Inhibition of invasion, angiogenesis, tumor growth, and metastasis by adenovirus-mediated transfer of antisense uPAR and MMP-9 in non–small cell lung cancer cells

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Abstract

Lung cancer is currently the leading cause of cancer deaths in the United States. Conventional therapeutic treatments, including surgery, chemotherapy, and radiation therapy, have achieved only limited success. The overexpression of proteases, such as urokinase-type plasminogen activator (uPA), its receptor (uPAR), and matrix metalloproteinases (MMP), is correlated with the progression of lung cancer. In the present study, we used a replication-deficient adenovirus capable of expressing antisense uPAR and antisense MMP-9 transcripts to simultaneously down-regulate uPAR and MMP-9 in H1299 cells. Ad-uPAR-MMP-9 infection of H1299 cells resulted in a dose- and time-dependent decrease of uPAR protein levels and MMP-9 activity as determined by Western blotting and gelatin zymography, respectively. Corresponding immunohistochemical analysis also showed that Ad-uPAR-MMP-9 infection inhibited uPAR and MMP-9 expression. As shown by Boyden chamber assay, Ad-uPAR-MMP-9 infection significantly decreased the invasive capacity of H1299 cells compared with mock and Ad-CMV (empty vector)–infected cells in vitro. Furthermore, Ad-uPAR-MMP-9 infection inhibited capillary-like structure formation in H1299 cells cocultured with endothelial cells in a dose-dependent manner compared with mock- and Ad-CMV-infected cells. Ad-uPAR-MMP-9 injection caused the regression of s.c. induced tumors after s.c. injection with H1299 lung cancer cells and inhibited lung metastasis in the metastatic model with A549 cells. These data suggest that Ad-uPAR-MMP-9 shows its antitumor activity against both established and early phases of lung cancer metastases by causing the destruction of the tumor vasculature. In summary, adenovirus-mediated inhibition of uPA-uPAR interaction and MMP-9 on the cell surface may be a promising anti-invasion and antimetastatic strategy for cancer gene therapy. [Mol Cancer Ther 2005;4(9):1399–408]

Introduction

Non–small cell lung cancers (NSCLC) have a rather unpredictable prognosis and account for ~80% of primary lung cancer (1). A crucial step during invasion and metastasis is the destruction of biological barriers, such as the basement membrane, which requires activation of proteolytic enzymes (2). Proteases contribute to each step beginning with the first breakdown of the basal membrane of a primary tumor to the extended growth of established metastases. Many studies have shown that enhanced production of members of the plasminogen activator pathway and matrix metalloproteinase (MMP) family contributes to tumor invasion, metastasis, and angiogenesis (3). The urokinase-type plasminogen activator (uPA) receptor (uPAR) focuses uPA activity on the cell membrane, thus regulating cell surface–associated proteolysis by uPA, which converts the zymogen plasminogen to plasmin (4). Plasmin is a broad-spectrum protease, which promotes extracellular matrix (ECM) degradation by directly degrading ECM components and activating latent collagenases and metalloproteases (5). The uPAR is also involved in the regulation of cell adhesion and migration independent of the enzymatic activity of its ligand (6). It is capable of transmitting uPA-mediated extracellular signals inside the cell probably through the association with different types of integrins (7) and with vitronectin (8), an ECM component (9). Predominant stromal expression of uPA correlated with tumor size and lymph node metastasis in a large series of lung tumors (10, 11). Plasminogen activator inhibitor-1 and uPA are strongly correlated and linked to tumor progression variables in lung cancer, suggesting a synergistic effect on tumor cell migration (12). Consequently, inhibition of uPA-uPAR interaction on the cell surface might be a promising anti-invasion and antimetastatic strategy. It has been shown that inhibition of uPA catalytic activity...
and its binding to the receptor by various strategies, such as neutralizing antibodies, competitive peptides, recombinant molecules, and antisense oligonucleotides, can inhibit tumor growth, invasion, and metastasis both in vitro and in vivo (13).

Another important family of proteinases responsible for the ECM destruction involved in cancer progression is the MMP family. Among the many MMPs that have been identified, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are thought to be key enzymes because they degrade type IV collagen, the main component of ECM (14). Increased expression of MMP-2 and MMP-9 was shown to correlate with an invasive phenotype of cancer cells (15). Several recent reports confirmed lung neoplastic cells produce both matrix metalloproteases and their inhibitors (16–18). MMP-9 is a Mr 92,000 gelatinase that degrades type IV collagen and was found to be significantly associated with survival in lung cancer patients (19–21). The prognostic effect of homogeneous MMP-9 expression was shown to be independent from possible prognostic joint effects of pT status, pN status, tumor histology, and tumor grading, thus suggesting possible joint effects of pT status, pN status, and MMP-9 expression (22).

Many potential genes and gene therapy strategies that have been tested in vitro and in vivo have shown tumor suppression. We previously showed efficient reduction of metastasis with ex vivo treatment of lung cancer cells with adenovirus encoding antisense transcript for uPAR (23). Here, we show the therapeutic potential of adenovirus expressing antisense transcripts against two genes (a protease receptor, uPAR, and MMP-9) in lung cancer. We show that an adenovirus bicistronic construct carrying antisense message for uPAR and MMP-9 (Ad-uPAR-MMP9) significantly decreased in vitro invasion through Matrigel-coated membranes and pre-established tumor growth and lung metastasis in vivo after s.c. injection of H1299 lung cancer cells and in a metastatic model using A549 lung cancer cells.

Materials and Methods

Cell Lines and Culture Conditions

A549 and H1299 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in 100 mm tissue culture plates in RPMI 1640 (American Type Culture Collection) supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA), 50 units/mL penicillin, and 50 μg/mL streptomycin (Life Technologies, Inc., Frederick, MD), hereafter called complete medium. Human microvascular endothelial cells were obtained from Cambrex BioScience Rockland, Inc. (Rockland, Maine) (CC-2616) and maintained in large vessel endothelial medium supplemented with basic fibroblast growth factor/heparin, epidermal growth factor, and cortisol in the presence of amphotericin/gentamicin. Virus constructs were diluted in serum-free culture medium to the desired concentration and added to cell monolayers or spheroids and incubated at 37°C for 1 hour. The necessary amount of complete medium was then added and cells were incubated for the desired periods.

Adenoviral Production

Construction of adenoviral vector expressing antisense sequences for human uPAR and MMP-9 was done as described previously (24). The adenovirus contains a cytomegalovirus promoter followed by a truncated 300-bp antisense message complementary to the 5' end of the uPAR gene, a 520-bp antisense message complementary to the 5' end of the MMP-9 gene, and a bovine growth hormone polyadenylation signal. Following previously established methods, suspensions of recombinant adenovirus were prepared by amplification in 293 cells followed by purification using three consecutive CsCl gradients (25). Viral titers were estimated using absorbance at 260 nm and standard plaque assay.

Gelatin Zymography

MMP-9 expression in conditioned medium of lung cancer cells infected with mock, empty vector [Ad-EV; 100 multiplicities of infection (MOI)], or the indicated doses of Ad-uPAR-MMP9 was analyzed by gelatin zymography as described previously (24). Briefly, the conditioned medium was resolved by 10% SDS-PAGE in the presence of 1 mg/mL gelatin. The resulting gel was washed in 10 mmol/L Tris (pH 8.0) containing 2.5% Triton X-100 and was then incubated for 16 hours in a reaction buffer [50 mmol/L Tris (pH 8.0), 0.5 mmol/L CaCl2, 10⁻⁴ mol/L ZnCl2] at 37°C. After staining with Coomassie brilliant blue R-250, gelatinases were identifiable as clear bands.

Western Blot

Lung cancer cells were infected with mock, the indicated doses of Ad-EV (100 MOI), Ad-uPAR, or Ad-uPAR-MMP9. Four days after infection, the medium was replaced with serum-free medium and the cells were further incubated for an additional 15 to 16 hours. At the end of incubation, the conditioned medium was collected, cells were harvested, and cellular proteins were extracted with lysis buffer [40 mmol/L HEPES-NaOH (pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl] containing Complete Mini, a mixture of protease inhibitors (Roche, Indianapolis, IN). Total proteins from the medium for MMP-9 and cell lysates (for uPAR) were electrophoresed on SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). After blocking with 5% nonfat, dry milk and 0.1% Tween 20 in TBS, membranes were incubated with the mouse monoclonal uPAR or MMP-9 (Biomed, Foster City, CA) or the mouse monoclonal anti-actin (Biomed) antibody. The membranes were then developed with peroxidase-labeled antibodies (Amersham Pharmacia, Piscataway, NJ) and chemiluminescence substrate (Pierce, Rockford, IL). Actin protein levels were used as a control for equal protein loading.

Invasion Assays

The ability of lung cancer cells to migrate through Matrigel-coated filters was measured using Transwell chambers (Costar, Cambridge, MA) with 12.0 μm pore
polycarbonate filters coated with 30 μg Matrigel in the top side of the filter (Collaborative Research, Bedford, MA). H1299 cells were infected with mock, Ad-EV, Ad-uPAR (100 MOI), or Ad-uPAR-MMP-9 (50 MOI). After 5 days, the cells were trypsinized, resuspended in serum-free medium, seeded on the top compartment of the chamber, and incubated for 24 hours. At the end of incubation, the cells were stained and the cells and Matrigel on the top surface of the filter were carefully removed with a cotton swab. The invasive cells adhering to the bottom surface of the filter were quantified under a light microscope (×20). The data are presented as the average number of cells attached to the bottom surface from randomly chosen fields. Each treatment condition was assayed using triplicate filters and filters were counted at five areas.

Migration Assay
Spheroids were initiated by inoculating 1 × 10⁵ exponentially growing H1299 cells onto 96-well low attachment plates (Corning Costar no. 3471) with 200 μL culture medium and incubated in a shaker at 200 rpm at 37°C for 72 hours. A single spheroid formed in each well. The medium was replaced and spheroids were cultured for another 5 days. Single spheroids were pelleted by centrifugation, resuspended in trypsin solution, and incubated at 37°C for 10 minutes. After the addition of complete medium, cell suspensions were passed twice through an 18-gauge needle to disrupt cell clumps and the mean cell number per spheroid was determined. The spheroids were infected with mock, Ad-EV (100 MOI), or the indicated concentrations of Ad-uPAR and Ad-uPAR-MMP-9 for 48 hours. The spheroids were then removed from low attachment plates, a single spheroid was placed in each well of an eight-well chamber slides, and slides were incubated for an additional 72 hours. Then, the spheroids were stained using a Hema-3 stain kit and photographed using a light microscope.

Coculture Assay
H1299 lung cancer cells were plated in eight-well chamber slides (5 × 10⁵) and infected with mock, Ad-EV (100 MOI), and various doses of Ad-uPAR and Ad-uPAR-MMP-9 for 4 days. The culture medium was replaced and human endothelial cells (2 × 10⁵) were plated and cocultured for 72 hours. After incubation, cells were fixed in 3.7% formaldehyde and blocked with 3% bovine serum albumin for 1 hour and endothelial cells were probed with antibody for factor VIII antigen (factor VIII antibody, DAKO Corp., Carpinteria, CA). The cells were washed with PBS and incubated with FITC-conjugated secondary antibody for 1 hour. The cells were then washed and examined under a laser scanning confocal microscope. Photomicrographs were subjected to a customized routine-based image analysis tool.

Animal Experiments
SCID mice (Harlan Sprague Dawley, Indianapolis, IN) were purchased germ-free at 2 to 3 weeks of age and maintained in a specific pathogen-free environment throughout the experiment. Animals were kept in sterile cages (maximum of five mice per cage) bedded with sterilized soft wood granulate and fed irradiated rat chow ad libitum with autoclaved water at a 12-hour light/dark cycle. Animals were kept at least 1 week before experimental manipulation. All manipulations were done in a laminar flow hood. Before tumor inoculation and radiographic examination, mice were anesthetized with an i.p. mixture of 50 mg/kg ketamine and 10 mg/kg xylazine. For euthanasia, animals were given a lethal dose of ketamine and xylazine.

Subcutaneous Tumors
A suspension of H1299 cells (2.5 × 10⁶ in 0.1 mL RPMI 1640) was s.c. injected into the left side of at least five nude mice per condition. Tumors were allowed to grow to ~5 to 6 mm and the mice were separated into three groups. Each group received Ad-EV (5 × 10⁸) or 5 × 10⁸ or 5 × 1⁰⁸ plaque-forming units (pfu) of Ad-uPAR-MMP-9 injections around the tumor on days 10, 15, and 20. We monitored the effect of tumor formation on the mice by looking for behavioral changes, such as inactivity and lameness, and by observing possible weight loss. All of the animals remained healthy and active during the experiment. Tumor sizes were measured every 5 days and mice were sacrificed at day 60. Tumor volumes were determined according to the formula: \[ V = 0.4 \times A \times B^2 \]
where A is the largest dimension of the tumor and B is the smallest dimension.

Metastasis Model
A549 cells (2 × 10⁶ in 100 μL serum-free medium) were injected into the left flank of 15 mice. The mice were separated into three groups composed of five animals each. After 3 weeks, when the tumors had reached 8 to 10 mm in size, the mice received i.v. injections of PBS or 5 × 1⁰⁸ pfu of either Ad-EV or Ad-uPAR-MMP-9 thrice at 5-day intervals. The mice were sacrificed after 12 weeks. The s.c. tumors and the lungs were immediately dissected and fixed in 4% paraformaldehyde in PBS at 4°C for 24 hours. The lungs were embedded in paraffin and evaluated using H&E staining. The sections were screened and evaluated by a pathologist blinded to treatment condition.

Results
Ad-uPAR-MMP-9 Infection Efficiently Down-Regulates uPAR and MMP-9 Protein Levels in Lung Cancer Cells
We examined two lung cancer cell lines (H1299 and A549) for uPAR and MMP-9 expression. uPAR protein levels and MMP-9 activity were assessed using Western blotting and gelatin zymography, respectively. Cells were infected with mock, Ad-EV, Ad-uPAR (100 MOI), or various doses of Ad-uPAR-MMP-9. Immunoblotting indicated that Ad-uPAR-MMP-9 infection inhibited uPAR expression in a dose- and time-dependent manner. Densitometric quantitation of uPAR protein bands indicated a 20% decrease in uPAR expression in cells infected with 10 MOI of Ad-uPAR-MMP-9. This decrease was ~90% in cells infected with 50 MOI of Ad-uPAR-MMP-9 compared with mock and Ad-EV-infected controls. Cells infected with the
Adenovirus expressing antisense message to only uPAR (Ad-uPAR at 100 MOI) decreased uPAR expression by ~40% compared with the controls (Fig. 1A). Kinetics of uPAR expression within cells infected with 50 MOI of Ad-uPAR-MMP-9 indicated that protein levels decreased significantly by day 4 and were inhibited by >90% by day 5 (Fig. 1B).

Similarly, Ad-uPAR-MMP-9 infection inhibited the levels of MMP-9 activity in a time- and dose-dependent manner as determined by gelatin zymography. Densitometric scanning of the bands indicated that MMP-9 was inhibited by 15% in cells infected at 10 MOI of Ad-uPAR-MMP-9 and 54% in cells infected at 25 MOI. This decrease reached 92% in cells infected with 50 MOI of the virus when compared with mock and Ad-EV controls. Ad-uPAR had a very minimal effect on MMP-9 expression (Fig. 1C). Progression of MMP-9 inhibition in cells infected with 50 MOI of Ad-uPAR-MMP-9 indicated a significant decrease by day 3 and reached ~90% by day 5 compared with mock and Ad-EV-infected H1299 cells (Fig. 1D). A similar pattern of Ad-uPAR-MMP-9 effect was also observed in A549 lung cancer cells (data not shown).

**Ad-uPAR-MMP-9 Infection Could Remarkably Inhibit the Invasive Capacity of Lung Cancer Cell Lines In vitro**

The invasive potential of the lung cancer cells after infection with Ad-uPAR-MMP-9 was determined using the Matrigel invasion assay. Figure 2A shows decreased staining of the invaded cells through the Matrigel with Ad-uPAR and Ad-uPAR-MMP-9 infection compared with the mock and Ad-EV controls. Quantitative analysis indicated that Ad-uPAR-MMP-9 was more effective in decreasing the invasiveness of these cells compared with Ad-uPAR. Tumor cell invasion decreased ~50% in cells infected with 100 MOI of Ad-uPAR and >90% in cells infected with 50 MOI of Ad-uPAR-MMP-9 (Fig. 2B).

**Ad-uPAR-MMP-9 Inhibits Spheroid Migration**

The cleavage of ECM components is a key requirement for cell migration; proteolysis allows release of growth factors and other signaling molecules from ECM, which in turn aid in migration (26). Multicellular spheroids composed of transformed cells are known to mimic tumor growth characteristics. H1299 cells were allowed to form spheroids and were infected with mock, Ad-EV, Ad-uPAR, or Ad-uPAR-MMP-9. Outgrowth and cell density were the variables chosen to evaluate migrational capacity. There was extensive outgrowth of cells in spheroids infected with mock and Ad-EV (Fig. 3A and B). Spheroids infected with 100 MOI of Ad-uPAR (Fig. 3C) showed less migration of the cells from the center. However, this inhibition in the outgrowth of cell from the spheroids was more distinct with Ad-uPAR-MMP-9 and was in a dose-dependent manner (Fig. 3D–F).

**Ad-uPAR-MMP-9 Inhibits Tumor Cell–Induced Angiogenesis**

Because tumor growth and proliferation depend on neovascularization, we evaluated the antiangiogenic potential of Ad-uPAR-MMP-9 in vitro using human microvascular endothelial cells. In this system, endothelial cells

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**Figure 1.** Ad-uPAR-MMP-9 decreased uPAR and MMP-9 expression in lung cancer cells. **A,** Western blot analysis of uPAR protein expression in cell lysates from H1299 infected with mock, Ad-EV (100 MOI), or the indicated doses of Ad-uPAR and Ad-uPAR-MMP-9. **C,** conditioned medium was collected from the H1299 cells. Protein (60 μg) from these samples was mixed with Laemmli sample buffer and run on 10% SDS-PAGE gels containing 0.1% gelatin (gelatin zymography). Densitometric quantitation of uPAR and MMP-9 was done. **Columns,** average values from three separate experiments. uPAR (**B**) and MMP-9 (**D**) expression in H1299 cells infected with 50 MOI of Ad-uPAR-MMP-9 at the indicated time points.
migrate to form tubular structures in the presence of tumor cells. Human microvascular endothelial cells were cultured in the presence of lung cancer cells and infected with various doses of Ad-uPAR-MMP-9. A very distinct capillary network was visualized following factor VIII staining of endothelial cells cultured in the presence of mock and Ad-EV-infected lung cancer cells. In contrast, Ad-uPAR-MMP-9 infection of the lung cancer cells inhibited the cancer cell–induced capillary network formation in a dose-dependent manner (Fig. 4A). Image analysis indicated that Ad-uPAR-MMP-9 treatment decreased the number of branch points as well as the length of vascular tubules in a dose-dependent manner (Fig. 4B and C). Lung cells infected with 100 MOI of Ad-uPAR

Figure 2. Ad-uPAR-MMP-9 inhibits lung cancer cell invasiveness through Matrigel. H1299 cells were trypsinized and counted 3 d after infection with mock, Ad-EV, or the indicated doses of Ad-uPAR and Ad-uPAR-MMP-9. Invasion assays were carried out in 12-well Transwell units (1 × 10^5 cells per treatment condition in triplicate). After a 24-h incubation period, the cells that had passed through the filter into the bottom wells were fixed and photographed (A). The percentage of invasion was quantitated as described in Materials and Methods (B). Columns, mean of five different experiments; bars, SD.

Figure 3. Ad-uPAR-MMP-9 inhibits cell migration from lung cancer cell spheroids. H1299 spheroids were infected with mock, Ad-EV, or the indicated doses of Ad-uPAR and Ad-uPAR-MMP-9. After 3 d, single spheroids were placed in the center of wells in 8-well chamber slides and cultured at 37°C for 48 h. At the end of the migration assay, spheroids were fixed, stained, and photographed.

A  Mock  B  Ad-EV  (100 MOI)  C  Ad-uPAR  (100 MOI)

D  E  F

10 MOI  25 MOI  50 MOI

Ad-uPAR-MMP-9
caused a 25% decrease in the length and a 50% inhibition in the number of branch points. However, Ad-uPAR-MMP-9 had a more dramatic inhibition in the tumor-induced angiogenesis. Five MOI of this virus inhibited the length of the capillaries by ~50% and the number of branch points was reduced to 24 compared with 59 in mock and 56 in the Ad-EV-infected cells. This decrease was more pronounced with increasing MOI of Ad-uPAR-MMP-9.

**Ad-uPAR-MMP-9 Inhibits Tumor Growth *In vivo* and Significantly Reduces the Metastatic Capacity of Lung Cancer Cells *In vivo***

**Subcutaneous Tumor Model.** H1299 cells formed rapidly growing primary tumors. We compared the tumor inhibitory effect of two different concentrations of Ad-uPAR-MMP-9. Three sets of mice with five animals per group received three doses of either $5 \times 10^8$ pfu of Ad-EV or $5 \times 10^8$ or $5 \times 10^9$ pfu of Ad-uPAR-MMP-9 i.t. All the tumors receiving Ad-uPAR-MMP-9 showed regression starting on the ninth day after injection. Analysis of tumor size revealed that tumors injected with $5 \times 10^8$ pfu of virus displayed a more sluggish response with respect to tumor regression than compared with those mice, which received $5 \times 10^9$ pfu of Ad-uPAR-MMP-9 virus. However, both concentrations of the virus regressed tumor growth ~90% in all the mice by the end of the experiment (Fig. 5A).

**Lung Metastasis Model.** A549 cells formed lung metastases in the mice that received s.c. inoculation. We examined the *in vivo* effect of three i.v. injections of Ad-uPAR-MMP-9 given on the 10th, 15th, and 20th day after the tumor inoculations on metastasis of lung tumors formed by the injection of A549 cells. Twelve weeks after s.c. inoculation of tumor cells, at which point the primary tumor had reached its maximum volume, the animals were sacrificed. There was a significant difference in the weight of the tumor volumes in the control mice when compared with the tumors from the mice that received Ad-uPAR-MMP-9 injections. The average tumor volume of the mice that received Ad-uPAR-MMP-9 was only 25% than the average tumor volumes of the mice that received PBS and Ad-EV (Fig. 5B). We did a histologic analysis of metastatic foci among the lungs from the control mice and those that received Ad-uPAR-MMP-9. Analysis of the metastases in serial lung sections was done under light microscopy. Lung metastases were observed in the control and Ad-EV-treated mice. The cancer cells varied in size and shape (polygonal, circular, or irregular). The nuclei also differed in size, displayed notable atypia and pathologic mitosis, and stained variably. However, no lung metastases were observed until the end of the experiments in mice treated with Ad-uPAR-MMP-9, with the exception of a single cell in one mouse (Fig. 5C). The results show the remarkable

![Figure 4](image-url)
inhibition of metastatic nodules in the lungs of animals treated with Ad-uPAR-MMP-9, suggesting that uPAR and MMP-9 influence the metastatic capacity of lung cancer cells in vivo.

Discussion
The ability of neoplastic cells to migrate, invade, and metastasize to other organs presents a major hurdle to successful therapeutic intervention. The degradation of basement membranes by tumor cells involves secretion and activation of proteinases, including the MMPs and those involved in the plasminogen activation system. Moreover, recent evidence has shown multiple functions for MMPs, rather than simply degrading ECM, which include the mobilization of growth factors and processing of surface molecules. Antiproteolytic approaches are valuable alternatives to conventional therapies. Using plasmin-deficient mice, Bugge et al. (27) showed that all the steps involved in the growth and dissemination of a malignant tumor could take place in the complete absence of plasmin-mediated proteolysis. Similarly, systemic administration of a synthetic gelatinase inhibitor showed very similar effects on the dissemination of Lewis lung carcinoma as those of Plg deficiency (i.e., a moderate reduction in primary tumor growth and metastasis) and slightly increased survival (28). These studies led us to hypothesize whether the combined elimination of several cancer-associated matrix-degrading proteases could possibly have a synergistic or additive effect on impeding tumor dissemination. In recent years, much attention has been focused on antimetastatic agents constructed by genetic engineering techniques for the therapy of malignant tumor cells. Here, we analyzed the efficiency of adenovirus-mediated synergistic expression of antisense sequences to uPAR and MMP-9 at inhibiting the invasive capacity, tumor growth, and metastasis of lung cancer cells.

![Inhibition of metastatic nodules](image)
First, expression of uPAR and MMP-9 was characterized in H1299 and A549 lung cancer cell lines. The uPAR- and MMP-9-bearing malignant lung tumor cells have greater access to the high concentrations of uPA and MMP-9, which facilitate matrix degradation and result in tumor cell invasion. Several components of the uPA system are potential targets for anti-invasive, antiangiogenic, and antimetastatic strategies (6, 29). Increased expression of MMP-9 mRNA and MMP-9 protein have been found in many solid tumors (30), including NSCLC (16, 19, 31). Higher levels of MMP-9 mRNA have been found in stage III NSCLC compared with stage I and II (16). The expression of either MMP-9 or MMP-2 confers a worse prognosis in early stage adenocarcinoma of the lung (19). Infection with Ad-uPAR-MMP-9 decreased the expression of these two molecules by >90% in both lung cancer cells. It is interesting to note that only 50 MOI of Ad-uPAR-MMP-9 inhibited ~90% of the uPAR protein expression compared with Ad-uPAR infection, which carried the antisense message for uPAR alone and achieved this effect but at a much higher dose. Correspondingly, the modified Boyden chamber test in vitro showed that reduced invasiveness of these cells through a reconstituted basement membrane at a lower concentration of this bicistronic gene construct compared with Ad-uPAR.

Next, migration of these cells was determined using a spheroid migration model. Multicellular spheroids composed of transformed cells are known to mimic the growth characteristics of tumors. It has been shown that cells in spheroid culture (32) and even plateau-phase monolayer culture (33) respond differently to a variety of signals compared with the monolayer cultures. Inhibition of uPAR and MMP-9 inhibited cell migration from tumor spheroids. Growth factors, such as insulin-like growth factor-I, insulin-like growth factor-II, hepatocyte growth factor, epidermal growth factor, and stem cell factor, induce migration of human NSCLC cells in the presence of ECM components (34). The ECM is considered a reservoir of growth factors, which are released by matrix protease-mediated ECM degradation (35). Growth factor activation could in turn be driven by the same matrix proteases (36). Growth factors were shown to promote invasiveness through their ability to enhance the expression and activity of matrix-degrading MMP-2 and MMP-9 in NSCLC cells (37). Furthermore, activation of plasminogen-induced proteolysis is involved in the release and activation of numerous growth factors as well as transforming growth factor-β, which are all involved in invasion and angiogenesis. The capacity of lung cancer cells to invade was increased in vitro after treatment with colony-stimulating factor. In addition, this enhanced invasive behavior of the cancer cells stimulated by colony-stimulating factors correlated with increases in MMPs and uPA activities (38).

I.t. and i.v. injection of Ad-uPAR-MMP-9 inhibited s.c. tumor growth and lung metastases in mice implanted with lung cancer cells in animal models. The uPA/uPAR interaction has been shown to have an important role in tumor metastasis. A recombinant adenovirus encoding the noncatalytic activating transcription factor of mouse uPA prevents lung carcinoma metastasis (39) and protects mice in a liver metastasis model of human colon carcinoma (40). In our previous study, Ad-uPAR (adenovirus expressing an antisense message to uPAR) could partially inhibit lung metastasis when H1299 cells were treated ex vivo with this virus and injected i.v. (23). First, this may be because several factors in the uPA system, including uPAR-binding capacity, the amount of uPA available for binding, endogenous uPA inhibitor plasminogen activator inhibitor-1, and the ratio of potent active uPA and inactive ligands occupied on uPAR, are involved in determining the tumor’s ability to invade and metastasize. Second, this may also be because other proteases, including metalloproteases, may compensate for a lack of uPA activity and uPAR protein. Other studies have shown that cooperation between uPA/uPAR and metalloproteases is required to complete the step for intravasation and consequently for metastasis (41). Up-regulation of secreted MMP-9 also correlated with an increase in tumor growth kinetics and angiogenesis compared with cells expressing low MMP-9 levels (mock) or active MMP-9 on the tumor cell surface (MMP-9-LDL) in a breast cancer model. This enhancement was partially inhibited by doxycycline, indicating that these effects require the proteolytic activity of the secreted MMP-9 form (42). Several MMP inhibitors are currently being investigated in clinical trials to assess their efficacy in maintenance of remission after other treatment modalities or in combination with standard chemotherapy (43). MMP inhibitors that have been studied in NSCLC include batimastat (BB-94), marimastat (BB-2516), prinomastat (AG-3340), and BMS-275291 (44). Yamamoto et al. (45) reported that ONO-4817, a third-generation MMP inhibitor, inhibited progression of established lung micrometastasis by tumor cells expressing MMPs and that therapeutic efficacy was further augmented when combined with docetaxel. Liu et al. (46) also suggest that the administration of prinomastat (AG3340) in combination with carboplatin may prolong survival in NSCLC patients. However, these inhibitors act against all MMPs. These sometimes lead to a negative response in the treatment of cancer because some MMPs could be expressed as a protective response and therefore play an important role in the host defense during tumorigenesis (47). In view of the role of MMP-9 in tumor progression and angiogenesis, we constructed an adenovirus that expresses antisense message for uPAR and MMP-9 to determine if down-regulation of two different proteases have an improved effect in inhibiting lung metastasis. In the present study, we could not detect any lung nodules in mice treated with Ad-uPAR-MMP-9 in the metastasis model. Histologic examination revealed only one or two cells, which appeared to be neoplastic (blind reviewed by a pathologist). Therefore, our strategy to specifically down-regulate two proteases simultaneously has achieved maximal tumor growth inhibition and has significant therapeutic potential.

To further characterize the relationship of uPAR and MMP-9 inhibition on tumor growth, we assessed the effect...
of Ad-uPAR-MMP-9 on tumor-induced angiogenesis in vitro. Tumor growth depends on the development of blood vasculature to bring in nutrients critical to sustain growth (48). The cell-associated plasminogen activator system is known to play a crucial role in the angiogenesis process by modulating the adhesive properties of endothelial cells in their interactions with ECM and in the degradation of ECM (49–52). Interference with the activities of the uPA system has been shown to cause inhibition of angiogenesis in vitro in some cases (49, 53–55) and in vivo (39, 54). Activating transcription factor–based uPAR antagonists are known to have antiangiogenic function mainly by blocking uPA/uPAR interaction (39, 53). Recently, the recombinant kringle domain of urokinase (UKI) has been shown to present antiangiogenic activity in vitro and in vivo (41). There is abundant evidence that MMPs, such as MT1-MMP, MMP-2, and MMP-9, are essential for angiogenesis (56, 57). In addition to the breakdown of connective tissue and vascular migration, MMPs have also been thought to regulate endothelial cell attachment and proliferation (58). A strong correlation between microvessel density and tumor level of MMP-9 was shown in pulmonary adenocarcinoma (59). Our results show that uPAR inhibition in the cancer cells moderately inhibited the tumor-induced angiogenesis. In our study, inhibition of uPAR and MMP-9 expression had a more pronounced effect in inhibiting capillary formation in endothelial cells compared inhibiting uPAR alone. This result is supported by several studies that show that the cooperation between uPA/uPAR and MMP-9 is required for breaching of the vascular wall, a rate-limiting step for intravasation, and consequently for metastasis. Angiostatin, a plasmin-derived angiogenesis inhibitor, was shown to mediate the suppression of metastasis by Lewis lung carcinoma (60). However, loss of angiostatin in plasmin-deficient mice was not associated with an obvious increase in tumor neovascularization within primary tumors and metastases, as assessed by qualitative microscopic analysis using an endothelial cell marker, or on the growth rate of primary tumors or metastases (27). Kim et al. (41) have shown that mice treated with the MMP inhibitor marimastat reduced intravasation by >90% and also that uPA/uPAR and MMP-9 are required to break the vascular wall in order for tumors to metastasize.

Pericellular proteolysis seems more and more as a crucial event controlling the local environment surrounding normal and tumor cells. It is accomplished by a cooperative interaction between several proteases. Their functions have been extended from pericellular proteolysis and control of cell migration to cell signaling, control of cell proliferation, and regulation of multiple stages of tumor progression, including growth and angiogenesis. The degradation of the ECM during tumor invasion and angiogenesis is likely to release active molecules stored in the matrix and/or to generate active fragments of matrix components, which promote tumor growth, invasion, and angiogenesis (61–63). A functional overlap between the plasminogen activator and MMP systems was shown in the dissection of the fibrin-rich provisional matrix by migrating keratinocytes (64). These findings showed that the effective arrest of cancer progression would require the combined use of inhibitors of MMPs and inhibitors of the plasmin/Plg activation system. Given the multiple roles of uPAR and MMP-9 in multiple biological events, the reduction in tumor growth and inhibition of lung metastasis in Ad-uPAR-MMP-9–treated mice in our study has significant therapeutic implications.

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References


Correction: Inhibition of Invasion, Angiogenesis, Tumor Growth, and Metastasis by Adenovirus-Mediated Transfer of Antisense uPAR and MMP-9 in Non–Small Cell Lung Cancer Cells

In this article (Mol Cancer Ther 2005;4:1399–408), which was published in the September 2005 issue of Molecular Cancer Therapeutics (1), the authors discovered errors in three figure panels.

The image originally published as Fig. 2A was from the wrong xenograft tumor tissue. The authors completed two cell lines–derived xenografts at the same time and mismatched the data. As a result, Fig. 2A is incorrect. In the corrected Fig. 2A, experiments were performed with A549 and H1299 cell lines under identical conditions as indicated before. Invasion assays were performed for H1299 lung cancer cells. The new data were generated to confirm and verify the effect of Ad-uPAR and Ad-uPAR-MMP-9 on invasion. The data obtained reconfirm the results originally reported in the article (the authors state they observed more than 85% suppression) on cell invasion in both the cases. Figure 2B is corrected in order that the data in the graph correspond with the corrected Fig. 2A.

In Fig. 5, the experiments were performed with in A549 and H1299 and obtained identical results. Figure 5C image was represented incorrectly. The authors apologize for the errors, and present correct figures below.

Figure 2. Ad-uPAR-MMP-9 inhibits lung cancer cell invasiveness through Matrigel. H1299 cells were trypsinized and counted 3 days after infection with mock, Ad-EV, or the indicated doses of Ad-uPAR and Ad-uPAR-MMP-9. Invasion assays were carried out in 12-well Transwell units (1 × 10^6 cells per treatment condition in triplicate). After a 24-hour incubation period, the cells that had passed through the filter into the bottom wells were fixed and photographed (A). The percentage of invasion was quantitated as described in Materials and Methods (B). Columns, mean of five different experiments; bars, SD.
Reference


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Figure 5. Inhibition of subcutaneous tumor growth. A, nude mice were injected with H1299 cells (5 × 10^7) / 100 μL). When the tumor reached 5 to 6 mm in size, mice were injected intratumorally with Ad-EV (5 × 10^9) or Ad-uPAR-MMP-9 (5 × 10^8 or 5 × 10^9) thrice. Tumor size was measured using calipers as described in Materials and Methods. B and C, inhibition of subcutaneous tumor growth and lung metastasis. A549 cells were injected subcutaneous into nude mice. After 2 weeks, the mice received intravenous injections of PBS or 5 × 10^8 PFU of either Ad-EV or Ad-uPAR-MMP-9 thrice at 5-day intervals. Representative subcutaneous tumors are shown for each treatment condition (B). H&E staining of lung sections from the same mice (C).
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Inhibition of invasion, angiogenesis, tumor growth, and metastasis by adenovirus-mediated transfer of antisense uPAR and MMP-9 in non–small cell lung cancer cells


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