

# Antisense-mediated Suppression of Human *Heparanase* Gene Expression Inhibits Pleural Dissemination of Human Cancer Cells<sup>1</sup>

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## ABSTRACT

Heparan sulfate proteoglycans is a major component of the cell surface and extracellular matrix and functions as a barrier against cationic molecules and macromolecules. Heparanase is an endoglucuronidase capable of specifically degrading heparan sulfate, and its activity is associated with the metastatic potential of tumor cells. To inhibit human heparanase expression in human cancer cells, we constructed an adenoviral vector carrying a full-length human heparanase cDNA in an antisense orientation (Ad-AS/hep). Increased heparanase expression in T.Tn human esophageal cancer cells and A549 human lung cancer cells after infection with an adenovirus vector expressing the human *heparanase* gene (Ad-S/hep) was specifically inhibited by simultaneous infection with Ad-AS/hep in a dose-dependent manner. A modified Boyden chamber assay demonstrated that infection with Ad-AS/hep significantly inhibited *in vitro* invasion of A549 cells after Ad-S/hep infection. Moreover, intrathoracic administration of Ad-AS/hep reduced the number and size of heparanase-expressing A549 tumors implanted intrathoracically into BALB/c-*nu/nu* mice. Our results suggest that heparanase contributes to the invasive phenotype of tumor cells, and that antisense-mediated inhibition of heparanase activity may be efficacious in the prevention of pleural dissemination.

## INTRODUCTION

HSPG<sup>3</sup> is a major component of the cell surface and ECM, and provide a physical barrier to movement of cells into tissues (1). The ability of HS to interact with ECM macromolecules, such as collagen, laminin, and fibronectin, and with different attachment sites on plasma membrane indicates that this proteoglycan is essential in the self-assembly and insolubility of ECM components as well as in cell adhesion and locomotion (2). Furthermore, HSPG binds various cytokines and growth factors such as fibroblast growth factor, hepatocyte growth factor, insulin-like growth factor, and transforming growth factor- $\beta$  and functions as a store of these factors (3). Malignant tumor cells can modulate the expression of HSPG by releasing degradative enzymes, such as heparanase, or cytokines that influence the turnover and biosynthesis of host-cell HSPG (4).

Heparanase is an endoglucuronidase that cleaves HS at specific interchain sites, thus facilitating the release of several protein modulators of cell function, including migration, adhesion, and angiogenesis (5). Heparanase activity correlates with the metastatic potential of tumor cells in animal models (6) and, moreover, is increased in the sera and urine of human patients with metastatic cancer. Although the importance of heparanase function has long been recognized, the

mammalian heparanase has remained an elusive target. However, several laboratories recently purified human heparanase and have isolated the cDNA encoding this activity. The human heparanase enzyme is expressed as a 543-amino acid precursor protein that is modified by proteolytic cleavage to remove the first 157 amino acids (7–10). Treatment with heparanase inhibitors considerably reduced the incidence of lung metastasis in animal models (11–13).

These observations led us to hypothesize that abrogation of heparanase expression could reduce pleural dissemination by inhibiting the tumor cell invasion to the basement membrane. Here we demonstrate that an adenoviral vector expressing an antisense to a full-length human *heparanase* gene specifically inhibits heparanase expression as well as its invasive potential *in vitro*, leading to the reduction of pleural dissemination in an orthotopic murine model *in vivo*.

## MATERIALS AND METHODS

**Cells and Culture Conditions.** The human esophageal squamous cell carcinoma cell line T.Tn and the human non-small cell lung cancer cell line A549 were propagated in monolayer culture in RPMI 1640 with 25 mM HEPES medium and DMEM:Ham's F-12 supplemented with 10% FCS, 100 units/ml penicillin and 100 mg/ml streptomycin. The transformed embryonic kidney cell line 293 was grown in DMEM with high glucose (4.5 g/l), supplemented with 10% FCS, 100 units/ml penicillin, and 100 mg/ml streptomycin. The 293 cells were used for the production of adenovirus vectors.

**Recombinant Adenoviruses.** Replication-deficient, E1- and E3-deleted recombinant Ad5 was used as the viral backbone. A human heparanase cDNA of 1758 bp was inserted, as a *EcoRI/XhoI* fragment, in sense and antisense orientation into the cosmid vector pAxCat (Adenovirus Expression Vector Kit; TaKaRa Biomedicals, Tokyo, Japan) to generate the pAxCat-S/hep and pAxCat-AS/hep constructs, respectively. These constructs were cotransfected with restriction enzyme-digested DNA-TPC into 293 cells to isolate recombinant adenovirus. The resultant adenoviruses were named Ad-S/hep and Ad-AS/hep (Fig. 1A). The E1A-deleted adenovirus vector d1312 was used as the control vector. Recombinant virus was purified by ultracentrifugation in cesium chloride step gradients, and its titer was determined by pfu on the 293 cells.

**RT-PCR.** Total RNA was isolated from cells using RNAzol (Cinna/Bio-Tecx, Friendswood, TX) in a single-step phenol-extraction method and used as templates. Reverse transcription was performed at 22°C for 10 min and then at 42°C for 20 min, and PCR was performed with specific primers in volumes of 50  $\mu$ l according to the protocol provided by the manufacturer (PCR kit; Perkin-Elmer/Cetus, Norwalk, CT). The following specific primers were used: for heparanase, sense (Hep-S: 5'-TTC GAT CCC AAG AAG GAA TCA AC-3') and antisense (Hep-AS: 5'-GTA GTG ATG CCA TGT AAC TGA ATC-3'); for the sequence just after the inserted fragment in the vector (Ad5 pax: 5'-ATC GAT TCT AGA CTA GTT TAA TTA ATT T-3'); for MMP-2, sense (5'-TTT TCT CGA ATC CAT GAT GG-3') and antisense (5'-CTG GTG CAG CTC TCA TAT TT-3'); and glyceraldehyde-3-phosphate dehydrogenase, sense (5'-CAG CCG AGC CAC ATC-3') and antisense (5'-TGA GGC TGT TGT CAT ACT TCT-3'). The amplification reaction involved denaturation at 95°C for 45 s, annealing at 52°C for 1 min, and 72°C for 1 min using a thermal cycler (Perkin-Elmer, Foster City, CA). The PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining.

**Western Blot Analysis.** Cells were collected by trypsinization and washed twice in cold PBS. Cells then were dissolved in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors

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<sup>3</sup> The abbreviations used are: HSPG, heparan sulfate proteoglycans; ECM, extracellular matrix; HS, heparan sulfate; pfu, plaque-forming unit; RT-PCR, reverse transcription-PCR; MOI, multiplicity of infection; MMP, matrix metalloproteinase.

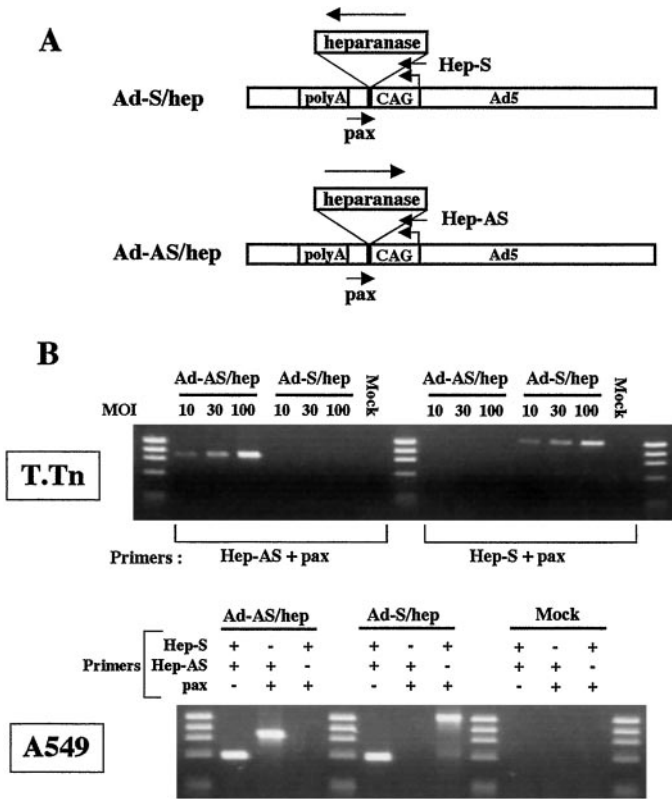


Fig. 1. A, structure of adenovirus vectors. Ad-S/hep and Ad-AS/hep vectors contain human heparanase cDNA in sense and antisense orientation, respectively, driven by the CAG promoter. Sites to which PCR primers (Hep-S, Hep-AS, and Ad5 pax) are targeted are indicated. Two primer pairs of Hep-S/Ad5 pax and Hep-AS/Ad5 pax are designed to detect sense and antisense sequences of the human *heparanase* gene. B, expression of the human heparanase sense and antisense transcripts in human cancer cells. *Top panel*, human esophageal cancer cell line T.Tn was infected with either Ad-AS/hep or Ad-S/hep at the indicated MOIs and subjected to RT-PCR analysis 36 h after infection. Primers are indicated at the *bottom* of the panel. *Bottom panel*, RT-PCR analysis was performed in A549 human non-small cell lung cancer cells transduced with 30 MOI of Ad-AS/hep or Ad-S/hep at 36 h postinfection. Primers are indicated at the *top* of the panel.

(0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin, and 1  $\mu$ g/ml aprotinin). The lysis was carried out at 4°C for 30 min and centrifuged at 15,000 rpm. The protein concentration of the supernatant was determined using the Bio-Rad protein determination method (Bio-Rad, Hercules, CA). Equal amounts (60  $\mu$ g) of proteins were electrophoresed under reducing conditions on 12% (w/v)

polyacrylamide gels. Proteins were electrophoretically transferred to a Hybond- polyvinylidene difluoride transfer membranes (Amersham, Arlington Heights, IL) and incubated with primary antibodies against heparanase and then peroxidase-linked secondary antibody. An Amersham enhanced chemiluminescence chemiluminescent Western system (Amersham, Tokyo, Japan) was used to detect secondary probes.

**Immunohistochemistry.** Immunohistochemical staining was performed using a HISTOFINE SAB PO kit (Nichirei, Tokyo, Japan) according to the instructions provided by the manufacturer. Formalin-fixed, paraffin-embedded tissue sections were mounted on silanized slides, deparaffinized in xylene for 20 min, and rehydrated in graded ethanol solutions. Endogenous peroxidase was blocked by incubating the sections in 3.0% H<sub>2</sub>O<sub>2</sub> in methanol for 15 min. Antigen retrieval on paraffin sections was performed by heating three times in 10 mM citrate buffer solution (pH 6.0) in a microwave. After the blocking of nonspecific reactivity with rabbit serum for 10 min at room temperature, sections were incubated overnight at 4°C with the antihuman heparanase mouse monoclonal antibody raised against recombinant human heparanase. After rinsing, the slides were incubated with biotinylated rabbit antimouse IgG and then with avidin-biotin-peroxidase complex. Peroxidase activity was determined using DAB/H<sub>2</sub>O<sub>2</sub> solution (Histofine DAB substrate kit; Nichirei). The slides were counterstained with methyl green with Mayer's hematoxylin. As a negative control, some sections were subjected to normal serum blocking and omission of the primary antibody.

**Quantitative Real-Time RT-PCR.** Heparanase mRNA copy number in surgical specimens and cell lines were determined by real-time quantitative RT-PCR using a LightCycler instrument and a LightCycler DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Indianapolis, IN). Amplifications were done in glass capillary tubes using 20- $\mu$ l reaction containing 3 mM MgCl<sub>2</sub>, 0.5  $\mu$ M each primer, and 2  $\mu$ l of 10 $\times$  LightCycler FastStart DNA Master SYBR Green I. PCR amplification began with a 60-s denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 10 s, and extension at 72°C for 9 s. The oligonucleotides used as specific primers were 5'-GCG GTT ACC CTA TCC TTT TT-3' and 5'-GCA GCA ACT TTG GCA TTT C-3'. Copy numbers of mRNA were calculated from serially diluted standard curves (Fig. 3B) generated from purified cDNA template, which consisted of a human heparanase cDNA of 1758 bp inserted into the expression vector. Data analysis was performed using LightCycler Software (Roche Molecular Biochemicals).

**Invasion Assay.** Invasion of tumor cells was assessed by counting the number of cells that migrated through transwell inserts with 8- $\mu$ m pores (Becton Dickinson, Mountain View, CA), according to the protocol recommended by the manufacturer. Briefly, transwell membranes were coated with 100  $\mu$ l of Matrigel (Collaborative Research Inc., Boston, MA) at a final concentration of 0.1 mg/ml and dried. Tumor cells ( $4 \times 10^4$ ) in 200  $\mu$ l of medium from each treatment were added in triplicate wells and allowed to invade through Matrigel for 12 h at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells that migrated through the membranes into the lower wells were assessed by

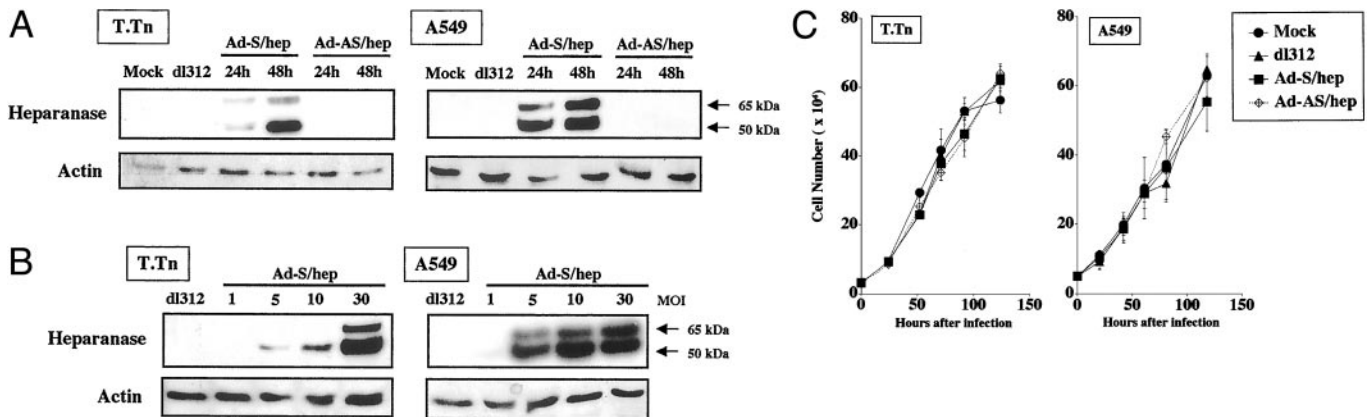


Fig. 2. A, Western blot analysis of human heparanase protein in T.Tn and A549 cells. Cells were infected with either Ad-S/hep or Ad-AS/hep at a MOI of 100 and analyzed for heparanase expression at the indicated times. Equal loading of samples was confirmed by stripping each blot and reprobing with antiactin antiserum. B, heparanase protein levels were determined by Western blot analysis 48 h after infection in T.Tn and A549 cells transduced with Ad-S/hep at MOIs of 1, 5, 10, and 30. Both inactive ( $M_r$  65,000) and active ( $M_r$  50,000) forms of heparanase proteins were detected. C, cell growth of heparanase-expressing cells. T.Tn and A549 cells cultured as a monolayer were infected with either dl312, Ad-S/hep, or Ad-AS/hep at a MOI of 30. Cell viability was determined by trypan blue staining on a daily basis. Each point represents the mean  $\pm$  SD of triplicate experiments.

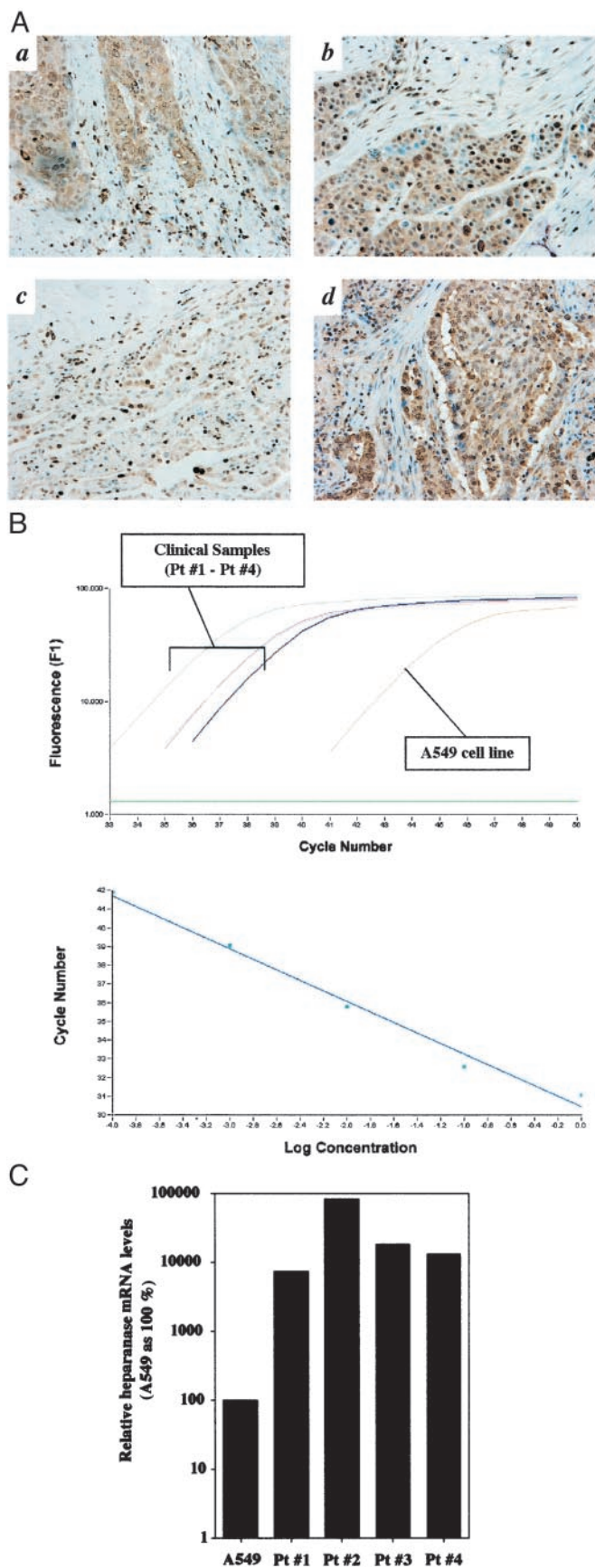


Fig. 3. A, immunohistochemical analysis of the heparanase protein in surgical specimens of human esophageal squamous cell carcinoma and lung adenocarcinoma. The tumor tissues were obtained from four patients with either esophageal squamous cell carcinoma (a and b) or lung adenocarcinoma (c and d). Positive staining is reddish-brown. Counterstain is blue-purple. Original magnification,  $\times 200$ . B, top panel, amplification

trypan blue uptake. Then the number of cells was counted within a field at  $\times 200$  under a light microscope. For each membrane, a total of five fields were selected at random, and the numbers were averaged.

**Animal Experiments.** The human non-small cell lung cancer cell line A549 was infected with Ad-S/hep at a MOI of 5, harvested 24 h after infection, and resuspended in HBSS at a density of  $4.0 \times 10^6$  cells/200  $\mu$ l. Female BALB/c *nu/nu* mice were intrathoracically injected with 200  $\mu$ l of cell suspension through a 27-gauge needle. After 24 h, either  $3.0 \times 10^8$  pfu/100  $\mu$ l of dl312 or  $3.0 \times 10^8$  pfu/100  $\mu$ l of Ad-AS/hep was injected into the thoracic space by the same technique. The procedure was repeated over 3 consecutive days. Thirty days after cell inoculation, mice killed, and their thoracic spaces were examined macroscopically for any growths. Tumors in the thoracic spaces were removed and weighed. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University School of Medicine, Okayama, Japan.

## RESULTS

**Generation of Adenovirus Construct Carrying the Human Heparanase Gene in Sense or Antisense Orientation.** To examine the relevance of human heparanase in tumor cell invasion, we constructed replication-defective recombinant adenoviruses Ad-S/hep and Ad-AS/hep expressing, respectively, sense and antisense RNA to a full-length human heparanase sequence as described in "Materials and Methods" (Fig. 1A). First we examined, by strand-specific RT-PCR assay, the expression of the sense and antisense constructs in human esophageal and lung cancer cell lines T.Tn and A549, respectively (Fig. 1B). The antisense transcript was detected with specific primers (Hep-AS and Ad5 pax) in Ad-AS/hep-infected cells, whereas RT-PCR with another set of primers (Hep-S and Ad5 pax) detected heparanase mRNA expression in cells transduced with the Ad-S/hep vector. Both sense and antisense transcripts could be seen when Hep-AS and Hep-S primers were used. This analysis also confirmed the dose-dependent expression of the transcripts in T.Tn cells.

**Heparanase Expression in Human Cancer Cells after Ad-S/hep Infection.** Western blot analysis was carried out to examine the effect of Ad-S/hep and Ad-AS/hep infection on heparanase protein levels in T.Tn and A549 cells. As shown in Fig. 2A, proheparanase ( $M_r$  65,000) as well as cleaved, active heparanase ( $M_r$  50,000) protein expression was detected in both cell lines as early as 24 h after infection, but not in parental and dl312- or Ad-AS/hep-infected cells. Experiments using various concentrations of Ad-S/hep showed that infection at a MOI of 5 was sufficient to exhibit a detectable heparanase protein expression in T.Tn and A549 cells, although the protein level was much higher in A549 cells compared with that in T.Tn cells (Fig. 2B). In addition, infection with dl312, Ad-S/hep, or Ad-AS/hep vectors at a MOI of 30 had no apparent effect on the *in vitro* growth of T.Tn and A549 cells (Fig. 2C).

**Heparanase Expression in Human Tumor Tissues and Human Cancer Cell Line.** Heparanase has been reported to be expressed in many types of neoplastic tissues (6). We examined heparanase protein expression by immunohistochemistry in surgical specimens of human esophageal squamous cell carcinoma and lung adenocarcinoma. Sections of human tumor tissues were obtained from the Okayama University Medical School. As shown in Fig. 3A, heparanase protein expression was present in the tumor cells in all of tumor specimens examined. Although some cells in the stromal regions adjacent to the tumor cells exhibited heparanase immunoreactivity, the staining was less intense than the tumor cells. These results suggest that heparanase

profiles with fluorescence data of human tumor tissues of esophageal cancer and A549 lung cancer cell line generated by the LightCycler instrument. Bottom panel, calibration curve for heparanase expression. C, relative expression of heparanase mRNA in surgical specimens and the A549 cell line analyzed by real-time RT-PCR assay.

**A**

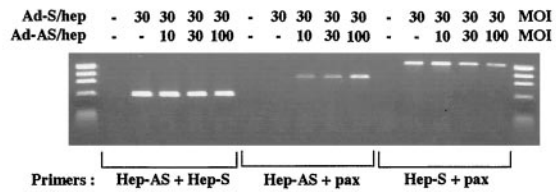
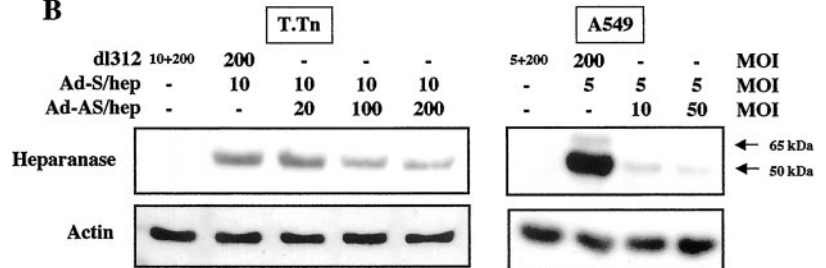


Fig. 4. Effect of antisense heparanase construct on heparanase mRNA and protein expression. **A**, T.Tn cells were simultaneously infected with 30 MOI of Ad-S/hep and increasing MOIs of Ad-AS/hep. RT-PCR analysis was performed 48 h after infection with specific primer pairs indicated at the bottom of the panel. Note the dose-dependent induction of antisense transcripts and the diminution of sense transcripts. **B**, Western blot analysis of heparanase protein in T.Tn and A549 cells. Simultaneous infection with Ad-AS/hep vector at different concentrations, as indicated, reduced heparanase protein expression of T.Tn and A549 cells transduced with 10 and 5 MOI of Ad-S/hep, respectively, in a dose-dependent manner 48 h after infection.

**B**



expression in the tumor tissues is derived primarily from the tumor cells.

To compare expression levels of heparanase message between tumor tissues and cell lines, we next performed a quantitative real-time RT-PCR assay using the LightCycler instrument (Fig. 3B). The assay demonstrated that heparanase mRNA expression was easily detectable in clinical samples of human esophageal and lung cancers, which is consistent with previous reports; human esophageal and lung cancer cell lines, however, expressed relatively low levels of heparanase mRNA. The relative expression of heparanase mRNA in primary tumors obtained from four patients as well as in A549 lung cancer cells was shown in Fig. 3C. The median expression was ~300-fold higher in tumor tissues than in the A549 cell line.

**Infection with Ad-AS/hep Decreased Heparanase mRNA and Protein Levels in T.Tn and A549 Cells.** We next evaluated the efficacy of Ad-AS/hep in inhibiting heparanase expression. However, having verified the presence of low expression levels of heparanase in T.Tn and A549 cell lines, we selected cells infected with Ad-S/hep for these experiments. T.Tn cells were infected with Ad-S/hep at a MOI of 30 and then Ad-AS/hep infection at various MOIs. RT-PCR analysis with specific primers demonstrated expression of the antisense transcript as well as a diminution of heparanase mRNA levels in a dose-dependent manner (Fig. 4A). For Western blot analysis, T.Tn and A549 cells were infected with Ad-S/hep at MOI of 10 and 5, respectively, and then Ad-AS/hep infection at various MOIs. The assay showed significantly decreased levels of heparanase protein in a dose-dependent fashion with an increase in MOI (Fig. 4B).

**Inhibition of Heparanase-induced Invasion of A549 Cells by Ad-AS/hep Infection.** To investigate the importance of heparanase expression in tumor cell invasion, the Matrigel invasion assay was performed using A549 cells in a modified Boyden chamber system. As shown in Fig. 5A, A549 cells infected with Ad-S/hep at 5 MOI showed aggressive invasion characteristics compared with dl312-infected cells, which could be significantly inhibited upon simultaneous infection with 100 MOI of Ad-AS/hep vector. These results suggest that the antisense construct selectively inhibited the heparanase-induced invasive properties of A549 cells.

We also evaluated the therapeutic effect of Ad-AS/hep injection against Ad-S/hep-transduced A549 cells growing intrathoracically in a thymic *nu/nu* mice. Preliminary experiments revealed that, when  $4 \times 10^6$  A549 cells transduced with Ad-S/hep at 5 MOI were inoculated into the thoracic space, dissemination of tumors appeared

in all mice 30 days after tumor injection. Injection of Ad-AS/hep vector ( $3.0 \times 10^8$  pfu/100  $\mu$ l) into the thoracic cavity over 3 consecutive days reduced the incidences of tumor cell dissemination and tumor nodules compared with mice that received intrathoracic dl312 injections (Fig. 5B). In addition, Ad-AS/hep-treated animals showed a reduced weight of tumor nodules compared with dl312-treated animals.

**Inhibition of MMP-2 Expression in Heparanase-expressing A549 Cells by Ad-AS/hep Infection.** MMP is a neutral proteinase that mediates ECM degradation and potentiates the invasiveness and metastatic potential of malignant tumors (14). To determine whether heparanase expression affects other invasion-related enzyme expression, we carried out semiquantitative RT-PCR analysis with specific primer pairs for type IV collagenases: MMP-2 and MMP-9. Densitometric quantification revealed that Ad-S/hep infection at a MOI of 5 caused a 2-fold increase of MMP-2 mRNA expression (Fig. 5C), whereas there were no changes in MMP-9 mRNA levels (data not shown). When A549 cells were simultaneously infected with Ad-AS/hep at a MOI of 50, MMP-2 mRNA expression decreased by 1.3-fold compared with that in cells transduced with 5 MOI of Ad-S/hep alone.

**DISCUSSION**

Inhibition of tumor invasion is an attractive approach for the treatment of highly malignant tumors (15). Tumor cell invasion is characterized by the secretion of enzymes that facilitate tumor cell spread by degrading the ECM surrounding tumors and solubilizing the vascular basement (16). Thus, heparanase, which degrades the HSPG in the ECM, is an attractive target for the development of new antimetastatic drugs because of considerable evidence implicating the enzyme in tumor cell invasion. In the present study, we demonstrated that overexpression of human heparanase enhanced tumor cell invasiveness *in vitro*, and that this invasive ability was significantly reduced by inhibiting heparanase expression using an adenovirus-mediated antisense gene-delivery strategy. Furthermore, injection of an antisense adenovirus construct against human heparanase into the pleural space inhibited the growth of human non-small cell lung cancer cells implanted in the pleural cavity of *nu/nu* mice.

Many compounds, such as laminarin sulfate, phosphomannopentose sulfate (17), and maltohexaose sulfate (18), are potent inhibitors of heparanase activity; these molecules are, however, difficult to target to specific tissue sites, and their pleiotropic interactions with

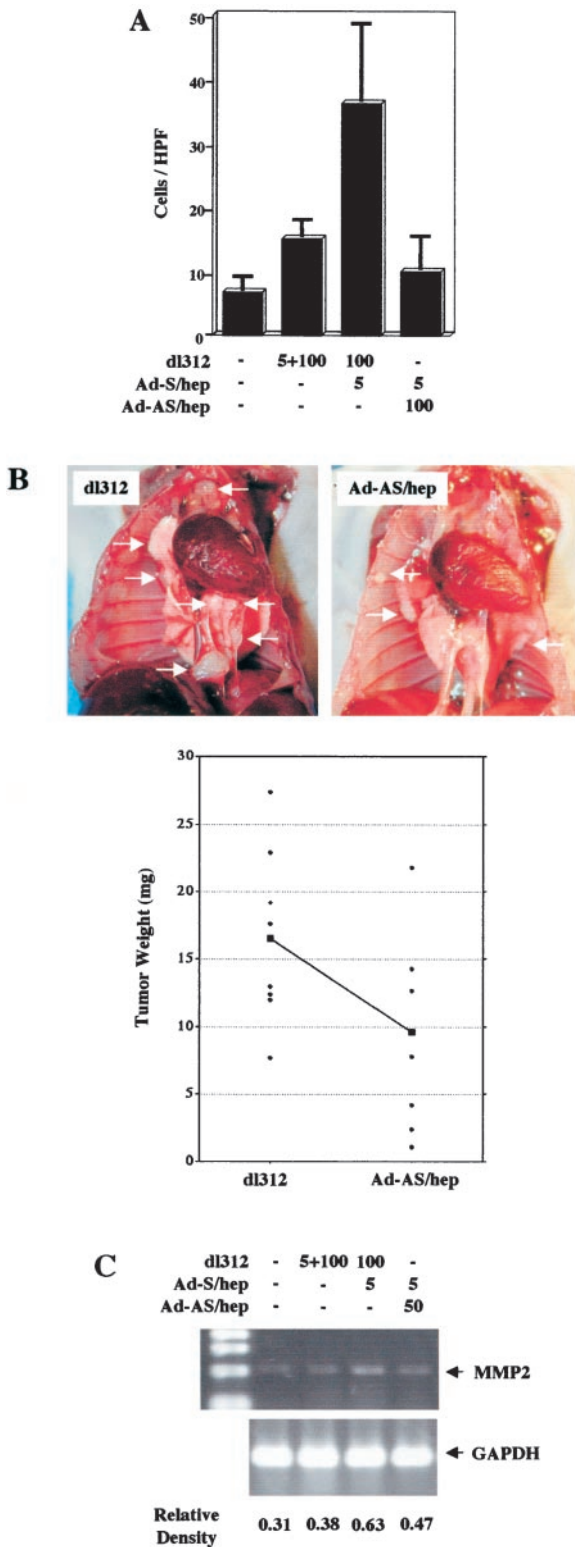


Fig. 5. A, invasion of A549 cells infected with Ad-S/hep and/or Ad-AS/hep. A549 cells were simultaneously treated with the vectors as indicated at the bottom of the figure. The cells were placed in 8- $\mu$ m pore transwells coated with Matrigel 36 h after infection, allowed to invade for an additional 12 h, and the cells that passed through transwells were then quantitated as described in "Materials and Methods." B, effect of Ad-AS/hep on the pleural dissemination of A549 cells. Female BALB/c *nu/nu* mice were intrathoracically treated with  $3.0 \times 10^8$  pfu of dl312 or Ad-AS/hep for 3 consecutive days, 24 h after the implantation of  $4 \times 10^6$  A549 tumor cells infected with 5 MOI of Ad-S/hep into the pleural space. Top panel, gross appearance of A549 tumors grown orthotopically in the thoracic spaces. After 30 days of tumor cell inoculation, the animals were killed, and pleural dissemination of the tumor cells was assessed. Bottom panel, the weight of each tumor nodule found in the thoracic spaces was also evaluated. C, RT-PCR analysis of MMP-2 in A549 cells. A549 cells were treated with the vectors as indicated at the top of

HSPG might produce undesired effects. Therefore, to selectively block heparanase expression, we used an antisense strategy. Expression of antisense RNA can directly influence the expression of a given gene of interest, although the precise mechanism of inhibition remains unclear (19). In addition, to ensure efficient transfer and high expression of antisense heparanase, we generated an adenovirus construct carrying human heparanase cDNA in an antisense orientation (Ad-AS/hep; Fig. 1). First, we examined whether Ad-AS/hep infection could suppress endogenous heparanase expression in human esophageal and lung cancer cell lines. However, unexpectedly, our quantitative real-time RT-PCR assay demonstrated that a specific heparanase transcript amplification was undetectable or extremely low in the cell lines tested in our study, although it was readily detected in surgical specimens (Fig. 3). We examined at least 10 clinical tissues of esophageal and lung cancer and detected heparanase expression in all of them by either RT-PCR assay or immunohistochemistry. Possible explanation for such a discrepancy is attributable to the differences of the microenvironment. Neoplastic cells may interact with surrounding cells *in vivo* to sustain their malignant potentials (e.g., metastatic melanoma cells and astrocytes; Ref. 20), which supports the concept that lack of heparanase expression in established cell lines results from the absence of microenvironmental stimuli.

Several groups have reported that tumor cells transfected with heparanase cDNA acquire a highly metastatic phenotype *in vivo* (21). Consistent with these findings, tumor cells adenovirally transduced with the human *heparanase* gene showed heparanase protein expression (Fig. 2, A and B) as well as heparanase activity (data not shown), which contributed to the highly invasive nature of the infected cells (Fig. 5A). The finding that overexpression of heparanase had no apparent effect on the proliferation of tumor cells *in vitro* indicates its important roles for invasive properties in the microenvironment (Fig. 2C). With the use of tumor cells expressing the ectopic *heparanase* gene, we showed successful transduction of an antisense construct with Ad-AS/hep, resulting in decreases of mRNA transcripts (Fig. 4A) and protein contents (Fig. 4B). In addition, we have noted complete inhibition of enhanced invasiveness in cells expressing heparanase by Ad-AS/hep infection (Fig. 5A), suggesting that Ad-AS/hep has significant biological consequences.

Our data also indicate the therapeutic potential of molecular manipulation of heparanase expression in an orthotopic model of pleural dissemination. Intrathoracic administration of Ad-AS/hep vector reduced the incidence of tumor formation in the thoracic cavity (Fig. 5B). Our preliminary experiments revealed that injection of adenovirus expressing the wild-type *p53* gene, which has been known to induce apoptosis in certain tumor cells, had no significant antitumor effect in this animal model (data not shown). Thus, approaches that selectively block expression of molecules implicated in cellular invasion may be clinically more relevant for the prevention of tumor cell dissemination than directly inducing apoptosis of tumor cells. In fact, selective inhibition of other degradative enzymes such as MMP-9 (22) and urokinase-type plasminogen activator receptor (23) by antisense gene transfer has been reported to impair tumor invasion as well as metastasis. We also found that Ad-AS/hep infection suppressed heparanase-induced up-regulation of MMP-2 expression (Fig. 5C), suggesting that Ad-AS/hep may have direct and indirect effects on the expression of heparanase and MMP-2, respectively, thereby being more clinically beneficial.

Although additional experiments using freshly isolated primary

the panel and applied for RT-PCR assay 24 h after infection. The relative density of MMP-2 mRNA expression was normalized by dividing by the glyceraldehyde-3-phosphate dehydrogenase signal for each sample shown at the bottom of the panel.

human cancer cells that consistently express endogenous heparanase are required, to the best of our knowledge, this is the first demonstration of antisense-mediated inhibition of heparanase expression as well as invasive properties by using an adenovirus construct. Our results suggest that heparanase is a promising target for the prevention of pleural dissemination of human cancer.

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