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Retroviral Gene Transfer Is Inhibited by Chondroitin Sulfate Proteoglycans/Glycosaminoglycans in Malignant Pleural Effusions*

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Raj K. Batra‡, John C. Olsen, Diana K. Hoganson, Bruce Caterson§, and Richard C. Boucher

From the Division of Pulmonary Diseases, Department of Medicine, University of North Carolina, Chapel Hill, North Carolina, and the §Connective Tissue Biology Laboratory, University of Wales, Cardiff, CF1 3US, United Kingdom

Gene therapy may be an important adjuvant for treating cancer in the pleural space. The initial results of retroviral gene transfer to cancer cells in malignant pleural effusions revealed that transduction was markedly inhibited, and studies to characterize the inhibitory factor(s) were performed. The inhibition was contained within the soluble, rather than cellular, components of the effusions and was demonstrated with amphotropic, gibbon ape leukemia virus, and vesicular stomatitis virus-glycoprotein pseudotyped retroviral vectors. After excluding complement proteins, a series of studies identified chondroitin sulfates (CSs) as the inhibitory substances. First, treatment of the effusions with mammmalian hyaluronidase or chondroitinases, but not *Streptomyces* hyaluronidase, abolished the inhibitory activity. Second, addition of exogenous CS glycosaminoglycans mimicked the inhibition observed with pleural effusions. Third, immunoassays and biochemical analyses of malignant pleural effusion specimens revealed CS in relevant concentrations within pleural fluid. Fourth, proteoglycans/glycosaminoglycans isolated from the effusions inhibited retroviral gene transfer. Analyses of the mechanism of inhibition indicate that the chondroitin sulfates interact with vector in solution rather than at the target cell surface. These results suggest that drainage of the malignant pleural effusion, and perhaps enzymatic pretreatment of the pleural cavity, will be necessary for efficient retroviral vector mediated gene delivery to pleural metastases.

Malignant pleural effusions represent a terminal stage in a disease process for which only symptomatic therapy exists (1, 2); therefore, new therapeutic strategies, including gene therapy, appear warranted. We conducted a series of pilot experiments that compared the transduction efficiencies of adenoviral and retroviral vectors in various lung cancer subtypes and determined that when indexed to multiplicity of infection (infectious particles per cell), retroviral vectors are more efficient than adenoviral vectors in transducing lung adenocarcinoma cells (3). Because most malignant effusions from lung primaries result from metastatic adenocarcinoma, we focused upon retroviruses as the likely vector for this therapeutic indication.

Malignant effusions are often bloody due to neovascularization and capillary leak associated with malignant cellular infiltration of the pleural surface. The fluid component of these exudative effusions is often turbid and viscous, reflecting the contributions of cellular debris and plasma proteins, as well as secreted proteoglycans (PG),[1] their glycosaminoglycan (GAG) catabolites, and hyaluronic acid (2, 4, 5). Glycosaminoglycans are long unbranched polysaccharide chains composed of repeating disaccharide units, linking an aminosugar (typically sulfated) with a uronic acid residue (in all cases except keratan sulfate), which identifies the GAG chain as hyaluronic acid (nonsulfated), chondroitin (or dermatan) sulfate, heparin, or heparan sulfate. Except for hyaluronic acid, GAGs are found associated with a core protein as proteoglycans (6, 7).

Preclinical studies testing gene transfer to primary cancer cells in native malignant pleural effusions indicated that cells in pleural fluid were poorly transduced by retroviral vectors when compared with cells in media. Since the target cells in these studies exhibited proliferation markers suggesting cell replication (a requirement for retroviral transduction), the inhibition to transduction was suspected to be due to components within the pleural fluid. To study the effect of pleural effusions on the transduction efficiency of retroviral vectors *in vitro*, target cell types that are highly transducible with amphotropic retroviral vectors were used. This report provides the identification of novel factors that inhibit retroviral transduction of cells in pleural fluid, a finding that may have relevance to the generally poor transduction efficiency observed *in vivo* using retroviral vectors.

EXPERIMENTAL PROCEDURES

Cells—Mv1Lu cells, a mink lung epithelial cell line that is highly permissive for gene transfer by amphotropic RV vectors (30–70% of the cells are reproducibly transduced at multiplicities of infection of 1–5 (3)), were obtained from the ATCC and maintained in minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, nonessential amino acids, and penicillin (100 units/ml)/streptomycin (100 μg/ml) (M_{10}). H1437, a human lung adenocarcinoma cell line derived from intrapleural metastases; H28, a human malignant mesothelioma cell line; and H226, a human lung squamous cell carcinoma cell line derived from pleural metastases, were kind gifts from Dr. Herbert Oie at NCI, National Institutes of Health (Bethesda, MD). These cell lines were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and penicillin/streptomycin (R_{10}). CFT1 cells (8), a human bronchial epithelial cell line that is efficiently transduced by gibbon ape leukemia virus (GALV) and

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‡ To whom correspondence should be addressed: Div. of Pulmonary Diseases, Dept. of Medicine, 7011 Thurston-Bowles Bldg., University of North Carolina, Chapel Hill, NC 27599-7248. Tel.: 919-966-7049 (ext. 1077); Fax: 919-966-7524; E-mail: rkbatra@med.unc.edu.

[1] The abbreviations used are: PG, proteoglycans; GALV, gibbon ape leukemia virus; VSV-G, vesicular stomatitis virus-glycoprotein; RV, retroviral; LNPOZ, retroviral construct encoding *neu* and *lacZ* genes in the amphotropic or GALV pseudo-typed vectors; HIT-LZ, retroviral construct encoding *lacZ* in the VSV-G pseudo-typed vectors; FDG, fluorescein di-β-D-galactopyranoside; R_{10}, RPMI medium with 10% fetal calf serum; M_{10}, minimal essential medium with 10% fetal calf serum; RBC, red blood cell; BTH, bovine testicular hyaluronidase; S-Hya, *Streptomyces* hyaluronidase; CS, chondroitin sulfate; CSA, chondroitin 4-sulfate; GAG, glycosaminoglycan; AP, alkaline phosphatase; BSA, bovine serum albumin.

amphotropic retroviral vectors (9), were maintained in defined serum-free medium.

Pleural Effusions—Pleural effusions were harvested using aseptic technique from patients with a known or suspected pleurally based malignancy at the time of hospital admission. The effusion fluid used for this study was in "excess" of the specimen required for diagnostic purposes, and the experimental protocol was approved by the University of North Carolina Committee on the Protection of the Rights of Human Subjects. All effusions studied exhibited exudative characteristics, and the final diagnostic cytopathology suggested metastases from the following primary cancers: non-small cell lung cancer (seven), breast adenocarcinoma (three), ovarian cystadenocarcinoma (one), malignant thymic carcinoid (one), and parotid adenocarcinoma (one). The remaining five effusions had suspicious but nondiagnostic cytopathology, and data from these effusions were also included in the final analyses.

Transduction studies using these effusions were generally performed within 60 min of their removal from patients, with whole (unfractionated) or effusion supernatants being used as vector diluents in transduction efficiency assays (see below). The cellular debris was removed from the effusions by centrifugation at 250 × *g* for 15 min, and aliquots of the supernatants stored at −70 °C were later used for detailed characterization of the inhibitory factors. Complement was inactivated by heating the effusions for 1 h at 60 °C, and all enzymatic treatments of effusions were performed overnight at 37 °C on an orbital shaker at 200 rpm.

Vectors—All vectors used were based upon the Moloney murine leukemia virus. LNPOZ (10) is a bicistronic retrovirus vector that uses a poliovirus internal ribosome entry site sequence to express both the *neo* and *lacZ* genes from a single mRNA. HIT-LZ[2] is a lacZ-containing vector with an immediate early CMV promoter/enhancer fused to the retroviral R-U5 region in the 5′-LTR (11). Amphotropic LNPOZ was generated from the helper-free cell line PA-LNPOZ.1 (12). LNPOZ, pseudotyped with the GALV envelope glycoproteins, was produced from PG.LNPOZ.21, a clonal producer line derived from PG13 cells (13). HIT-LZ, pseudotyped with the vesicular stomatitis virus (VSV) glycoprotein, was transiently produced from 293 cells using the three-plasmid co-transfection system (11). Viral vectors were filtered through 0.2 μM cellulose acetate filters and stored at −70° until use. Prior to use, virus stocks were tested for the passive transfer of β-galactosidase ("pseudotransduction") from virus producer cells (14) by analyzing cells for *lacZ* expression immediately following infection. Under these conditions, no β-galactosidase activity was detected in transduced cells.

Red Blood Cell (RBC) Isolation—Ten ml of whole blood were collected in heparinized syringes from three healthy volunteers by venipuncture on three separate occasions. The RBC mass was measured in an aliquot of whole blood using a Coulter Counter (UNC Hospitals hematology laboratories), and RBCs were isolated by Ficoll-Hypaque gradient separation (Sigma). Following isolation, RBCs were co-incubated with the LNPOZ vector at hematocrits ranging from 0 to 15% for 30 min at 37 °C/5% CO_2, following which the RBCs were removed by centrifugation (250 × *g*) prior to vector delivery to target cells.

Retroviral Transduction Protocol and Analysis of Gene Transfer Efficiency—Cells were exposed to vector in 2 ml of vector containing (transduction) media in 3.5-cm tissue culture wells (Costar). Unfractionated (whole) pleural effusions, effusion supernatants, or modified effusion supernatant specimens (heated, enzymatically treated) were mixed with 1 ml of the LNPOZ vector to constitute 1, 10, or 50% of a final volume of 2 ml, with M_{10} and 8 μg/ml polybrene constituting the remainder of the volume. The control transduction medium, used to gauge the maximal achievable transduction in the absence of effusion factors, was 1 ml of vector stock admixed with 1 ml of M_{10}. Target cells were transduced for 2 h at 37 °C and 5% CO_2. Following this interval, vector was aspirated, the cells were washed once with phosphate-buffered saline, growth medium (M_{10}) was added, the cells were incubated at 37 °C in 5% CO_2, and cells were harvested 48 h later for transgene expression analyses.

Transduction efficiency of RV-transduced cells was quantified using flow cytometry (FAC-Scan; Becton-Dickinson Immunocytometry Systems) by measuring intracellular FDG (Molecular Probes) hydrolysis. Briefly, cells were detached from the tissue culture plates (1% trypsin, 1 mM EDTA), sedimented (250 × *g*), and resuspended into 100 μl of growth medium. Next, the cells were loaded with FDG during a brief exposure to hypotonic shock (1 min at 37 °C in the presence of 1 mM FDG in 1% Me_2SO/H_2O). FDG loading was terminated by the addition of 400 μl of ice-cold M_{10} containing 1 μg/ml propidium iodide. The cells were then placed on ice in the dark until fluorescence-activated cell sorting analysis, in which 16,000 viable cells were evaluated for β-galactosidase expression. Nonviable cells, defined as the population that had incorporated propidium iodide, were excluded in real time as red fluorescent cells. Negative (cells that were not exposed to the vector but were loaded with FDG) and positive (cells transduced in medium alone) controls were included in all experiments. Cells displaying green fluorescence exceeding the 99th percentile of the negative control population constituted the transduced cell population.

The percentage of inhibition to transduction was calculated as (1 − (% of transduction of the test cell population − % of transduction of the negative control population)/(% of transduction of the positive control cell population − % of transduction of the negative control population)) × 100. If the test cell population exhibited a transduction efficiency greater than or equal to the positive control, the percentage of inhibition was reported as zero. Similarly, if the experimental population exhibited a transduction efficiency less than or equal to the negative control (*i.e.* ≤1%), the data were reported as 100% inhibition.

Alternatively, transduction efficiency of CFT1 cells by *lacZ*-expressing RV pseudotypes was quantitated by counting 200–400 cells following histochemical staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (5 Prime → 3 Prime, Inc., Boulder, CO) in three separate experiments (15).

PG/GAG Protocols—Hyaluronic acid, chondroitin sulfate A (also referred to as CSA and chondroitin 4-sulfate), and chondroitin sulfate B (also referred to as dermatan sulfate) were derived from bovine tracheal mucosa (Sigma). Chondroitin sulfate C (also referred to as chondroitin 6-sulfate) was derived from shark cartilage (Sigma Biochemical). Each glycosaminoglycan was reconstituted in phosphate-buffered saline (1 mg/ml), and transduction efficiency was measured following exposure of vector admixed with GAG at varying concentrations. In separate experiments, glycosaminoglycans (100 μg/ml) were preexposed to target cells for 2 h and aspirated off before LNPOZ infection.

Enzyme Protocols—The concentration and conditions for the enzymatic digestion of pleural fluid were modifications of procedures used for the isolation of PG/GAG from synovial fluid, vitreous humor, and cartilage (16–22). Excessive amounts of all enzymes were utilized because their relative activities in biological fluids is unknown. Bovine testicular hyaluronidase (BTH), a mammalian hyaluronidase (Sigma), and *Streptomyces* hyaluronidase (S-Hya), a bacterial hyaluronidase (Seikagaku Amano Pharmaceutical Company, Rockville, MD), were reconstituted in 50 mM sodium acetate, pH 6.0. Chondroitinase AC (*Flavobacterium heparinum*), and chondroitinase ABC (CABC) (*P. vulgaris*) were acquired from Sigma and reconstituted in 50 mM sodium acetate/phosphate-buffered saline, pH 7.4. Heparinase (*F. heparinum*), heparitinase (*F. heparinum*), and keratanase (*Pseudomonas* sp.) were purchased as lyophilized preparations from Seikagaku and reconstituted in 50 mM sodium acetate/phosphate-buffered saline, pH 7.4.

Enzymatic treatments of pleural fluid were performed overnight at 37 °C, and retroviral transduction of target cells in the presence of treated or untreated effusions was compared. To evaluate the effect of BTH treatment on cell transduction, a variety of cell types were pretreated with various concentrations of BTH at 37 °C for 4 h prior to RV exposure.

Protocols for the Isolation and Identification of PG/GAG in Pleural Fluid—PG/GAG were purified from 10 ml of six effusion supernatants (representing a range of inhibition to retroviral transduction) by ultracentrifugation (100,000 × *g* for 48 h) in a dissociative (4 M guanidine HCl) CsCl density gradient. Following ultracentrifugation, the tubes were snap frozen at −80 °C and cut into three equal aliquots. The recovered fractions possessed mean specific densities of 1.6 g/ml (bottom fraction, D1), 1.45 g/ml (middle fraction, D2), and 1.34 g/ml (top fraction, D3). These fractions were dialyzed (molecular weight cut-off of 3500) against distilled, deionized water at 4 °C for 2 days (five exchanges). Following dialysis, the fractions were lyophilized, and the PG/GAGs in each fraction were resolubilized in 10 ml of water. The uronic acid content in each fraction was quantitated by the modified carbazole method (23, 24), and the sulfated GAG concentration was measured by the dimethylmethylene blue (DMMB) assay (25). Estimates of the total hexuronic acid and sulfated GAG content in the effusions were made by adding the PG/GAGs isolated in the D1 and D2 fractions derived from each effusion.

Next, the reconstituted D1 and D2 fractions were evaluated for their effect on RV transduction by infecting target cells with LNPOZ in the presence of the isolated PG/GAG. In addition, the D1 fractions isolated from each of three effusions were treated with a panel of enzymes that degrade an array of PG/GAGs to determine whether any of the observed

[2] J. C. Olsen, unpublished observations.

inhibition could be abolished by this procedure.

To size-fractionate the chondroitin PG/GAGs in malignant pleural effusions, 2-ml aliquots of *Streptomyces* hyaluronidase-treated effusions were gel-filtered through a 20-ml Sepharose CL-6B column (Pharmacia Biotech Inc.) equilibrated with 4 M guanidine HCl. Two-ml fractions were collected using 4 M guanidine HCl, 50 mM Tris, and fractions containing chondroitin sulfates were identified with a dot blot immunoassay (see below). The void volume was determined by chromatography of blue dextran (approximately 2 million daltons; Fluka, New York), and the bed volume (salt peak) was determined by chromatography of the bromphenol blue dye (approximately 700 daltons; Sigma) from the column.

Identification of PGs in pleural effusions was carried out by immunoassay utilizing murine monoclonal antibodies with specificity for CSA, chondroitin 6-sulfate, or sulfated GAGs. The 2B6 monoclonal IgG antibody specifically detects nonreducing Δ-unsaturated disaccharides of CSA, and the 3B3 monoclonal IgM antibody reacts with a nonreducing Δ-unsaturated disaccharides in chondroitin 6-sulfate (16, 26) following chondroitinase treatment, which exposes the specific residues in native PGs. The 7D4 monoclonal IgM antibody recognizes a sequence of isomers with different sulfation patterns in native chondroitin sulfate (18). For these analyses, 100-μl aliquots of effusion supernatants, serially diluted in 50 mM Tris-HCl, 200 mM NaCl, 0.02% NaN_3 (Tris-salt buffer, pH 7.0), were loaded onto nitrocellulose membranes (0.2-μm pore; Schleicher and Schuell, Keene, NH) using a dot blot apparatus (Bio-Rad) at room temperature. The membranes were blocked using 5% BSA in Tris-salt buffer for 2 h, treated with chondroitinase ABC (0.01 units in 0.1 M Tris acetate, pH 8, for 1 h) to expose the epitopes for immunoassays using the 2B6 and 3B3 primary antibodies, washed with Tris-salt buffer, and incubated for 1 h with the primary antibody (1:1000 in 1% BSA). The 7D4 immunodetection was performed on native proteoglycans without chondroitinase treatment. Membranes were washed three times with Tris-salt buffer and incubated with the secondary antibody (1:7500 in 1% BSA, anti-mouse IgG conjugated to alkaline phosphatase (AP)) (Promega, Madison, WI) for 1 h. After three additional washes, the dot blots were exposed to AP substrate (Promega) for 15 min and analyzed colorimetrically. Bovine nasal cartilage core was used as the positive control for detection of chondroitin sulfate proteoglycan (2B6 and 3B3 antibodies), and aggrecan from shark cartilage was used as the positive control for detection of sulfated GAG (7D4 antibody).

Statistics—Data are reported as means ± S.E. with the overall statistical significance of differences within the groups determined using one-way analysis of variance. Kruskal-Wallis analysis of variance is initially performed on ranks, followed by the Student-Newman-Keuls or Bonferroni group comparisons. For Figs. 2 and 3, the statistical significance of the difference between the treated *versus* untreated groups was calculated using the two-tailed paired *t* test. For all statistical analyses, $p < 0.05$ was considered significant.

RESULTS

Inhibition of Retroviral Gene Transfer to Target Cells in Malignant Pleural Effusions—Unfractionated (whole) pleural effusions harvested from patients with suspected intrapleural malignancies inhibited retroviral transduction of Mv1Lu cells in a dose-dependent manner (Fig. 1*A*). An approximate 75% inhibition of amphotropic retroviral transduction was observed when these effusions were admixed 1:1 with the retroviral vector-containing medium (Fig. 1*A*). These initial observations suggest that there are factors inhibitory to amphotropic retroviral transduction contained within malignant pleural effusions and led first to studies to discriminate between the cellular *versus* soluble components as mediators of this effect.

We postulated that the amphotropic retroviral receptor (RAM-1) (27) may be expressed in the RBC plasma membrane and bind the LNPOZ vector prior to its interaction with the target cell. However, when RBCs were coincubated with retroviral vectors in the range of hematocrits typically present in malignant pleural effusions (0–1.5%), only a small inhibition of transduction was detected (Fig. 1*B*). At a hematocrit of 15% (RBC content one-third that present in blood), a 50% reduction in transduction efficiency was seen. Because RBC concentrations representing the maximal hematocrit in malignant pleural effusions (~1.5%) could only account for a small component

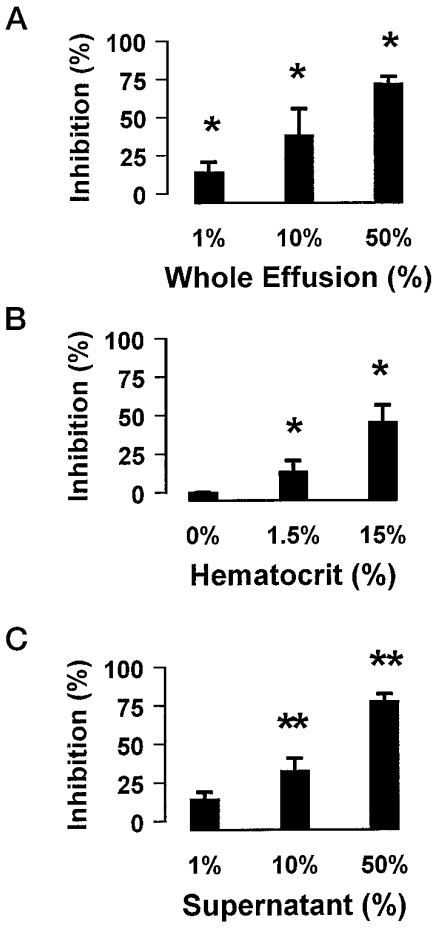
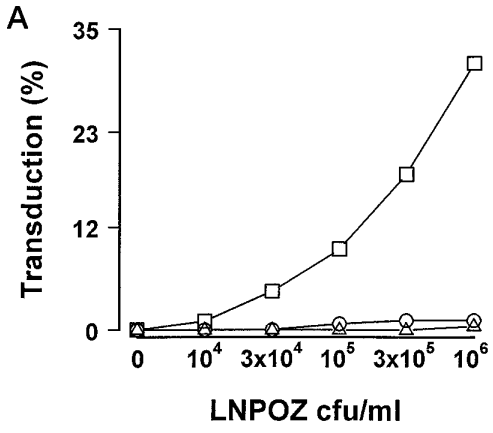


FIG. 1. **Comparison of the inhibition of amphotropic retroviral transduction efficiency in the presence of whole (unfractionated) pleural effusions (*A*), RBCs (*B*), and supernatants (*C*) fractionated from whole pleural effusions.** Mv1Lu cells were transduced with the LNPOZ amphotropic retroviral vector in the presence or absence (control) of freshly harvested malignant pleural effusions (n = 5), RBCs (n = 9, three separate experiments using RBCs from three subjects), or effusion supernatants (n = 16). The efficiency of reporter *lacZ* gene expression was quantified as the percentage of cells transduced by flow cytometry. Shown is the inhibition to transduction (compared with control) by the vector in the presence of increasing volume percentages of the effusions or hematocrits in LNPOZ-containing medium. Data are presented as means ± S.E. *, $p < 0.05$; **, $p < 0.001$, for a statistically significant difference from control by the Student-Newman-Keuls pairwise multiple comparison analysis.

(<20%) of the overall inhibition exhibited by whole effusions, other factors are responsible for the majority of the inhibition to retroviral transduction.

We next tested whether soluble factors within the effusions were blocking retroviral transduction. To test this hypothesis, cells and particulate debris were removed by centrifugation, and the effusion supernatants were evaluated in transduction efficiency assays. Fig. 1*C* shows a dose-dependent inhibition to retroviral transduction by the effusion supernatants in a pattern that is virtually identical to that observed with whole effusions (Fig. 1*A*). This observation suggests that the inhibition to retroviral transduction by malignant effusions is mediated by soluble factors present in the fluid component of those effusions.

To investigate whether this finding was relevant to target cells other than Mv1Lu and to other candidate retroviral vectors, the inhibition to transduction in the presence of effusion supernatant was tested for and observed when the human lung adenocarcinoma cell line H1437 (68 ± 13.4% inhibition) was used as the target cell type. Next, using the human bronchial



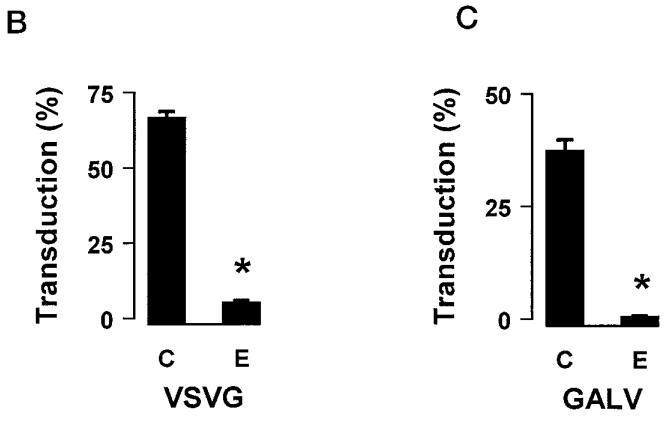


FIG. 2. **CFT1 transduction in the presence or absence of effusions over a range of amphotropic RV vector concentration (*A*) or using VSV-G (*B*) or GALV (*C*) pseudotyped retroviral vectors.** CFT1 cells were transduced with various vectors admixed (50%, v/v) with effusion supernatants or medium. 48 h after transduction, the efficiency of gene transfer was quantitated using the 5-bromo-4-chloro-3-indolyl β-D-galactoside histochemical assay and by microscopically counting the percentage of blue cells. Each data point in *A* represents the mean transduction efficiency of two groups of cells in the absence (□) or presence (○, △) of two different effusions. *B* and *C* show the mean ± S.E. transduction efficiency of three groups of cells exposed to the VSV-G or GALV pseudotyped vectors in the absence (*C*) or presence (*E*) of effusions. *, a statistically significant ($p < 0.01$) difference between the groups by a paired *t* test analysis.

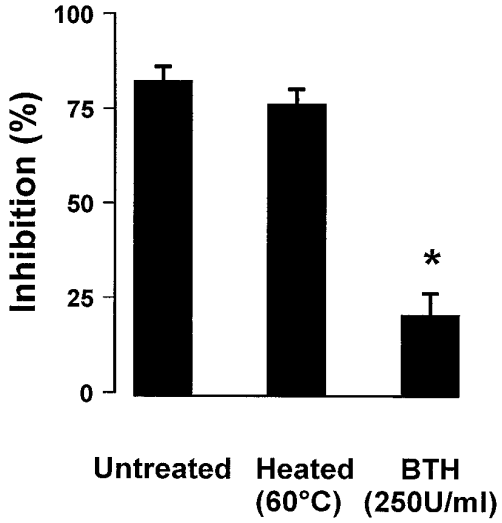


FIG. 3. **Comparison of the inhibition of amphotropic retroviral transduction by untreated pleural effusion supernatants, supernatants heated (60 °C for 1 h) to inactivate complement, or supernatants treated with bovine testicular hyaluronidase (250 units/ml BTH).** Shown is the inhibition to transduction of Mv1Lu cells by the LNPOZ amphotropic retroviral vector admixed (50%, v/v) with untreated effusion supernatants, heated effusion supernatants, or effusion supernatants treated with BTH. The reporter *lacZ* gene expression is quantified by flow cytometry. $n = 16$ for each group, data presented as means ± S.E., *, the two-tailed *p* value from a paired *t* test analysis where $p < 0.001$ for a statistically significant difference between the untreated and BTH-treated group.

epithelial cell line (CFT1) as the target cells, transduction efficiencies of amphotropic, VSV-G, and GALV pseudotyped vectors were tested in the presence or absence of effusion supernatants. In the presence of effusions, amphotropic RV (LNPOZ) transduction is inhibited over a broad range of amphotropic vector concentration (Fig. 2*A*). Furthermore, as demonstrated in Fig. 2, *B* and *C*, the inhibition of RV transduction extends to the VSV-G and GALV pseudotyped vectors as well.

Inactivation of Complement and Identification of the Inhibitory Factors Using Differential Enzymatic Digestion and the Addition of Exogenous GAGs—Because recent reports have attributed the reduced RV transduction into target cells in the presence of human serum to complement-induced lysis of the retroviral vector (28, 29), we first tested whether the observed inhibition to retroviral transduction was complement-dependent. Complement levels within the malignant pleural effusions were measured (C_3 and C_4 measured by rate nephelometry, and CH50 levels measured by complement-mediated sheep RBC lysis; UNC Hospital Labs) and did not correlate with the observed inhibition (data not shown). Furthermore, heating the effusions to inactivate complement components did not remove the block to transduction (Fig. 3).

Malignant pleural effusions are often highly turbid and viscous, reflecting in part the contribution of secreted PG/GAG. To test an alternative hypothesis that proteoglycans block retroviral transduction, pleural effusion supernatants were treated with BTH, a mammalian enzyme with a broad activity against PG/GAGs (7, 21). A marked reduction in the inhibitory activity of the effusions was observed following BTH treatment (Fig. 3), implicating either hyaluronic acid or other PG/GAGs as the putative factors mediating the inhibition to retroviral transduction.

A series of experiments were next performed to specifically identify the inhibitory glycoconjugates. First, effusions were pretreated with a more specific hyaluronidase derived from *Streptomyces* (S-Hya) (30, 31) to test whether hyaluronic acid was responsible for the observed inhibition. As shown in Fig. 4, S-Hya, at a range of effective concentrations (19, 22), did not affect the block to transduction, whereas the inhibitory factors within these effusions were still susceptible to BTH activity. The differential susceptibility of the inhibitory factor(s) to the mammalian BTH *versus* the bacterial S-Hya demonstrated that hyaluronic acid was not the component responsible for mediating the inhibition to retroviral transduction.

Based on data indicating that BTH cleaves chondroitin (and dermatan) sulfates in addition to hyaluronic acid (7), we tested whether the inhibitory factor(s) was a member of the chondroitin sulfate family of PG/GAGs by treating effusion supernatants with specific chondroitinases (20, 26). Pretreatment of effusions with chondroitinase AC or ABC was as effective as BTH (Fig. 5) in abolishing the inhibition of transduction with retroviral vectors, suggesting that chondroitin sulfates are the components inhibiting retroviral transduction in pleural effusions.

To verify the identity of the glycoconjugates responsible for inhibiting retroviral transduction, BTH-degradable glycosami-

noglycans were added into the vector supernatants at varying concentrations (Fig. 6). Hyaluronic acid did not inhibit retroviral transduction, whereas inhibition to transduction was observed when CSA, chondroitin 6-sulfate, or dermatan sulfate was added into retroviral stock at various concentrations. Amphotropic retroviral transduction was virtually abolished when concentrations of the sulfated GAGs exceeded 50 μg/ml in the LNPOZ-containing medium.

Immunodetection and Quantitative Biochemical Analysis of Chondroitin Sulfates in Malignant Pleural Effusions—We next tested whether the chondroitin sulfates identified as the inhibitory factors (Figs. 5 and 6) were present in pleural fluid. First, randomly selected effusion specimens were tested for the presence of sulfated GAGs, chondroitin 4-sulfate, and chondroitin 6-sulfate, using immunoassays. As shown in Fig. 7, the target proteoglycan epitopes were detected in every effusion tested at serial dilution of the effusions to 1:50,000, indicating that chondroitin sulfate proteoglycans are components of malignant pleural effusions. Gel filtration (Sepharose CL-6B) analysis of effusions showed that the CS moieties started eluting with the void volume and continued to elute until the salt peak was reached (data not shown), suggesting that chondroitin sulfates in malignant pleural effusions are a heterogeneous group represented by elements with molecular masses of over 1 million to smaller fragments of less than 10,000 daltons.

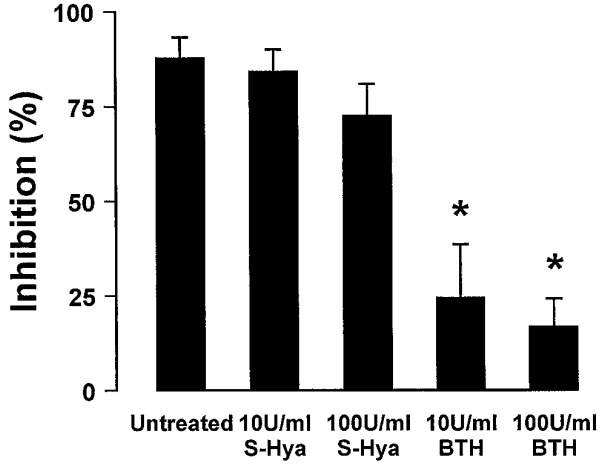


FIG. 4. **Comparison of the inhibition of amphotropic retroviral transduction in the presence of untreated effusion supernatants or in the presence of effusion supernatants treated with the mammalian BTH or the bacterial S-Hya.** Mv1Lu cells were transduced with the LNPOZ amphotropic retroviral vector in the presence (50%, v/v) of four effusion supernatants treated with BTH or S-Hya at equivalent unit concentrations, and the reporter *lacZ* gene expression was quantified. $n = 4$ effusion specimens, data presented as means ± S.E. *, $p < 0.05$ for a statistically significant difference from control by the Bonferroni pairwise multiple comparison analysis.

Hexuronic acid and total glycosaminoglycan sulfation content were used as markers in biochemical assays to test whether relevant concentrations of GAGs were present within pleural effusions. For these studies, PG/GAGs were purified directly from representative (moderately to completely inhibitory to retroviral transduction) effusion supernatants. The hexuronic acid concentrations in the D1 and D2 fractions of the CsCl density gradient-purified effusions ranged from 6.6 to 58.4 μg/ml (mean ± S.E. = 27.37 ± 8.69 μg/ml). Assuming that 40% of the isolated PG/GAGs is hexuronic acid (23, 24), the mean PG/GAGs concentration in the effusions was estimated to be 68 μg/ml. Similarly, the mean ± S.E. sulfated GAG concentration in the effusions was determined to be 30 ± 7.9 μg/ml. These concentrations of uronic acid and sulfated PG in the effusions fall within the range predicted to be inhibitory (Fig. 6).

Transduction Bioassays Using PG/GAG Isolated from Effusions—Following their purification by CsCl density gradient centrifugation from pleural effusions, the PG/GAGs were tested in bioassays measuring inhibition to retroviral transduction and found to be inhibitory (Fig. 8). For example, the inhibitory activity of the D1 fraction from three representative effusions mirrored the inhibition by the effusion supernatant and responded to enzymatic treatment with BTH or chondroitinases in an analogous manner. In contrast, heparinase, heparitinase, keratanase, and *Streptomyces* hyaluronidase had no impact upon the observed inhibition, indicating that heparin, heparan sulfates, keratan sulfate, and hyaluronic acid are not the inhibitory substances in this milieu and that chondroitin sulfates are the relevant inhibitory factors in malignant pleural effusions (Fig. 8).

Mechanism(s) of Inhibition of Retroviral Transduction by CS—Finally, we investigated the mechanism(s) of action for the PG/GAG-mediated vector inhibition. By incrementally increasing the polybrene concentrations in the vector/effusion admixtures up to 40 μg/ml (a 5-fold excess), only about 25% of the effusion-mediated inhibition could be reversed (data not

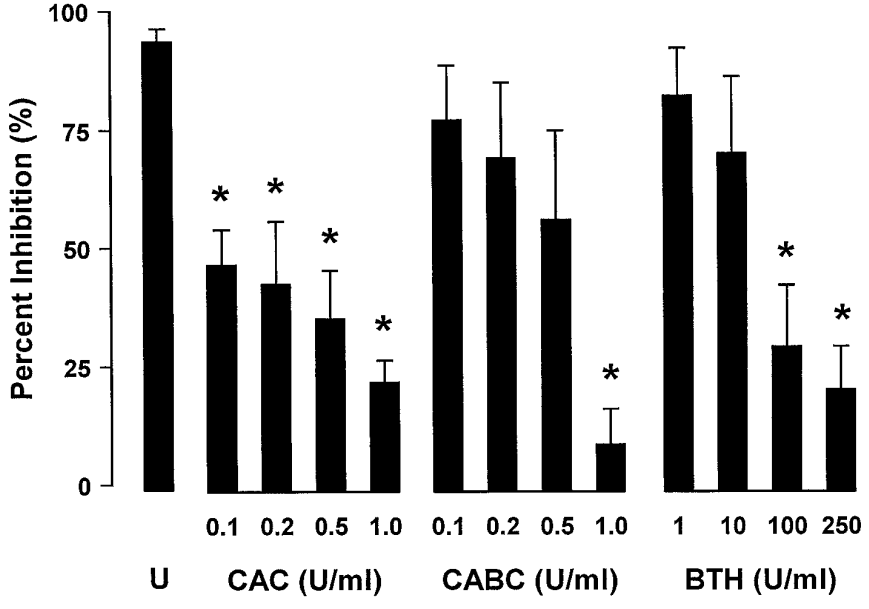


FIG. 5. **Inhibition of amphotropic retroviral transduction in the presence (50%, v/v) of untreated effusion supernatants (*U*) compared with effusion supernatants treated with varying concentrations of chondroitinase AC (*CAC*), chondroitinase ABC (*CABC*), or BTH.** Mv1Lu cells were transduced with the LNPOZ amphotropic retroviral vector in the presence (50%, v/v) of five separate effusion supernatants or of the same effusion supernatants treated with chondroitinase AC or CABC or BTH. The reporter *lacZ* gene expression is quantified as the percentage of cells transduced by flow cytometry. Shown is the relative inhibition to transduction by the vector in the presence of untreated *versus* treated effusion supernatants in LNPOZ-containing medium. $n = 5$ effusion specimens. Data are presented as means ± S.E. *, $p < 0.05$ for a statistically significant difference from control by the Bonferroni pairwise multiple comparison analysis.

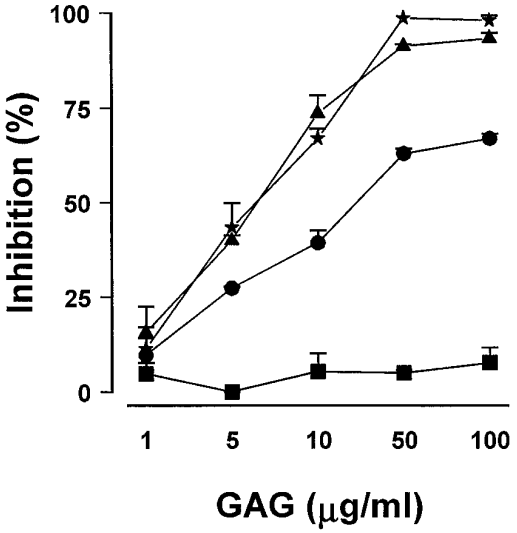


FIG. 6. **Inhibition of amphotropic retroviral transduction in the presence of exogenous glycosaminoglycans (GAG; μg/ml) admixed with vector containing medium.** Mv1Lu cells were transduced with the LNPOZ amphotropic retroviral vector in the presence of hyaluronic acid (■), chondroitin sulfate A (●), chondroitin sulfate B (▲), or chondroitin sulfate C (⋆) at concentrations ranging from 0 to 100 μg/ml GAG. The reporter *lacZ* gene expression is quantified as the percentage of cells transduced by flow cytometry. Shown is the relative inhibition of retroviral transduction (compared with control) as a function of increasing concentrations of exogenous GAGs in LNPOZ-containing medium (n = 3 separate experiments; data are presented as means ± S.E.).

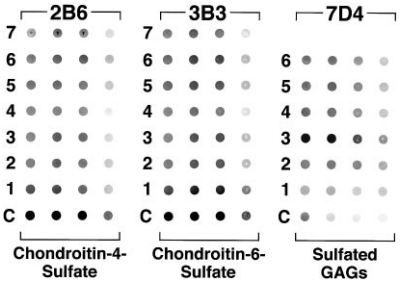


FIG. 7. **Dot blot immunoassays testing for the presence of chondroitin 4-sulfate (using the 2B6 antibody), chondroitin 6-sulfate (using the 3B3 antibody), and native chondroitin sulfate epitopes (using the 7D4 antibody) in malignant pleural effusion supernatants.** 100 μl of seven (1–7) serially diluted effusion specimens (1:1000, 1:5000, 1:10,000, and 1:50,000 in Tris-salt buffer) were assayed for the presence of chondroitin 4-sulfate and chondroitin 6-sulfate using bovine nasal cartilage core (at concentrations of 50, 10, 5, and 1 ng/ml) as the control (*C*) antigen. For these analyses, samples were applied to the nitrocellulose, blocked, then pretreated with chondroitinase ABC to generate epitopes recognized by antibodies 2-B-6 and 3-B-3 (16, 17, 47). Similarly, 100 μl of six serially diluted effusion specimens (1:1000, 1:2500, 1:10,000, and 1:25,000) were assayed for the presence of native chondroitin sulfate epitope (7D4 antibody) without chondroitinase pretreatment and using digested shark cartilage (50, 10, 5, and 1 ng/ml) as the control (*C*) epitope. The primary murine antibodies (diluted 1:1000 in 1% BSA) were recognized by a secondary (diluted 1:7500 in 1% BSA) anti-mouse IgG conjugated to AP and detected following exposure to the AP substrate.

shown). This finding suggested that the anionic nature of the PG/GAG is probably playing a minor role in the vector/proteoglycan interaction. To test whether mammalian hyaluronidase cleaves PG/GAGs associated with the target cell surface rather than in pleural fluid, Mv1Lu or the human tumor cell lines H226 and H28 were treated with various concentrations of BTH over a 4-h period, washed free of BTH, and then exposed to the LNPOZ vector. Fig. 9 shows that BTH pretreatment of cells does not effect transduction efficiency into cells. In a

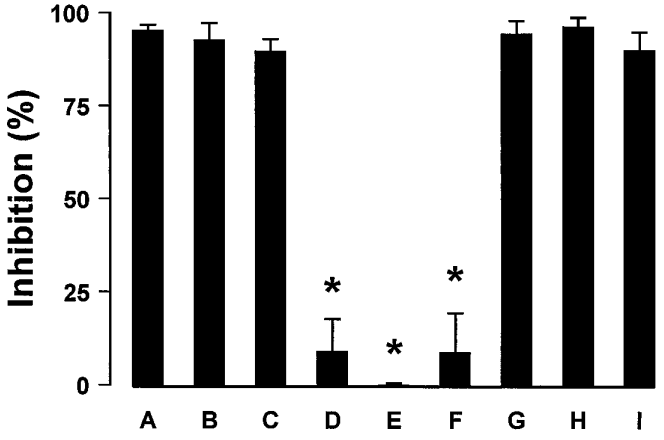


FIG. 8. **Comparison of the inhibition of amphotropic retroviral transduction by untreated effusion supernatants, PG/GAGs isolated from effusions, or PG/GAGs isolated from the effusions that have been enzymatically treated with a variety of specific enzymes.** Shown is the relative inhibition to transduction conferred by supernatants from three malignant effusions, the PG/GAGs isolated with the D1 fraction (specific gravity 1.6 g/ml) from those effusions, or the PG/GAGs isolated with the D1 fraction that have been treated with a panel of specific degradative enzymes. *A*, 50% (v/v with LNPOZ vector stock) effusion supernatant; *B*, 50% D1 fraction; *C*, 50% D1 fraction plus *Streptomyces* hyaluronidase (10 units/ml); *D*, 50% D1 fraction plus bovine testicular hyaluronidase (250 units/ml); *E*, 50% D1 fraction plus chondroitinase AC (0.5 units/ml); *F*, D1 fraction plus chondroitinase ABC (0.5 units/ml); *G*, 50% D1 fraction plus heparinase (5 units/ml); *H*, 50% D1 fraction plus heparitinase (5 units/ml); *I*, 50% D1 fraction plus keratanase (1 unit/ml). n = 3 effusion specimens; data are presented as means ± S.E. *, $p < 0.001$ for a statistically significant difference from control by the Student-Newman-Keuls pairwise multiple comparison analysis.

second series of experiments to test whether PG/GAGs coat the target cell surface and block retroviral attachment, we preincubated target cells for 2 h with effusion supernatants or exogenous glycosaminoglycans at a concentration of 100 μg/ml, and aspirated these components prior to vector exposure. In this analysis, the inhibition to the transduction efficiency into cells preincubated with hyaluronic acid, CSA, dermatan sulfate, chondroitin sulfate C, or patient effusions was small (ranging from 1 ± 1.2% (CSA) to 19.9 ± 12.7% (mean ± S.E. of five different effusions)) and not statistically different from the transduction efficiency of control cells that were not preincubated with PG/GAGs or effusions. Taken together, these results suggest that the mechanism of action of CS inhibition is not at the target cell surface, but rather in solution to inhibit RV gene transfer.

DISCUSSION

During preclinical evaluation of a gene therapy strategy for malignant pleural disease using retroviral vectors, we observed that the transduction efficiency of amphotropic retroviral vectors was markedly impaired or completely nullified in the presence of malignant pleural effusions (Fig. 1*A*). This inhibition to transduction constitutes a significant barrier to the therapeutic goal, and consequently, we sought to identify and neutralize the inhibitory factors in the pleural effusions responsible for this effect.

Initially, we investigated whether the inhibitory factors were contained within the cellular or soluble components of the pleural effusion. We found minimal inhibition attributable to RBCs when present in the range of hematocrits present in pleural effusions (Fig. 1*B*); however, our analysis predicts that RBCs (or serum factors that co-sedimented with the red cells in a Ficoll-Hypaque gradient) would be inhibitory to retroviral transduction when present at a volume percentage found in blood. Since the RBC-mediated block to retroviral transduction

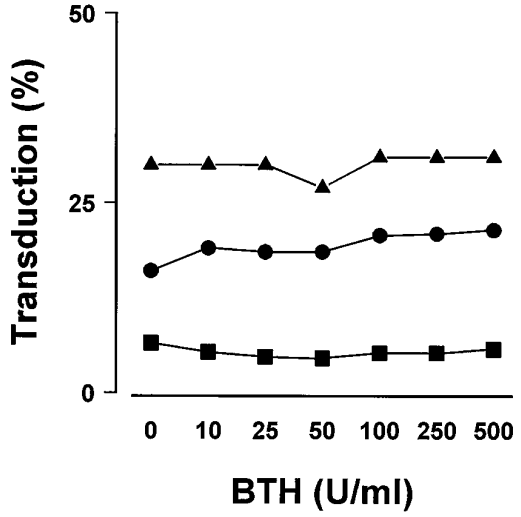


FIG. 9. **Transduction efficiency of LNPOZ into three cell lines (H28 (human malignant mesothelioma, ■), H226 (human lung squamous cell carcinoma, ●), and Mv1Lu (mink lung epithelium, ▲)) pretreated in culture with BTH (units/ml in maintenance medium).** Cells were transduced with the LNPOZ amphotropic retroviral vector following pretreatment of the cells with BTH and analyzed for *lacZ* expression by flow cytometry. (Each data point represents the mean efficiency of transduction from two measurements).

did not appear to be relevant in the context of malignant pleural effusions, the affinity (receptor) of the RBCs for vector and the reversibility of this interaction were not further studied.

Next, we determined that the inhibition to retroviral transduction observed in whole unfractionated effusions was reproduced by the acellular (supernatant) component of the effusions (Fig. 1*C*). In fact, as demonstrated in Fig. 2, the inhibition was present over a broad range of the amphotropic RV-vector concentrations and was observed using other (VSV-G, GALV) RV pseudotypes with potential applications for gene therapy. These data suggest that soluble factors within the milieu of malignant pleural effusions adversely affect retroviral transduction in a generic manner. Inactivation of complement proteins, known mediators of retrovirolysis (32), did not significantly affect the inhibition to retroviral transduction (Fig. 3); however, treatment with a mammalian hyaluronidase (BTH) to degrade PG/GAGs, markedly reduced the inhibitory activity of the effusions (Fig. 3). The elimination of the inhibitory activity with enzymatic pretreatment by BTH suggested that hyaluronic acid and/or chondroitin sulfates were the PG/GAGs responsible for the inhibitory effect. To distinguish between these possibilities, a series of studies were performed. First, effusions treated with a more specific bacterial hyaluronidase (S-Hya) maintained their inhibitory activity, suggesting that hyaluronic acid was an unlikely candidate for mediating this effect (Fig. 4). This inference was supported by the observation that the addition of exogenous hyaluronic acid (up to 500 μg/ml; data not shown) had no effect upon the transduction efficiency of retroviral vectors (Fig. 6). In contrast, the inhibitory activity in effusions was abolished by specific chondroitinases (Fig. 5), and inhibition to retroviral transduction was detected when exogenous CS glycosaminoglycans were added to the amphotropic RV vector (Fig. 6). These data indicate that chondroitin sulfates in malignant pleural effusions are the likely inhibitors of retroviral gene transfer.

To demonstrate the relevant molecules *in vivo*, pleural effusions were assayed for the presence of chondroitin sulfate moieties by biochemical and immunodetection techniques (Fig. 7). The immunoassays confirmed the presence of the relevant PG/GAGs in pleural fluid, and quantitatitive biochemical assays revealed them to be in the range of concentrations predicted to be inhibitory from previous studies (Fig. 6). In addition, the purified PG/GAGs isolated from the pleural effusions were found to inhibit retroviral transduction in a manner identical to that of whole effusions, and this inhibitory activity could be eradicated by enzymatic treatment with BTH or chondroitinases (Fig. 8). Importantly, enzymatic treatment of the fractions with heparinase, heparitinase, and keratanase had no effect on the inhibitory capacity of these isolates. Collectively, the results of biochemical, immunodetection, and differential enzymatic digestion analyses conclusively demonstrate that the inhibition to retroviral transduction in the pleural fluid can be accounted for by the activity of chondroitin sulfates in the effusion.

Although it is not possible to test directly, our data suggest that the PG/GAGs block RV transduction by binding or inactivating the vector in solution rather than adsorbing onto the cell surface. No effect upon transduction efficiency was observed when three cell lines were pretreated with various concentrations of BTH before infection with retroviral vector (Fig. 9), and preincubation of the target cells with exogenous GAG did not impair subsequent retroviral transduction, provided that the GAGs were aspirated from the cells prior to vector exposure.

The proteoglycan families (and their glycosaminoglycan catabolites) in malignant pleural effusions have not been extensively studied to date. Elevated concentrations of hyaluronic acid are known to be present in effusions from patients with malignant mesothelioma (33), and more recently, these GAGs have been characterized to include both hyaluronic acid and sulfated moieties (5). In addition, mesothelial cells have been recognized as a source of secreted hyaluronic acid and chondroitin GAG (34, 35). In this regard, the majority of sulfated GAGs produced by rat mesothelial cells *in vitro* are shown to be secreted into the media, and only 25% of the sulfated GAG is cell surface- or extracellular matrix-related. Elevated quantities of PG/GAGs have also been measured in tumor tissue and in pleural effusions of patients with inflammatory conditions (36). Our results suggest that the relevant molecules inhibitory to RV-transduction in malignant pleural effusions likely originate from parent proteoglycans that are secreted and have CS components (*e.g.* versican, and/or members of the biglycan or decorin families) (37). Furthermore, the source of these parent proteoglycans may be the mesothelial cell (34, 35) or tumor stroma *per se* (38).

PG/GAGs play diverse roles in different tissues, and their important interaction with certain infectious agents is increasingly being appreciated (39–41). For example, the cell surface heparan GAGs are the site for the initial viral attachment of herpesvirus, pseudorabies virus, and cytomegalovirus onto the cell surface (40, 42, 43). Likewise, several sulfated polysaccharides are potent and selective inhibitors of infection due to various enveloped viruses, including vesicular stomatitis virus (VSV, a rhabdovirus) and human immunodeficiency virus (HIV, a lentivirus) (44). Indeed, dextran sulfate and heparin (but not chondroitin sulfates) prevent HIV replication and syncytia formation *in vitro* (45, 46). In this report, we demonstrate that a variety of enveloped (amphotropic, GALV, VSV-G) RV vectors are inhibited from transducing target cells by soluble chondroitin sulfates. These results are compatible with prior observations and expand the role for connective tissue glycoconjugates in their interactions with infectious agents and potential vectors for gene therapy.

The mechanism(s) by which vector inactivation is taking place remains to be resolved. The interaction of the glycoconjugates with the viral vector may be a steric phenomenon or in

part a function of the polyanionic charge of the PG/GAGs. However, a more specific relationship is perhaps suggested by affinity of herpesvirus and cytomegalovirus only for cell-associated heparan sulfates and the inability of chondroitin sulfates to affect HIV infection. The implications of our findings for gene therapy of intrapleural malignancy are clear if amphotropic RV vectors are utilized in that vector neutralization by soluble PG/GAGs would likely occur in a pleural cavity containing an effusion. However, potential strategies to overcome this effusion-mediated inhibition to RV transduction are suggested by our study. Most simply, the effusion accompanying the pleural process should be drained completely to remove soluble proteoglycans/glycosaminoglycans from the chest cavity before the instillation of vector. In addition, pretreatment of the pleural cavity with mammalian hyaluronidase or specific chondroitinases may enhance retroviral gene transfer to the target cells *in vivo*.

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