humoral immunity, and removing immunoglobulin from MPE substantially reverses the inhibition to gene transfer (Fig. 3A). Similarly, because the estimated concentration of sCAR in MPEs was determined to be in the 10^{-9} M range in MPEs, and because CAR is known to interact with the wild-type Ad5 fiber knob with high affinity (an estimated Kd of ~0.5 to 1 \times 10^{-7} M; Refs. 51, 52), it was conceivable that sCAR was capable of inhibiting gene transfer at this concentration in vivo by binding with high affinity to the Ad fiber knob. However, experiments testing that prediction yielded disparate results. Whereas the depletion of sCAR by affinity chromatography suggested that this factor was contributing to the inhibition of gene transfer in MPEs (Fig. 3A), the addition of recombinant sCAR at the relevant concentrations into the transduction medium did not impair Ad gene transfer into susceptible target cells (Fig. 3B). In fact, the concentration of rCAR that was needed to mediate significant inhibition was approximately three orders of magnitude greater than the concentration predicted to be in MPEs (Fig. 3B). These results are consistent with previous reports, which cite that the extracellular domain of CAR (expressed in a baculoviral vector) inhibits Ad2-binding to Ramos cells (which express high levels of CAR but lack \alpha_v integrins), although the concentration of sCAR that was needed to mediate complete inhibition was 10 \mu g/ml (48). Collectively, these results seem to suggest that despite high-affinity binding, sCAR may not be an effective inhibitor of adenoviral infectivity unless the concentrations of the soluble receptor are sufficiently high in the target cell microenvironment. Thus, CAR-specific inhibition to Ad gene transfer may be relevant within tumor microenvironments in which CAR is not diluted by fluid (as in MPEs), and in which extracellular sCAR may achieve high tissue concentrations (e.g., within a solid tumor mass). The ineffectiveness of low concentrations of soluble CAR to competitively inhibit Ad gene transfer may also reflect differences in the relative avidity of the intact Ad vector with mCAR (where this interaction triggers associations with cellular coreceptors), versus the affinity of the isolated (knob and CAR or CAR-CAR homotypic interactions (47) recombinant protein domains in solution. In conclusion, although sCAR is present in MPE, its contribution to the observed inhibition to Ad gene transfer is unclear in that milieu. And, if sCAR does mediate inhibition to gene transfer in select situations in situ, then strategies that to overcome this block may also require the development of chimeric or specifically retargeted vectors.

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