Antimetastatic activity of insulin-like growth factor binding protein-3 in lung cancer is mediated by insulin-like growth factor–independent urokinase-type plasminogen activator inhibition

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Abstract

Insulin-like growth factor binding protein-3 (IGFBP-3), a major IGF-binding protein in human serum, regulates the growth of non–small cell lung cancer (NSCLC) cells through IGF-dependent and IGF-independent mechanisms. However, the role of IGFBP-3 in lung cancer metastasis is not well known. In the present study, we showed that noncytotoxic doses of adenoviral or recombinant IGFBP-3 significantly decreased the migration and invasion of H1299 and A549 NSCLC cells. Furthermore, treatment of human lung fibroblasts with recombinant IGFBP-3 suppressed their ability to stimulate the invasion of H1299 cells. Overexpression of IGFBP-3 markedly reduced lung metastasis of A549 cells in an experimental animal model system and prolonged the survival time of the animals. Urokinase-type plasminogen activator (uPA) inhibitor treatment or uPA small interfering RNA transfection of A549 and H1299 cells resulted in a significant decrease in invasion. Corresponding ELISA, Western blot, gelatin zymogram, and semiquantitative reverse transcription-PCR analyses revealed that IGFBP-3 reduced the expression of uPA mRNA through IGF-independent mechanisms. The specific role of uPA in anti-invasive activity of IGFBP-3 was further confirmed in NSCLC cells, in which uPA expression/activity was suppressed by the transfection with synthetic small interfering RNA or by the treatment with uPA inhibitor or induced by the infection with an adenoviral vector. IGFBP-3 also decreased the expression/activity of matrix metalloproteinase-2 through IGF-dependent but uPA-independent pathways. Taken together, our data suggest that IGFPB-3 effectively block uPA- and matrix metalloproteinase-2–stimulated invasion pathways, ultimately reducing lung cancer cell metastasis. Our findings indicate that IGFBP-3 may be a promising anti-invasive and antimetastatic therapeutic agent in lung cancer. [Mol Cancer Ther 2006;5(11):2685–95]

Introduction

Non–small cell lung cancer (NSCLC) represents ~80% of all pulmonary carcinomas. Despite surgical resection and advances in radiotherapy and chemotherapy, the high mortality rate of NSCLC has not been substantially reduced over the past years, largely due to the potential of tumor cells to invade and metastasize (1). There is thus a need for development of therapeutic agents that regulate signaling molecules involved in lung tumor cell invasion and metastasis.

It has long been established that cancer dissemination involves multiple processes, such as cellular adhesion to specific glycoproteins of the extracellular matrix, degradation of extracellular matrix components by tumor-associated proteases (serine proteinases, metalloproteinases, cathepsins, and plasminogen activators), migration through blood vessels, and local invasion (2, 3). The plasminogen activation system, which consists of the urokinase-type plasminogen activator (uPA), its cell surface receptor uPA receptor (uPAR), and their inhibitors plasminogen activator inhibitor-1 and plasminogen activator inhibitor-2, plays a key role in invasion and metastasis in a variety of cancers, including lung cancer (4–6). Cell surface uPA facilitates extracellular matrix degradation by converting plasminogen into the active serine protease plasmin, which then degrades fibronectin, laminin, collagen IV, and other noncollagenous extracellular matrix proteins. uPA itself is synthesized and secreted as an inactive, single-polypeptide proenzyme that is converted by plasmin and other proteases into its active form. A recent study indicated that plasma levels of uPA is significantly higher in NSCLC

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Note: S-H. Oh and O-H. Lee contributed equally to this work and should be considered as first authors.

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patients, especially those with advanced metastatic disease, than in control subjects (7). Elevated levels of uPA in tissue specimens obtained from NSCLC patients seem to be related to cancer cell invasion, tumor aggressiveness, and a poor clinical outcome (5, 8, 9).

The matrix metalloproteinase (MMP) family is another important group of proteinases that are responsible for extracellular matrix destruction. Among the many MMPs, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) have a critical function in the degradation of extracellular matrix (10). Increased levels of MMP-2 and MMP-9 expression have been well correlated with an invasive phenotype of cancer cells (11, 12). These findings led us to hypothesize that agents inhibiting expression and/or activity of uPA/uPAR and MMPs might be a promising antimetastatic strategy in lung cancer.

An increasing number of evidence has shown the crucial role of the insulin-like growth factor (IGF) pathway in cancer cell metastasis. IGF-I induces the secretion and activity of extracellular matrix–degrading enzymes, such as uPA, MMP-2, and MMP-9 (13–15). Moreover, the expression levels and function of the IGF-1 receptor (IGF-1R) correlated with the metastatic potential in a murine lung carcinoma model (16). IGF-binding proteins (IGFBPs) have been known to modulate the biological action of IGFs by sequestering them from their receptors (17). We previously showed that IGFBP-3, a major IGFBP in human serum, is a potent inducer of apoptosis in NSCLC cells *in vitro* and *in vivo* partly because it suppresses the phosphatidylinositol 3-kinase/Akt/protein kinase B and mitogen-activated protein kinase signaling pathways (18). However, to our knowledge, no report has been published with regard to the effects of IGFBP-3 on invasion and metastasis of NSCLC cells. This study was designed to determine the effects of IGFBP-3 on the metastasis of human NSCLC cells and to investigate the action mechanisms of IGFBP-3. The data presented here reveal that IGFBP-3 has antimetastatic therapeutic activities *in vitro* and *in vivo* by inhibiting expression/activity of uPA and MMP-2 through mechanisms that are either IGF independent or dependent.

Materials and Methods

Materials, Cell Culture, and Animals

The human NSCLC cell lines H1299 and A549 and mouse NIH/3T3 fibroblasts were purchased from the American Type Culture Collection (Rockville, MD). Human lung fibroblast cells were obtained from Dr. Reuben Lotan (M.D. Anderson Cancer Center, Houston, TX). NSCLC cells were cultured in RPMI 1640 supplemented with 10% FCS (Life Technologies, Gaithersburg, MD); NIH3T3 and human lung fibroblast cells were maintained in Earle's MEM supplemented with 10% fetal bovine serum (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO_2. Mouse embryonic fibroblast cell line (R^-) derived from IGF-1 receptor (*Igf1r*) knockout embryos was kindly provided by Dr. R. Baserga (Thomas Jefferson University, Philadelphia, PA). The adenoviral vector expressing human IGFBP-3 (Ad-BP3) and control empty vector (EV) were amplified as previously described. Recombinant human IGFBP-3 protein (rbBP-3) was obtained from Insmed (Glenn Allen, VA). The adenovirus expressing human uPA (Ad-uPA) was kindly provided by Dr. Richard H. Simon (University of Michigan Health Science Center, Ann Arbor, MI). IGF-1R, uPA, and nonspecific control small interfering RNA (siRNA) was obtained from Dharmacon (Chicago, IL). Six-week-old female athymic nude mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). The uPA inhibitor uPA-STOP and the MMP-2 inhibitor MMP-2 inhibitor III were purchased from American Diagnostica (Stamford, CT) and Calbiochem (La Jolla, CA), respectively. Bovine serum albumin, gelatin, DMSO, SDS, plasminogen, fibrinogen, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were obtained from Sigma-Aldrich (St. Louis, MO).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was conducted as described previously. Briefly, 1×10^3 per well of H1299 or A549 cells were seeded in 96-well plates. After 1 day, cells were treated with 10 μg/mL rbBP-3 or infected with EV or Ad-BP3 at 10 or 50 plaque-forming units (pfu)/cell. After 2 days of incubation, cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and dissolved in DMSO, and formazan crystal formation was measured at wavelengths of 550 and 630 nm.

Adhesion Assay

For treatment of uPA and MMP-2 inhibitors, A549 cells were pretreated with 0.1, 0.5, or 1 μg/mL uPA-STOP or 0.5 or 5 μmol/L MMP-2 inhibitor III for 1 day. The uPA- or MMP-2 inhibitor–treated cells (5×10^5/mL) were resuspended in RPMI 1640 containing 0.1% bovine serum albumin before being seeded into 96-well microtiter plates coated with 100 μL of collagen type I (1 μg/mL), fibronectin (5 μg/mL), or laminin (10 μg/mL). After 60 minutes of incubation at 37°C, the plates were washed thrice with PBS to remove nonadherent cells, fixed, and stained with 0.04% crystal violet in PBS. The fixed cells were lysed in DMSO, and the absorbance was measured at 570 nm on a microtiter plate reader (MR5000; Dynatech Laboratories, Chantilly, VA).

Migration and Invasion Assays

The ability of NSCLC cells to migrate and invade was examined in 24-well cell culture chamber inserts (6.5 mm diameter, 8 μm pore size; Corning Costar, Cambridge, MA). H1299 and A549 tumor cells were infected with Ad-BP3 or EV (10 and 50 pfu/cell) for 1 day (control cells were not infected), harvested by trypsinization, washed, and resuspended in phenol red–free medium containing 0.1% (w/v) bovine serum albumin. The number of living cells was counted using 0.4% trypan blue (Life Technologies) exclusion. Only cells with viability ≥95% were used for further experiments.

For the migration assay, filters were coated with a 0.1 mg/mL solution of collagen type IV (Trevigen, Gaithersburg, MD) in distilled water and incubated for 24 hours at 37°C. About 7×10^4 tumor cells in 200 μL of medium were added to the upper chamber. The lower compartments were filled with 700 μL of conditioned medium, used as a chemoattractant, collected from 3×10^6 NIH/3T3 fibroblasts incubated overnight in growth medium containing 10% FCS. The invasion assay was done the same except for coating the Transwell units with Matrigel (Becton Dickinson Labware, Bedford, MA) at a concentration of 50 μg/mL in PBS, as described elsewhere. In some experiments, we added conditioned medium from human lung fibroblasts cultured in the absence or presence of 10 μg/mL rbBP-3. When indicated, rbBP-3 was added to the upper chamber. After 24 hours of incubation at 37°C under 5% CO_2, the migrated or invaded cells were fixed and stained with H&E, and four independent fields were counted under a microscope (Carl Zeiss, Jena, Germany). The results are expressed as the percentage of cells that migrated or invaded compared with that of control cells.

Measurement of uPA Levels by ELISA

Supernatants were obtained from cultures of NSCLC cells treated with adenoviral or recombinant IGFBP-3 and analyzed for secretion of uPA with an IMUBIND uPA ELISA kit (American Diagnostica, Stamford, CT) according to the manufacturer's instructions. Each sample was assayed in triplicate. The values obtained were within the linear portion of the standard curve generated by the use of uPA standards 0.0, 0.10, 0.25, 0.50, 0.75, and 1.0 ng/mL uPA provided from the company.

Western Blot Analysis

Lysates from exponentially growing H1299 and A549 NSCLC cell lines were prepared in buffer containing 20 mmol/L Tris-HCl (pH 7.6), 1 mmol/L EDTA, 140 mmol/L NaCl, 1% NP40, 1% aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium vanadate. The total protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Conditioned medium used for Western blot analysis was obtained from serum-free cultures of NSCLC cells and concentrated using an Amicon Ultra-4 centrifugal filter device (Millipore, Bedford, MA). Equivalent amounts of sample proteins were resuspended in SDS buffer [100 mmol/L Tris-HCl (pH 8.8), 0.01% bromophenol blue, 36% glycerol, 4% SDS, and 1 mmol/L DTT], boiled for 5 minutes, separated by SDS-PAGE, and transferred to Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were probed with an antibody directed against the B-chain of human uPA (American Diagnostica, Stamford, CT), anti-IGFBP-3 antibody (Diagnostic Systems Laboratories, Webster, TX), anti-IGF-1R antibody (Cell Signaling Technology, Beverly, MA), anti-uPAR antibody (American Diagnostica, Inc., Greenwich, CT), and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Coomassie blue and Ponceau S staining (Bio-Rad Laboratories, Hercules, CA) was used to confirm equal loading in samples when conditioned medium was used.

Gelatin and Fibrinogen/Plasminogen Zymography

The proteolytic activity of MMP-2/MMP-9 and uPA was assessed by SDS-PAGE using zymogram gels containing 0.1% (m/v) gelatin and fibrinogen/plasminogen, respectively. Equal volumes of serum-free culture medium were obtained from control uninfected H1299 and A549 cells or cells that had been infected with EV (10 or 50 pfu/cell) or Ad-BP3 (10 or 50 pfu/cell) for 2 days. Culture media were concentrated with Amicon Ultra-4 centrifugal device obtained from Millipore and loaded onto gels. Following electrophoresis at 4°C, the gels were incubated in Novex zymogram renaturation buffer for 30 minutes at room temperature and agitated with Novex zymogram development buffer overnight at 37°C. Clear bands indicative of gelatinolytic activity were visualized by staining the gels with Coomassie blue.

Experimental Lung Metastasis Models

To determine the effects of IGFBP-3 on the formation of metastatic lung tumors, experiments were done using mouse lung metastasis model. A549 cells with high incidence of lung metastasis were selected from the lungs of nude mice after the tail vein injection, as described elsewhere. We confirmed lung metastasis of the cells in 100% of animals after tail vein injection (data not shown). A549 cells seeded in tissue culture plates were treated with PBS, EV, or Ad-BP3 (50 pfu/cell). Two days later, the cells were harvested, washed, and resuspended at a density of 2×10^6 viable cells/100 μL of sterile PBS. Cells were injected into the tail veins of 6-week-old female nude mice. Seven mice were used in each of the two treatment groups (EV and Ad-BP3) and six in the control group (PBS). Four to 6 weeks after injection, animals were killed by CO_2 inhalation, and their lungs were excised. Lung tumor formation was observed, and tumor nodules were counted under a dissecting microscope.

For survival studies, two groups of seven athymic nude mice were injected with A549 cells infected with Ad-BP3 or EV (both 50 pfu/cell). A control group of six mice was given untreated A549 cells in PBS only. The lungs of each mouse were excised after they died without intervention and fixed in Bouin's solution for 24 hours at room temperature. On day 190, the remaining mice were killed and examined for lung metastasis as described above. The animal studies were done in the veterinary facilities at the M.D. Anderson Cancer Center in accordance with institutional guidelines.

Reverse Transcription-PCR

Total RNA was extracted from NSCLC and R^- cells using TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA for templates was synthesized from 1 μg of total RNA in a 50-μL reaction using the Taqman reverse transcriptase reagent according to the protocol provided by the manufacturer [Invitrogen; SuperScript First-Strand Synthesis System for reverse-transcription (RT-PCR)]. The reaction

was done at 42°C for 2 minutes and at 42°C for 50 minutes and then inactivated at 70°C for 15 minutes. After inactivation, the cDNA was aliquoted and stored at −20°C until use. RT-PCR was done by coamplifying the genes with the reference gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) using the cDNA templates and corresponding gene-specific primer sets. The gene-specific primer sequences were as follows: 5′-GAAGGGCGACACTG-CTTTTTC-3′ (sense) and 5′-CCAGCTCCAGGAAATGCTAG-3′ (antisense) for human *IGFBP-3*; 5′-CCAATTAGGAAGTG-TAAGCAGC-3′ (sense) and 5′-GCCAAGAAAGGGACATC-TATG-3′ (antisense) for human *uPA*; 5′-TGTCAGAACG-GAGGTGTATGCG-3′ (sense) and 5′-TGTTCTTCTGGTA-GATGGCTGC-3′ (antisense) for mouse *uPA*; 5′-CATGC-CAAGATGGATGTCATC-3′ (sense) and 5′-GAGGCAA-TGTACGTCGTCTTTC-3′ (antisense) for mouse *IGFBP-3*; 5′-ACGCCAATAAGTTCGTCCAC-3′ (sense) and 5′-TCCA-TCCTTGAGGGACTCAG-3′ (antisense) for *IGF-1R*; 5′-TCG-CCCATCATCAAGTTC-3′ (sense) and 5′-GTGATCTG-GTTCTTGTCC-3′ (antisense) for *MMP-2*; 5′-GGTGAAGG-TCGGTGTGAACGGATTT-3′ (sense) and 5′-AATGC-CAAAGTTGTCATGGATGACC-3′ (antisense) for human *GAPDH*; and 5′-TAAAGGGCATCCTGGGCTACACT-3′ (sense) and 5′-TTACTCCTTGGAGGCCATGTAGG-3′ (antisense) for mouse *GAPDH*. To avoid amplification of genomic DNA, the gene primers were chosen from different exons. PCR was carried out in a total volume of 25 μL containing 1 μL of cDNA solution and 0.2 μmol/L sense and antisense primers. The RT-PCR exponential phase was determined in 28 to 33 cycles to allow quantitative comparisons among the cDNAs developed from identical reactions. The thermocycler conditions used for amplification were 94°C for 6 minutes (hot start), 94°C for 45 seconds, 56°C to 60°C for 45 seconds, and 72°C for 1 minute. Control PCR was done in 26 or 27 cycles with 0.5 μL of cDNA solution to allow quantitative comparisons among the cDNAs developed from identical reactions with primers for GAPDH. Amplification products (8 μL) were resolved in 2% agarose gel, stained with ethidium bromide, visualized in a transilluminator, and photographed.

siRNA Transfection

For the IGF-1R and uPA knockdown experiments, 1×10^6 A549 or H1299 NSCLC cells were transiently transfected with 100 nmol/L (final concentration) anti-IGF-1R, anti-uPA, and nonspecific control siRNA using Oligofectamine (Invitrogen) according to the manufacturer's recommendations. After 12 hours of incubation, the cells were rinsed with PBS and maintained in RPMI 1640 supplemented with 10% FCS for an additional 48 hours before analysis. The extent of the specific gene knockdown was detected by Western blotting and RT-PCR analysis.

Statistical Analyses

The results of the *in vitro* studies were expressed as means ± SD or SE of at least three independent experiments done in triplicate. Student's *t* test was used to compare differences between groups. Median survival was compared with the Kaplan-Meier survival analysis. $P \leq 0.05$ was considered statistically significant for all experiments.

Results

Inhibition of Migration and Invasion of NSCLC Cells by IGFBP-3

To investigate whether IGFBP-3 inhibits NSCLC metastasis, we did *in vitro* migration and invasion assays in A549 and H1299 NSCLC cells, in which overexpression of IGFBP-3 was induced by an adenoviral vector (Ad-BP3). To exclude the antiproliferative effect of IGFBP-3 on NSCLC cells, we used low doses (10 or 50 pfu/cell) of Ad-BP3 that had no cytotoxic activities within 2 days (Fig. 1A), when the effects of Ad-BP3 on the migration and invasion of NSCLC cells were evaluated. As shown in Fig. 1B and C, migration (Fig. 1B) and invasion (Fig. 1C) of H1299 and A549 cells were significantly inhibited by the infection with Ad-BP3 (50 pfu/cell). In contrast, infection with EV had no significant effect on the migration and invasion of the cell lines. A representative result showing invasion of H1299 cells uninfected (*Con*) or infected with 50 pfu/cell EV or Ad-BP3 is shown (Fig. 1C, *bottom*). To exclude any artificial effects of Ad-BP3 on cancer cells induced by the adenovirus, we examined the effects of recombinant IGFBP-3 (rbBP-3) on invasion of H1299 cells. Incubation of the cells with 10 μg/mL of rbBP-3 did not induce cytotoxic effects but inhibited invasion of H1299 cells by 50% compared with untreated cells (*Con*; $P < 0.001$; Fig. 1D). Because epithelial-mesenchymal interactions have been suggested to contribute to cancer progression, we further tested whether the addition of rbBP-3 regulates the invasion-stimulating effect of human lung fibroblast cells on NSCLC cells. Conditioned medium obtained from human lung fibroblasts treated with rbBP-3 was placed into the lower compartment of the Transwell units, and an invasion assay was done. As shown in Fig. 1E, the H1299 cells in conditioned medium from rbBP-3-treated human lung fibroblasts showed a significant reduction in cell invasion when compared with the H1299 cells in the conditioned medium from untreated control lung fibroblasts. All of these findings provide *in vitro* evidence of anti-invasive activities of IGFBP-3 in NSCLC.

Inhibition of Lung Metastasis and Prolonged Survival of Nude Mice by IGFBP-3 in Experimental Lung Metastasis Model

Because IGFBP-3 suppressed the migration and invasion of NSCLC cells *in vitro*, we next used mouse lung metastasis model to investigate whether IGFBP-3 can inhibit metastasis of lung cancer cells *in vivo*. A549 cells pretreated with PBS, EV, or Ad-BP3 (50 pfu/cell) were injected into the tail veins of the mice. Six weeks later, the mice were killed, and the total number of visible lung tumor nodules per mouse was quantified. Lung metastases were present in 100% of the mice injected with PBS- or EV-infected A549 cells (Fig. 2A). In contrast, only 1 of 7 (14%) mice injected with Ad-BP3–infected cells developed lung nodules, which were significantly fewer in number (mean = 0 nodule) compared with control (8 nodules) or EV-infected (5.5 nodules) mice ($P < 0.001$). Similar results were observed in repeated experiments.

In a separate experiment, we investigated the survival of 6-week-old female nude mice i.v. injected with A549 cells pretreated with PBS, EV, or Ad-BP3. The animals were observed daily until their death, at which point their lungs were excised and stained with Bouin's solution before examination. Five of 6 (83%) mice injected with uninfected cells and 5 of 7 (71%) mice injected with EV-infected A549 cells died by day 140 (Fig. 2B). In contrast, all of the mice injected with Ad-BP3–infected cells were alive at day 140, and only one (14%) of the Ad-BP3–infected mice died with lung tumors during the next 50 days (at day 160). On day 190, all surviving mice were killed; their lungs were excised and stained with Bouin's solution; and the incidence of tumor nodules was examined macroscopically. As shown in Fig. 2C, the formation of nodules was abundant in the animals injected with control and EV-infected cells. In contrast, the lungs of the mice injected with Ad-BP3–infected cells showed no detectable tumors except in one animal, and that mouse developed substantially fewer lung nodules than did the mice from the other two groups.

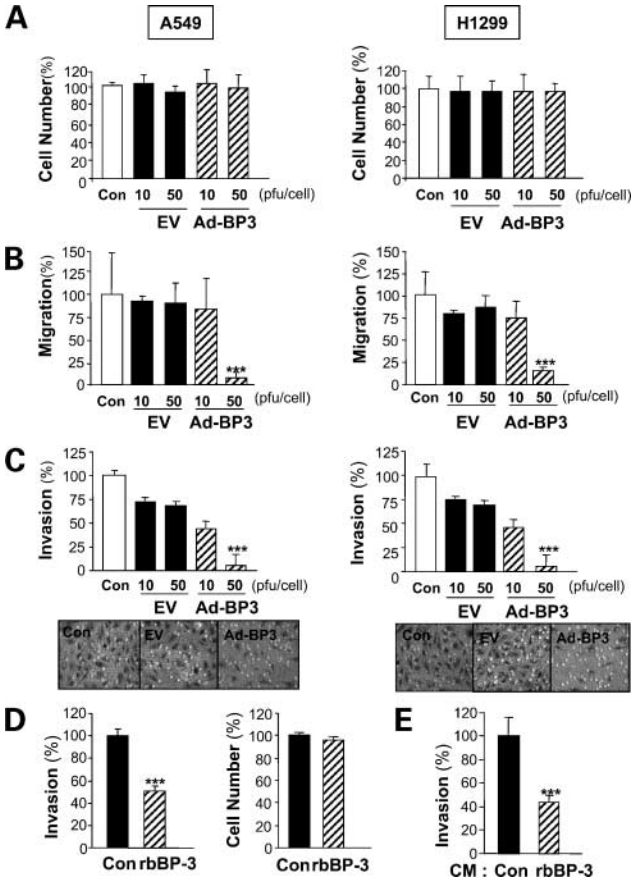

Figure 1. Effects of IGFBP-3 on viability, migration, and invasion of A549 (*left*) and H1299 (*right*) NSCLC cell lines treated with PBS (*Con*) or infected with Ad-BP3 or EV. For the effect of IGFBP-3 on viability of NSCLC cells (**A**, 48 h), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine numbers of cells uninfected or infected with EV or Ad-BP3. For the migration (**B**, 24 h) and invasion (**C**, 48 h) assays, the filters were coated with a solution of collagen IV (0.1 mg/mL) and Matrigel (50 μg/mL PBS), respectively. *Bottom,* representative H1299 and A549 cells that invaded through the Matrigel (**C**). H1299 cell invasion was also analyzed in the presence of rbBP-3 (**D**) or conditioned media (*CM*) obtained from overnight incubation of lung fibroblasts with 10 μg of rbBP-3 (**E**) in the lower compartment of the Transwell unit, respectively. *Columns,* mean percentages of three independent experiments done in triplicate; *bars,* SD. $P < 0.01$; ***, $P < 0.001$ compared with EV-infected cells (**B** and **C**) or with control cells (**D** and **E**).

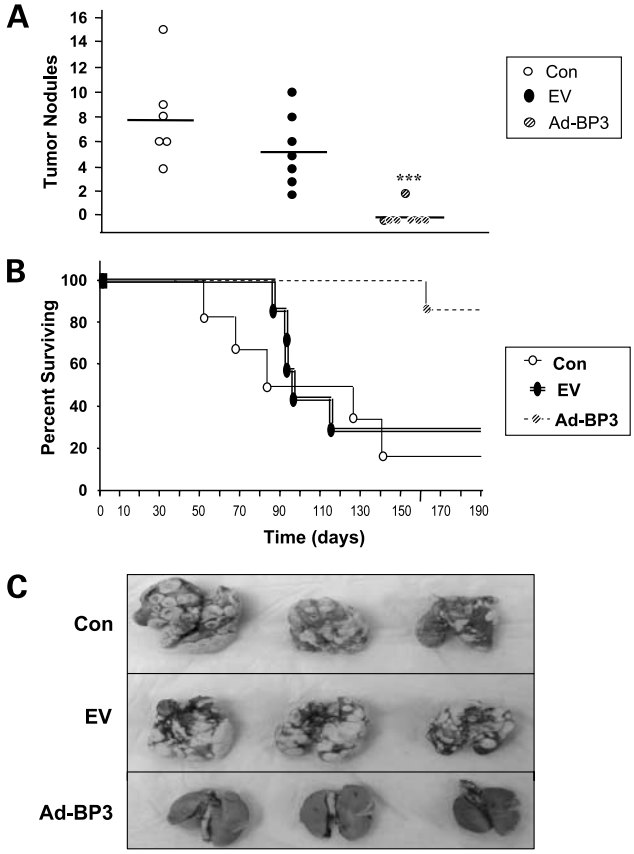

Figure 2. Effect of IGFBP-3 on metastasis of tumor cells and survival of mice bearing NSCLC cells using mouse lung metastasis model. **A,** nude mice were injected with A549 cells treated with PBS (*Con*; $n = 6$), EV (50 pfu/cell; $n = 7$), or Ad-BP3 (50 pfu/cell; $n = 7$). The total number of visible nodules per mouse was determined after 4 to 6 wks by counting under a microscope. *Points,* number of nodules per mouse; *bars,* median. ***, $P < 0.001$ compared with Con. **B,** Kaplan-Meier survival curves of nude mice bearing NSCLC cells expressing IGFBP-3. Nude mice were injected with A549 cells treated with PBS (*Con*; $n = 6$), EV (50 pfu/cell; $n = 7$), or Ad-BP3 (50 pfu/cell; $n = 7$). **C,** The lungs of each mouse was macroscopically examined after death or on day 190.

Role of uPA in IGFBP-3-Mediated Inhibition of NSCLC Cell Invasion

To understand the molecular mechanisms by which IGFBP-3 inhibits NSCLC cell invasion, we first analyzed the effects of Ad-BP3 on the uPA/uPAR system, which plays an important role in tumor cell invasion and metastasis (4–6). The results of ELISA using the conditioned media from H1299 and A549 cells indicated that infection with Ad-BP3 decreased the secretion of uPA from

the cells (Fig. 3A). Using the zymography analysis, we confirmed a considerably decreased uPA activity in the conditioned media from the Ad-BP3–infected H1299 and A549 cells (data not shown).

To test the effects of IGFBP-3 on uPA expression, Western blot analysis was done on EV- or Ad-BP3–infected H1299 and A549 cells. The levels of uPA B-chain decreased concomitantly with increases in IGFBP-3 expression in H1299 and A549 cells (Fig. 3B). rbBP-3 also induced a dose-dependent decrease in uPA expression (Fig. 3C). In contrast, the levels of uPAR protein were not affected by the rbBP-3 treatment.

We then investigated the specific role of uPA in antimetastatic activities of IGFBP-3 in NSCLC cells by the use of the synthetic inhibitor of uPA (uPA-STOP; ref. 19), uPA siRNA specific to uPA, and adenoviral vector expressing uPA (Ad-uPA; ref. 20). Regulation in uPA activity in the A549 and H1299 cells treated with uPA-STOP, transfected with uPA-specific siRNA, or infected with Ad-uPA were confirmed by zymogram analysis using conditioned medium (data not shown). We determined the effects of the treatment with uPA-STOP or transfection with uPA siRNA on the invasion of NSCLC cells. As shown in Fig. 4A, uPA-STOP–treated or uPA siRNA–transfected A549 and H1299 cells showed a significantly low level of invasion through the Matrigel-coated membrane compared with the untreated or scrambled (scr) siRNA–transfected cells. Moreover, overexpression of uPA by the infection with adenovirus expressing uPA (Ad-uPA) significantly increased invasion of the A549 and H1299 cells. Whereas, inactivation of uPA did not alter adhesion of A549 cells to extracellular matrix components, including collagen type I, fibronectin, and laminin (Fig. 4B), suggesting an important role of uPA in invasion, but not adhesion, of NSCLC cells.

To test the specific role of uPA in the inhibitory mechanism of IGFBP-3 in NSCLC cell invasion, Ad-uPA–infected H1299 or A549 cells were treated with rbBP-3. We found that the anti-invasive effects of rbBP-3 were obviously abrogated when the cells were infected with Ad-uPA– compared with control virus (EV)–infected cells (Fig. 4C). These data imply that the role of uPA in the action mechanisms of IGFBP-3 to suppress the invasion of NSCLC cells.

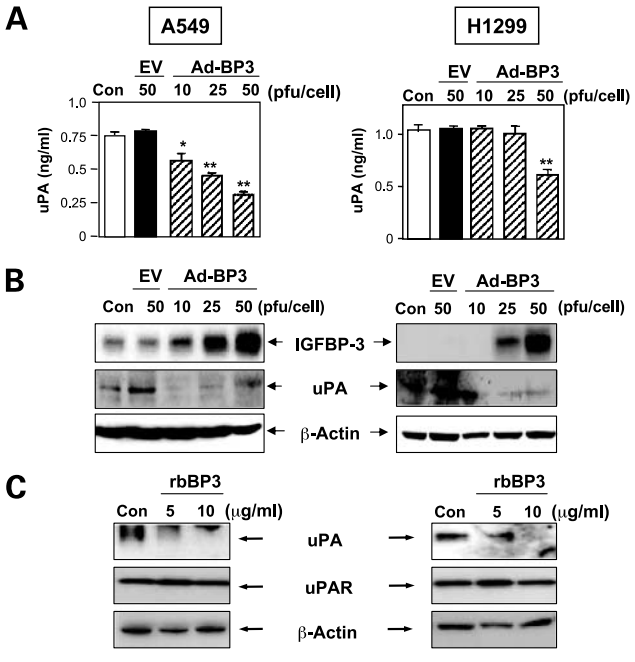

Figure 3. Effects of IGFBP-3 on uPA expression in NSCLC cells. The expression of uPA was determined by ELISA (**A**) and Western blot analyses (**B**), respectively, in lysates of A549 (*left*) and H1299 (*right*) cells. **C,** effects of rbBP-3 on uPA and uPAR expression in A549 (*left*) and H1299 (*right*) cell lines were examined by Western blotting. The cells were treated with PBS (*Con*) or infected with EV or Ad-BP3 at a concentration of 10, 25, or 50 pfu/cell for 48 h. *Columns,* mean secreted level of uPA protein of three independent experiments done in triplicate; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$ compared with EV-infected cells. β-Actin served as a loading control.

uPA-Independent Suppression of MMP-2 Activity by IGFBP-3 in NSCLC Cells

The plasminogen activation system is responsible for the extracellular matrix degradation at least in part via by stimulating MMP-2 activity (4). Moreover, IGF-1R pathway has been known to regulate MMP-2 expression (21). Hence, we hypothesized that the MMP-2 is involved in the anti-invasive effects of IGFBP-3 on NSCLC cells. To test this hypothesis, we explored the effects of IGFBP-3 on MMP-2 expression/activity by gelatin zymogram assay using conditioned media from the rbBP-3-treated cells. As shown in Fig. 5A, conditioned media from the rbBP-3-treated cells at concentrations >5 μg/mL showed a reduced amount of active MMP-2. Short staining of the gel showed decreases in the level of pro-MMP-2 expression (data not shown). However, there was no change in the expression/activity of MMP-9 after the rbBP-3 treatment (data not shown). We then tested the effects of MMP-2 inhibitor on the invasion of A549 and H1299 cells. As shown in Fig. 5B, treatment with MMP-2 inhibitor decreased the invasive potential of A549 and H1299 cells, indicating that regulation of MMP-2 expression/activity contributed at least in part to the anti-invasive effects of IGFBP-3 on NSCLC cells. Based on previous findings showing the implication of plasminogen activation system in the activation of MMP-2 activity (4), we reasoned that the inactivation of MMP-2 by IGFBP-3 could be caused by decreased levels of uPA activity. However, the gelatin zymography of the conditioned media from H1299 cells, in which uPA expression was knock downed or overly induced through transfection with siRNA (si-uPA) or adenoviral vector (Ad-uPA) revealed no detectable change in the expression/activity of MMP-2 (Fig. 5C). Moreover, overexpression of IGFBP-3 by the Ad-BP-3 infection consistently decreased MMP-2 expression/activity in the H1299 cells that were transfected with control (si-scr) or uPA siRNA (si-uPA). We had similar results with A549 cells transfected with si-uPA or Ad-uPA (data not shown). These results indicated that IGFBP-3 regulated MMP-2 expression/activity via uPA-independent mechanisms.

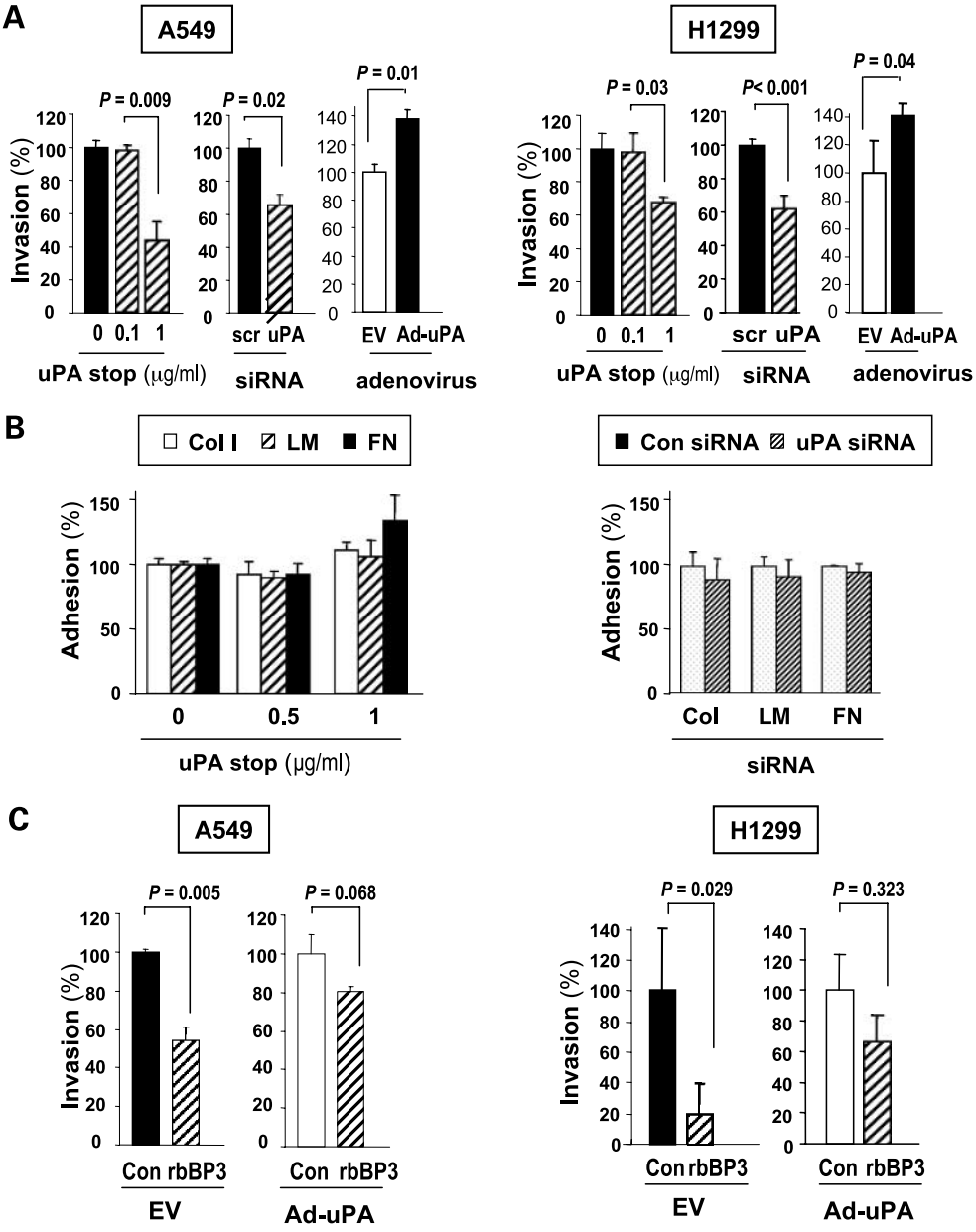

Figure 4. Role of uPA in IGFBP-3-mediated inhibition of NSCLC cell invasion. **A,** treatment of uPA inhibitor, knockdown of uPA gene by siRNA, or overexpression of uPA by Ad-uPA regulate A549 and H1299 NSCLC cell invasion. **B,** adhesion activity of A549 cells pretreated with uPA inhibitor for 1 d or transfected with uPA siRNA were measured by incubation of cells onto collagen type I (*Col*)–, laminin (*LM*)–, or fibronectin (*FN*)–coated well. **C,** A549 and H1299 cell invasion was analyzed after treatment of 10 μg/mL rbBP-3 following infection with EV or Ad-uPA (100 pfu/cell). *Columns,* mean cell invasion of three independent experiments done in triplicate; *bars,* SE.

Role of IGF-1R in Regulation of uPA and MMP-2 by IGFBP-3

We asked whether the effects of IGFBP-3 on the expression/activity of uPA and MMP-2 required IGF-1R using embryonic fibroblasts (R^-) from *Igf1r* knockout mouse. Both the biological activity of human IGFBP-3 in mouse and the high degree of sequence homology (81% amino acid homology) between human and mouse IGFBP-3 were previously verified (22). After the loss of IGF-1R expression was confirmed in the R^- cells (data not shown), semiquantitative RT-PCR analysis was done using RNAs from the *Ifg1r*$^{-/-}$ fibroblasts infected with EV or Ad-BP3 (5, 10, or 50 pfu/cell). As shown in Fig. 6A, Ad-BP3 infection decreased mRNA and protein expression of uPA in the R^- cells, indicating IGF-independent regulation of uPA mRNA expression by IGFBP-3. In contrast, mRNA expression of MMP-2 was not altered by Ad-BP3. The gelatin zymogram analysis of the conditioned medium also showed no obvious change in the level of pro- and active form of MMP-2 in the R^- cells after the Ad-BP3 infection, suggesting the IGF-dependent regulation of MMP-2 by IGFBP-3.

We further investigated whether the regulation of uPA and MMP-2 by IGFBP-3 required the IGF-mediated signaling pathway in H1299 cells, in which IGF-1R expression was knocked out by the use of IGF-1R siRNA. The transfection of IGF-1R siRNA effectively inhibited the expression of IGF-1R mRNA and protein but had no effect on GAPDH mRNA or α-tubulin protein expression (Fig. 6B). Infection with Ad-BP3

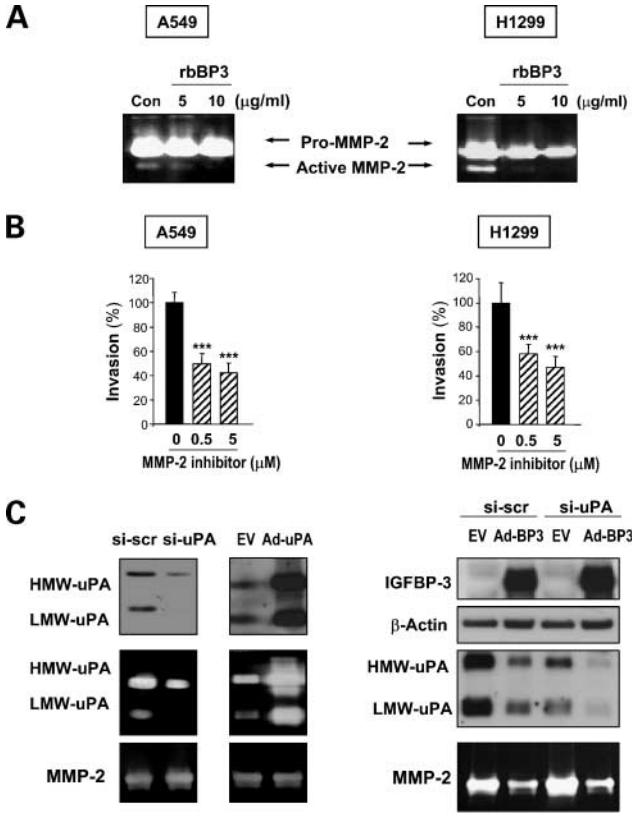

Figure 5. uPA system-independent inhibition of MMP-2 activation by IGFBP-3. **A,** effect of rbBP-3 on activation of MMP-2. The treatment with rbBP-3 inhibited formation of active MMP-2 in A549 and H1299 cells. **B,** invasion of A549 and H1299 cells were inhibited by the treatment of MMP-2 inhibitor. *Columns,* mean; *bars,* SE. ***, $P < 0.001$ compared with Con. **C,** uPA-independent regulation of MMP-2 expression/activation by IGFBP-3. H1299 cells were transfected with control (scr) or uPA siRNA or infected with EV or Ad-uPA. Otherwise, H1299 cells were transfected with control (scr) or uPA siRNA and then infected with EV or Ad-BP3. After an additional 2 d, expressions of IGFBP-3 and uPA were measured using Western blotting. uPA activity and MMP-2 expression/activation were determined by zymography. β-Actin was included as a loading control.

(10 or 50 pfu/cell) decreased uPA mRNA levels in the IGF-1R siRNA–transfected H1299 cells. Similar to the findings in the R^- cells, the levels of MMP-2 mRNA remained unchanged after the Ad-BP3 infection. These findings suggest that uPA expression might be regulated by IGFBP-3 in an IGF-1R-independent manner. Conversely, IGF-1R is essential for IGFBP-3-blocked MMP-2 expression and/or activation.

Discussion

We have shown for the first time that IGFBP-3 suppresses the invasive ability of NSCLC cells at least in part by inhibiting the expression/activity of uPA and MMP-2 through IGF-independent and IGF-dependent signaling pathways, respectively. This inhibition resulted in a decrease in lung cancer metastasis and an increase in survival of the mice injected with NSCLC cells.

Because IGF-1R signaling has long been known to be associated with cellular transformation and more recently with angiogenesis, invasion, and metastasis (23–28), we predicted that the IGF signaling pathway would offer a therapeutic target for lung cancer. We then reasoned that IGFBP-3, a natural IGF-binding protein, could be effective for blocking this pathway, for several reasons. First, IGFBP-3 has a major role in regulating the amount of free IGF-1 available to extravascular tissues. Second, clinical studies have identified high plasma IGF-1 levels as a potential risk factor for lung cancer (29). Third, loss of IGFBP-3 expression is a frequent abnormality in stage I NSCLC and is strongly associated with poor prognosis (30, 31). Finally, IGFBP-3 has shown antitumor activities in NSCLC by inhibiting IGF-induced pathways (18). Hence, we set out to determine the antimetastatic activities of IGFBP-3 in NSCLC.

Tumor metastasis involves enormously complex processes, including cancer cell release from the primary

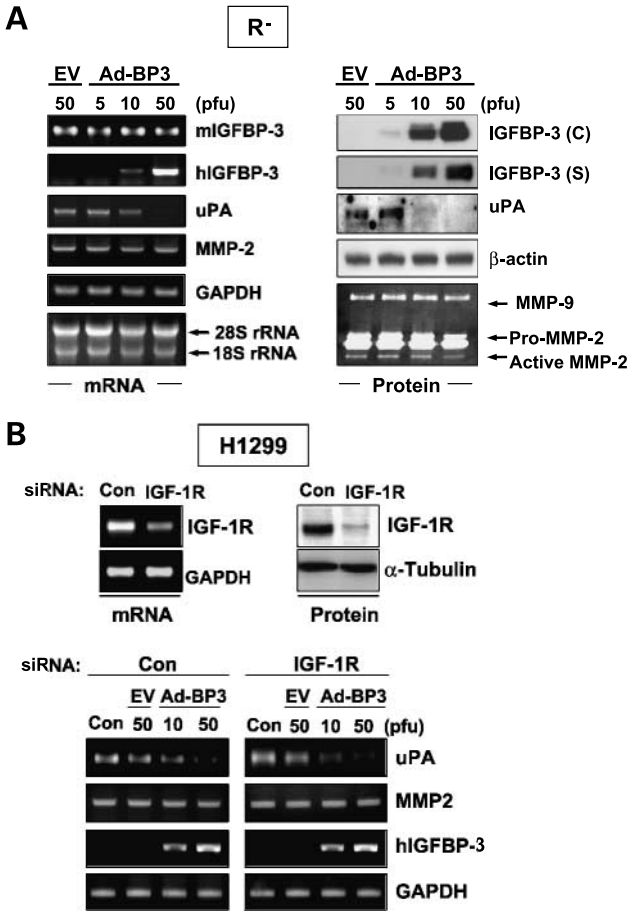

Figure 6. IGF-1R-dependent regulation of MMP-2 activation and IGF-1R-independent inhibition of uPA expression by IGFBP-3. **A,** effect of IGFBP-3 on expression of uPA, MMP-2, and MMP-9 in mouse embryonic fibroblasts derived from *Igf1r* knockout embryos. **B,** IGFBP-3 regulates uPA in H1299 cells even after transfection with IGF-1R siRNA. Levels of MMP-2, uPA, human IGFBP-3 (*hIGFBP-3*), and mouse IGFBP-3 (*mIGFBP-3*) mRNA were analyzed by semiquantitative RT-PCR after infection of the cells with EV (50 pfu/mL) or Ad-PB3 (5, 10, or 50 pfu/mL) for 2 d. GAPDH mRNA expression served as loading control. Levels of cellular (*C*) IGFBP-3, secreted (*S*) IGFBP-3, uPA, and IGF-1R were analyzed by Western blotting. β-Actin or α-tubulin was used as a loading control.

tumor and adhesion to extracellular matrix, invasion of the surrounding tissues, and movement into the lymphatic system or blood vessels. Those cells that survive within the circulation migrate into parenchymal tissue at distant sites and establish metastatic proliferation (32). Invasion into these distant sites requires degradation of extracellular matrix components, which may be mediated by uPA systems or MMPs (4, 33).

Our *in vitro* data provided several indications that IGFBP-3 has antimetastatic activities in NSCLC. First, overexpression of IGFBP-3 significantly reduced the ability of H1299 and A549 NSCLC cell invasion through Transwell membrane coated with Matrigel. Second, treatment of human lung fibroblasts with rbBP-3 suppressed its stimulating effects on NSCLC cell invasion through Matrigel. Finally, we confirmed the *in vitro* antimetastatic effects of IGFBP-3 using the mouse lung metastasis model. Injection of A549 NSCLC cells infected with adenoviral IGFBP-3 into nude mice resulted in significantly fewer lung tumor nodules compared with the mice injected with EV-infected cells. Furthermore, the mice injected with IGFBP-3-overexpressing NSCLC cells survived significantly ($P = 0.041$) longer than did control mice. Because the doses of IGFBP-3 used to suppress migration and invasion of NSCLC cells did not inhibit cell proliferation, the antimetastatic activities of IGFBP-3 were not likely due to cell cytotoxicity. These findings suggested the potential of IGFBP-3 as an antimetastatic therapeutic agent in NSCLC.

We further investigated the mechanism underlying the antimetastatic action of IGFBP-3 in NSCLC cells and found that IGFBP-3 inhibited the expression/activity of uPA and MMP-2 in A549 and H1299 cells. uPA and MMP-2 have a critical role in extracellular matrix degradation and invasion of cancer cells (34). MMP-2-deficient mice had 77% fewer colonies of Lewis lung carcinoma compared with wild-type mice (35). Moreover, injection of lung cancer cells expressing antisense uPAR reduced the incidence of lung metastasis in nude mice (6). Therefore, it was plausible that IGFBP-3-mediated antimetastatic activities were caused, at least in part, by decreases in the activity of uPA and MMP-2. In support of our hypothesis, inhibitors of uPA and MMP-2 significantly inhibited the invasive potential of A549 and H1299 cells with no effects on the adhesion of the NSCLC cells to extracellular matrix components, indicating direct involvement of uPA and MMP-2 in invasion, but not adhesion, of NSCLC cells.

We further investigated the mechanism of IGFBP-3-mediated regulation of uPA and MMP-2 expression and activity. Given the roles of the IGF-1R signaling pathway in regulating the expression/activity of uPA and MMPs (14, 21, 36), the inhibitory effects of IGFBP-3 on these proteins could have been the result of reduced availability of IGF. However, the regulatory effects of IGFBP-3 on uPA mRNA expression were unchanged in *Igf1r*$^{-/-}$ fibroblasts and in H1299 cells, in which IGF-1R expression was knocked down by transfection with IGF-1R siRNA, suggesting the presence of as yet unidentified IGF-independent mechanisms responsible for the IGFBP-3-mediated regulation of uPA expression. In contrast, the effects of IGFBP-3 on MMP-2 expression disappeared in the cells with loss of IGF-1R expression, indicating the dependence of IGFBP-3 on the IGF-1R signaling pathway for regulating MMP-2 expression/activity. Recent findings have suggested MT1-MMP in as a major mediator of MMP-2 activation; in this study, highly invasive Lewis lung carcinoma subline H-59 cells used MT1-MMP to activate their major extracellular matrix-degrading proteinase-MMP-2. When these cells were treated with IGF-I, the promoter of MT1-MMP was activated and MT1-MMP protein synthesis was augmented through IGF-1R (37). These finding indicated a potential involvement of MT1-MMP in the IGFBP-3-mediated regulation of MMP-2 in NSCLC cell lines tested in our study. Indeed, Ad-BP3–infected A549 cells showed an impaired expression of MT1-MMP (data not shown), suggesting that IGF-dependent regulation of MT1-MMP expression by IGFBP-3 could have caused a decreased MMP-2 activation. These findings present evidence that IGFBP-3 regulates expression and/or activity of uPA and MMP-2 through mechanisms that are either IGF dependent or independent.

The uPA/uPAR and plasminogen activator inhibitors have received extensive attention because their roles are not limited to their proteolytic activation of plasminogen to plasmin. uPAR has been shown to interact with and modify the function of β_1 and β_2 integrin, thereby inducing cell adhesion to vitronectin (38). Czekay et al. showed that the binding of uPA to uPAR promotes cell adhesion by increasing the affinity of uPAR to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (39). Thus, the inhibitory effects of IGFBP-3 on uPA expression could have caused a reduced adhesion of NSCLC cells to extracellular matrix components. However, we could not detect any change in the adhesion ability of H1299 or A549 cells, in which uPA expression or activity were blocked by the use of uPA siRNA or small molecule inhibitor, suggesting that uPA is not directly involved in the adhesion of NSCLC cells. However, given the environmental complexity of *in vivo* system, we cannot rule out the potential implication of uPA/PAR/plasminogen activator inhibitor-1 system in the NSCLC cell adhesion process.

In summary, we used *in vitro* and *in vivo* lung metastasis models to show for the first time that IGFBP-3 suppresses the expression and/or activity of uPA and MMP-2 through IGF-independent or IGF-dependent mechanisms resulting in the inhibition of invasion and metastasis of NSCLC cells. In light of the dual role of IGFBP-3 in regulation of uPA and MMP-2, manipulation of IGFBP-3 expression could also provide effective therapy for a variety of cancers. The importance of targeting uPA and MMP-2 for cancer therapy is supported by epidemiologic studies in which high levels of uPA and MMP expression are associated with metastasis and are correlated with poor prognosis in patients with various types of tumors, including lung cancer (40, 41). The ability of IGFBP-3 to down-regulate

MMP-2 is perhaps of additional significance because IGFBP-3 is degraded by MMP-2 (11, 42–44); thus, suppression of MMP-2 in tumor cells would result in even higher levels of inhibitory IGFBP-3. Although antimetastatic cancer treatment is still largely experimental, our studies provide a strong rationale for development of IGFBP-3 as a cancer therapeutic agent targeting cancer cell invasion and metastasis. Ongoing work is directed toward delineating the optimal protocol for clinical studies of the activity of IGFBP-3 observed *in vitro* and *in vivo*. Further studies are warranted to determine the mechanisms that mediate the IGF-independent regulation of uPA expression by IGFBP-3. In addition, because gene therapy is not going to be done in the way described in the current study, the effects of recombinant IGFBP3 on lung metastasis need to be tested in a physiologically more relevant mouse model of metastasis.

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