Adenovirus-mediated Antisense Urokinase-Type Plasminogen Activator Receptor Gene Transfer Reduces Tumor Cell Invasion and Metastasis in Non-Small Cell Lung Cancer Cell Lines


ABSTRACT

The urokinase-type plasminogen activator (uPA) and its receptor (uPAR) play an important role in the proteolytic cascade involved in the metastasis of lung and other cancers. We report that the reduction in uPAR levels produced by an antisense strategy using an adenovirus construct (Ad-uPAR) in H1299 cells, an invasive human lung cancer cell line that produces high levels of uPAR, resulted in a decrease of uPAR levels to 80–90% of those seen in cells infected with mock or adenovirus (Ad)-cytomegalovirus vector controls. In addition, increasing the multiplicity of infection from 25 to 200 caused a corresponding decrease in the level of uPAR protein within 5 days of treatment, as shown by Western blot analysis. Furthermore, the in vitro translation of total RNA levels of Ad-uPAR-infected H1299 cells in a rabbit reticulocyte lysate system caused a 50–70% decrease in uPAR immunoprecipitate in Ad-uPAR-infected cells relative to the levels in cells of mock and vector controls. The Matrigel invasion assay showed the invasion of H1299 cells and A549 cells infected with Ad-uPAR to be decreased by 70% relative to mock- and vector-infected controls. Infection of tumor cells with Ad-uPAR before implantation significantly reduced the incidence of lung metastasis by 85% as compared with the control virus-infected cells injected into nude mice through the tail vein. Our collective results show that the uPAR system is a potential target of treatment for lung cancers.

INTRODUCTION

Metastasis requires the dissolution of basement membranes and other extracellular protein structures, and this destruction has been attributed to the activity of proteolytic enzymes (1–3). Among the proteases implicated in tumor cell dissemination is uPA, a serine protease that converts plasminogen to plasmin on the surface of the cancer cells (4). Several studies have shown that uPA is localized on the cell surface through a high-affinity receptor (uPAR), a $M_r$ 55,000–60,000, glycosylphosphatidylinositol-anchored membrane protein (5, 6).

Both uPA and uPAR play a critical role in tissue remodeling in normal cells; production of these proteins, however, is seen in many invasive tumor cell lines and during tumor growth (7). In fact, the uPAR levels appear to be prognostically significant during the progression of tumors in breast, colon, prostate, lung, and brain (8–14). The receptor is particularly abundant in those areas where tumor cells are invading normal tissue (12, 13, 15).

The components of the plasminogen-activation system present in NSCLC have been studied by different techniques, and all of the components are present at different levels in lung cancer tissue (16). For example, uPA and uPAR mRNA were detected in the fibroblast-like cells present in confined fibrotic areas in adenocarcinoma, which suggests the involvement of the uPA system in the progression of lung fibrosis (17). In addition, high levels of uPA and uPAR receptor were detected in human NSCLC tissue (18). Of particular note from the standpoint of the study reported here is the finding that the uPAR level is of prognostic importance because it surges the degree of local proteolysis (11).

Because uPAR is a prognostic marker in NSCLC, we studied its role in the invasion and metastasis of human lung...
cancer cell lines after the administration of an adenoviral vector carrying antisense uPAR. We observed, both in vivo and in vitro models, that the down-regulation of uPAR in the lung cancer cell lines after adenoviral administration of antisense uPAR greatly diminished the invasive behavior of the cells and the formation of metastatic foci.

MATERIALS AND METHODS

The recombinant virus Ad-uPAR was obtained by cotransfection of an adenovirus vector, pAdΔE1sp1A, containing 300-bp of DNA at the 5’ end of the uPAR gene in the antisense orientation with the pJM17 vector into human embryonic kidney 293 cells, as described elsewhere (19). Ad-CMV was used as a control virus. It is identical to Ad-uPAR, except that it does not carry the antisense uPAR expression cassette.

Cell Lines and Infection Conditions. The human lung cancer cell lines H1299 and A549, which are migratory in vitro and metastatic in vivo, were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented with 10% FCS in a humidified atmosphere containing 5% CO2 at 37°C. Viral stocks were suitably diluted in serum-free medium to obtain the desired MOI or plaque-forming units, added to cell monolayers or tumor cell spheroids (1 ml/60-mm dish or 3 ml/100-mm dish), and incubated at 37°C for 30 min. The remaining necessary amount of culture medium was then added, and the cells were incubated for the desired times.

Western Blot Analysis. Total cell lysates were prepared in extraction buffer containing 0.1 M Tris (pH 7.5), Triton X-114 and protein A-agarose beads, which were subsequently washed on glass fiber filters and dried, after which the radioactivity was measured in a liquid scintillation counter and the immunoprecipitate band was resolved by SDS-PAGE (19).

In Vitro Translation. Total RNA was translated in a rabbit reticulocyte lysate system according to the manufacturer’s protocol (Promega Corp., Madison, WI). Sodium phosphate solution specific to uPAR (#399; American Diagnostica, Inc., Greenwich, CT) was used to immunoprecipitate the uPAR along with protein A-agarose beads, which were subsequently washed with glass fiber filters and dried, after which the radioactivity was measured in a liquid scintillation counter and the immunoprecipitate band was resolved by SDS-PAGE (19).

Measurement of the Incorporation of [35S]Methionine into uPAR. H1299 and A549 cells were infected with Ad-uPAR or Ad-CMV at increasing MOIs for 5 days. Cells were then starved overnight in methionine-free medium, after which 50 μCi of [35S]methionine (3000 Ci/mol) was added, and incubation continued for 16 h. The total amount of [35S]methionine incorporated was determined, and equal counts (5 × 106 cpm) from the Ad-CMV- and Ad-uPAR-infected cell extracts were used for immunoprecipitation with uPAR-specific polyclonal antibodies and protein A-agarose beads.

Animal Experiments. The human NSCLC tumor cell lines H1299 and A549 were used for determining the inhibitory effects of Ad-uPAR on the establishment of metastatic tumors. Briefly, H1299 and A549 tumor cells were infected with either Ad-uPAR or Ad-EV at 100 MOI in cell culture. Three days later, cells were harvested, washed with PBS, and resuspended in sterile PBS at a density of 1 × 106 viable cells/ml. Female severe combined immunodeficient/beige mice (for H1299) and female nude mice (for A549) were injected in the tail vein with Ad-uPAR- or Ad-EV-infected H1299 and A549 tumor cells. Eight animals were used in each group. Nine days after injection, animals were euthanized, injected intracardially with 15% India ink, and fixed in Fekete’s solution. Lung tumor formation was observed under a dissecting stereomicroscope, and the number of lung tumors was counted.

RESULTS

Ad-uPAR-stimulated Decrease in uPAR Protein Levels in H1299 Cells. Using Western blot analysis to study the effect of a replication-defective recombinant Ad-uPAR and Ad-
CMV in H1299 and A549 on the levels of uPAR protein, we observed that the uPAR protein band (M_r 55,000–60,000) was decreased in a dose-dependent manner (Fig. 1A). In addition, densitometry of the uPAR protein bands on the Western blots showed significantly decreased levels of protein in cells infected with Ad-uPAR at an MOI. Specifically, H1299 cells infected with Ad-uPAR at 100 MOI showed a 50% decrease in uPAR levels by day 3 and almost a 90% decrease by day 7 as compared with the levels in Ad-CMV-infected control cells (Fig. 1B). Quantitation of the uPAR synthesized in the system by immunoprecipitation with polyclonal antibodies specific for uPAR revealed a 70% decrease in the uPAR protein levels in Ad-uPAR-infected H1299 cells (Fig. 3B) as compared with the levels in cells infected with the control vector (P < 0.001). The addition of RNA from Ad-uPAR-infected cells to RNA from Ad-CMV-infected controls also inhibited the translation of uPAR protein.

**uPAR mRNA Levels after Infection of Lung Cancer Cell Lines with Ad-CMV and Ad-uPAR Lung Cancer Cell Lines.** Northern blot analysis with a cDNA probe labeled for uPAR was performed to determine whether Ad-uPAR infection affected the expression of uPAR mRNA. There was no significant change in the levels of uPAR mRNA in Ad-uPAR-infected H1299 and A549 cells compared with the levels in mock- and Ad-CMV-infected cells (Fig. 2). Densitometry of the uPAR mRNA band also showed no significant change.

**Ad-uPAR-stimulated Decrease in the Translation of the uPAR Protein.** Because of the decrease in the uPAR protein levels in H1299 and A549 cells infected with Ad-uPAR, despite the fact that there was no change in uPAR mRNA levels (Fig. 2), we studied the translation of RNA in an *in vitro* rabbit reticulocyte lysate system. The immunoprecipitated uPAR protein was much higher in Ad-CMV-infected H1299 and A549 cells compared with antisense Ad-uPAR and Ad-uPAR + Ad-CMV-transfected cell lines (Fig. 3A). Quantitation of the uPAR synthesized in the system by immunoprecipitation with polyclonal antibodies specific for uPAR revealed a 70% decrease in the uPAR protein levels in Ad-uPAR-infected H1299 cells (Fig. 3B) as compared with the levels in cells infected with the control vector (P < 0.001). The addition of RNA from Ad-uPAR-infected cells to RNA from Ad-CMV-infected controls also inhibited the translation of uPAR protein.

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**Fig. 1** A Western blot analysis of uPAR protein. A, H1299 cells infected with Ad-uPAR at increasing MOIs for 5 days and analyzed for uPAR protein on SDS-PAGE. B, H1299 cells infected with Ad-uPAR at 100 MOI for times indicated and analyzed for uPAR protein on SDS-PAGE. Densitometry of Western blot results was performed, and the data represent average values from four separate experiments; bars, SD. **Fig. 2** Northern blot analysis of uPAR mRNA levels after infection with Ad-CMV and Ad-uPAR in H1299 lung cancer cell lines. Total RNA was isolated, and 10 μg of RNA were electrophoresed in 1.2% agarose gels and blotted onto a nylon membrane. The membrane was hybridized with 32P-labeled uPAR cDNA specific for uPAR mRNA. After removal of the radiolabeled probe, the membrane blot was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to check the relative amounts of mRNA loaded onto the gel.
bars experiments; B, H1299 and A549 cells were infected with Ad-uPAR or Ad-CMV at 100 MOI for 5 days. The data represent average values from four separate A-Sepharose-4B beads at 4°C.

Fig. 3. uPAR protein translation. H1299 and A549 cells were infected with Ad-uPAR or Ad-CMV of 100 MOI for 5 days. The RNA from H1299 and A549 cells was extracted, and 10 μg were used for translation in 50-μl reaction volumes containing 35 μl of rabbit reticulocyte lysate according to the Promega protocol. Total incorporation of [35S]methionine was determined from a 2-μl aliquot of reaction mix by TCA (10%) precipitation. Equal counts (5 × 10⁶ cpm) were used for overnight immunoprecipitation of uPAR with polyclonal antibodies (American Diagnostica) and protein A-Sepharose-4B beads at 4°C. A, the immunoprecipitated uPAR protein band was resolved by SDS-PAGE as described in “Materials and Methods.” B, H1299 and A549 cells were infected with Ad-uPAR or Ad-CMV at 100 MOI for 5 days. The data represent average values from four separate experiments; bars, SD. * P < 0.001.

Inhibition of the Invasiveness of H1299 and A549 Cells by Ad-uPAR Infection. We used a Matrigel assay to study the invasion of lung cancer cells, one of the hallmark behaviors of this cancer. When cells were placed at a density of 0.5 × 10⁶ cells/ml in the upper chamber, the staining of Ad-uPAR-infected H1299 and A549 cells that invaded through the Transwell inserts precoated with reconstituted basement membrane Matrigel was significantly less intense than that of mock-infected and Ad-CMV-infected cells (Fig. 4A). This effect was dose dependent in Ad-uPAR-infected controls, but no change was seen in Ad-CMV-infected control cells. The 3-(4,5-dimethylthiazol-2-) -2,5-diphenyltetrazolium bromide assay (20) showed that 100% of mock-infected cells had invaded through the Matrigel. As shown in Fig. 4B, 60, 35, and 15% of H1299 cells infected at Ad-uPAR 25, 50, and 100 MOI, respectively, invaded through Matrigel when compared with controls (P < 0.001). A similar trend was observed for A549 cells, but the number invaded through the Matrigel was less when compared with H1299 (Fig. 4, C and D).

Inhibition of Metastasis in Mice by Ad-uPAR Infection. To examine whether the gene transfer of uPAR in the antisense orientation also influenced metastasis in vivo, H1299 and A549 cells infected with Ad-uPAR at different MOIs in vitro were implanted in severe combined immunodeficient/beige and nude mice, respectively. This led to a reduced incidence of metastasis to the lungs in both mouse models at 9 days, the earliest time when lung metastasis could be reproducibly detected in 100% of animals. As shown in Fig. 5, nodule formation was reduced in a dose-dependent manner in both A549 and H1299 cells. Fig. 5 shows a 60% reduction in tumor nodule formation in A549 cells infected at 25 MOI and >90% reduction in those infected at 100 MOI. Fig. 5 shows that H1299 cells infected with Ad-uPAR at higher MOIs exhibited a similar distinct decrease in the formation of metastatic foci in a dose-dependent manner (P < 0.001).

DISCUSSION

Recent studies from our laboratory have shown that the adenovirus-mediated gene transfer of uPAR in an antisense orientation into SNB19 cells did not produce tumors in nude mice (19). In addition, injection of the Ad-uPAR construct into previously established s.c. U87-MG tumors in nude mice caused regression of these tumors. These previous observations in other cancers were extended by the findings in the present study, which had as its aim determining the effects of this Ad-uPAR construct on the metastasis of two lung cancer cell lines in vivo and in vitro. In particular, infection of H1299 and A549 cells with Ad-uPAR effectively reduced the uPAR protein levels but did not change the uPAR RNA levels. In addition, immunoprecipitation with polyclonal antibodies specific for uPAR revealed a 70% reduction in the uPAR translation level in Ad-uPAR-infected H1299 cells relative to the level in Ad-CMV-infected controls. Furthermore, the addition of RNA from Ad-uPAR-infected cells to RNA from Ad-CMV-infected controls inhibited translation by ~60% relative to the vector-infected controls, and this was almost to the level seen in the Ad-uPAR-infected H1299 cells. These results were confirmed by the [35S]methionine incorporation into the uPAR protein. We propose that the arrest of hybridization during translation of the target protein is one of the major mechanisms of antisense action, apart from the degradation of RNA hybrids by specific nucleases (22). In the study reported here, we also examined the migration and invasion of the H1299 and A549 cells in response to Ad-uPAR infection. Migration of both these cell lines decreased in re-
sponse to Ad-uPAR infection. Invasion was decreased in vivo by almost 90% compared with the results in mock- or Ad-CMV-infected cells, and metastasis was decreased by >90%.

By promoting pericellular proteolysis and regulating cell adhesion and migration in a nonproteolytic fashion, the plasminogen activation system has a dual role in cell invasion. uPAR plays a central role in both these functions by engaging in complexes with β1, β2, and β3 integrins. In fact, uPAR has been shown recently to be a key facilitator of integrin signaling during states of accelerated cellular migration, such as during inflammation and tumorigenesis (23). uPA and uPAR were shown to be colocalized with the vitronectin receptor (24). More recently, (25) showed that the down-regulation of uPAR in such a scenario could disrupt the uPAR/integrin interaction and thereby reduce cellular migration. The binding of uPA to its receptor has also been observed to result in the de novo association of various intracellular proteins, such as nonreceptor tyrosine kinases, with uPAR (26–28) and uPA to initiate signal-transducing events (29, 30). Earlier, it was shown that uPAR serves as a cell-surface receptor for the extracellular matrix protein vitronectin by limiting PKC-dependent localization on αVβ3 to focal contacts (31). In addition, earlier work from our laboratory has shown that decreasing the uPAR levels using an antisense technique inhibited tumor cell invasiveness (20, 32).

Several components of the uPA system are potential targets for antiangiogenic, anti-invasive, and/or antimetastasis therapy, and various different approaches to interfering with the expression or reactivity of uPA or uPAR at the gene or protein level have proved successful (7, 33). For example, the down-regula-
tation of uPAR expression in response to antisense strategy produced a protracted period of dormancy in human epidermoid carcinoma cells (34), which was attributed to a low basal level of ERK1 and ERK2 in uPAR-poor cells (25). It is also possible, however, that this dormancy is attributable to the fact that the down-regulation of uPAR reduces ERK1 and ERK2 activity to a level less than that needed to sustain tumor growth in vivo. In another effort, the administration of a recombinant human uPAR antagonist resulted in increased latency of primary tumors and micrometastases from human tumor cells and drastically reduced the incidence of lung metastasis (35–37). In addition, Min et al. (38) reported that a mouse uPA-IgG recombinant uPAR antagonist inhibited angiogenesis in vitro and in vivo and increased the latency of primary mouse B16 BL6 tumors. Another possible target is the basic fibroblast growth factor, an angiogenic agent that is produced by endothelial cells in response to factors secreted by tumor cells and that in turn induces the production of uPAR and uPA in the endothelium (39). It has also been reported recently that the adenovirus-mediated delivery of a uPA/uPAR antagonist suppresses angiogenesis-dependent tumor growth and dissemination in mice (40). Conversely, reducing uPAR levels by using antisense oligonucleotides was also found to inhibit tumor growth, invasion, and metastasis in various cancers (20, 32, 41–43). With regard to safety, administering an antisense gene to uPAR may not have serious toxic consequences; transgenic mice lacking uPAR had no developmental defects (44). Down-regulating uPAR could have an added advantage over simply blocking the uPA-uPAR interaction because uPAR participates in cells independently of uPA (45). Taken together, our present data on Ad-mediated antisense therapy strongly support the therapeutic value of down-regulating the overexpression of uPAR in lung cancers.

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