Urokinase Receptor Antibody Can Reduce Tumor Volume and Detect the Presence of Occult Tumor Metastases in Vivo

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ABSTRACT

The serine protease urinary plasminogen activator or urokinase (uPA), produced in abundance by many malignancies, plays a key role in tumor cell invasion and metastasis. uPA is localized within the malignant cell milieu via its cell surface receptor [uPA receptor (uPAR)], which is expressed by tumor and tumor-associated cells. In the present study, we have used a syngeneic model of rat breast cancer to directly evaluate the role of uPAR as a diagnostic and therapeutic target in metastatic breast cancer. A polyclonal antibody against the ligand-binding NH2-terminal domain of rat uPAR (ruPAR) was developed. This antibody recognizes ruPAR by both immunofluorescence and Western blot analysis. Recombinant ruPAR and ruPAR IgG displaced the binding of 125I-labeled ruPAR IgG to rat prostate cancer cells (Dunning R3227 Mat Ly Lu) and breast cancer cells (Mat B-III) overexpressing ruPAR (Mat B-III-uPAR). ruPAR IgG also blocked the invasive capacity of these tumor cells in a dose-dependent manner. Mat B-III-uPAR cells were inoculated s.c. into the mammary fat pad of syngeneic female Fischer rats. On day 10 after tumor cell inoculation, animals were injected with 125I-labeled preimmune or ruPAR IgG and then sacrificed at timed intervals. Maximum 125I uptake was observed in primary tumors and in tissues commonly affected by tumor metastases (liver, spleen, lungs, and lymph nodes) at 12 h. Injection of 125I-labeled preimmune or ruPAR IgG into normal non-tumor-bearing animals resulted in minimal basal levels of uPAR expression and established the specificity of the ruPAR IgG. Similar results were obtained by Northern blot and PCR analysis of mRNA isolated from tissues of normal and tumor-bearing animals. To evaluate the effectiveness of this antibody in tumor progression, ruPAR IgG (50–100 µg/day) was injected s.c. for 7 days (day 1–7) at the site of tumor cell inoculation (mammary fat pad), and animals were sacrificed at various time points for evaluation of tumor growth and metastases. Animals receiving ruPAR IgG showed a marked decrease in tumor growth and metastases as compared with control tumor-bearing animals receiving the same dose of preimmune rabbit IgG. Histological analysis of experimental primary tumors showed marked tumor necrosis that was due to increased tumor cell apoptosis as determined by terminal deoxynucleotidyl transferase-mediated nick end labeling assay. Together, these studies demonstrate the ability of anti-uPAR antibody to decrease tumor volume and detect the presence of microscopic occult tumor metastases in malignancies where uPA/uPAR play a key role in tumor progression.

INTRODUCTION

The process of tumor invasion and metastasis is a multistep event that starts with the shedding of malignant cells from their primary site, transport through blood vessels, and seeding at distant organs, resulting in tumor-associated metastasis (1). These events in tumor progression are initiated after the degradation of the ECM by various proteolytic enzymes synthesized and secreted by tumor cells (2). Among these proteases are PAs that can convert the inactive proenzyme plasminogen to its active moiety plasmin, which plays a key role in clot lysis (3). This family of PAs consists of tissue-type PA and uPA, which are products of two separate genes and are involved in cell migration, growth, and invasion (4). However, uPA, produced by several tumor cells, is strongly implicated in tumor progression because of its ability to convert plasminogen to plasmin, which in turn activates procollagenases in cancer cells that can break down different components of the ECM and promote tumor invasion and metastasis (5). Increased uPA expression by a variety of human cancers, in particular breast, prostate, and colon cancer, has been shown to have a direct correlation with the higher invasive and metastatic potential of these malignancies. Determination of uPA levels has also proven to be a useful negative prognostic marker of disease progression (6).

In previous studies, we have demonstrated the abundant production of uPA and its NH2-terminal fragment by the human prostate cancer cell line PC-3 and identified it as a unique mitogen for cells of the osteoblast phenotype (7, 8). In gain of function experiments, overexpression of uPA by rat prostate Dunning R3227 cancer cells using gene transfer techniques resulted in increased tumor metastasis to several skeletal and extraskeletal sites. Due to the mitogenic and proteolytic effects of uPA on osteoblasts, these skeletal metastases were primarily of the osteoblastic variety (9).

More recently, we and others have isolated and characterized the cell surface receptor of uPA (uPAR) from human and rat cell lines (10, 11). This cysteine-rich, highly glycosylated receptor protein is linked to the cell surface through a glycophosphatidylinositol anchor (12). Several reports related to mRNA expression, enzyme kinetics, receptor binding, and cell invasion assays have demonstrated the key role of uPAR in the process of tumor cell invasion and metastasis (13). Taken together, these results demonstrate that increased expression of both uPA and uPAR by tumor and tumor-associated cells plays an important role in establishing malignant disease (14). Cells expressing elevated levels of uPA and its receptor are also associated with increased invasion in an in vitro model of chorioallantoic membrane (15). Furthermore, increased expression of uPAR on the surface of tumor cells allows for enhanced tumor cell contact with the surrounding matrix due to localization of uPA. This resulting plasminogen activation provides tumor cells with greater invasive and metastatic potential (16). Recent studies have provided compelling evidence that uPA, uPAR, and PAI-1 can serve as independent prognostic markers in patients with breast cancer (17, 18).

Due to species specificity of uPA/uPAR interactions, we have previously developed a syngeneic model of uPAR overexpression using the rat breast cancer cell line Mat B-III (Mat B-III-uPAR). These experimental cells have demonstrated a higher receptor binding and invasive capacity in vitro. Inoculation of Mat B-III-uPAR cells into the mammary fat pad of female Fischer rats resulted in the development of large tumors and tumor metastases to lymph nodes, liver, spleen, and kidney as compared with control animals inoculated with wild-type Mat B-III cells (16). In the present report, we have carried out a series of studies to detect tumor metastases and determine the bioavailability of ruPAR IgG. Toward these objectives, we have expressed the NH2-terminal region (aa 25–114) of rat uPAR in vivo.

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3 The abbreviations used are: ECM, extracellular matrix; uPA, urinary plasminogen activator or urokinase; uPAR, uPA receptor; ruPAR, rat uPAR; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PA, plasminogen activator; PAI-1, plasminogen activator inhibitor 1; aa, amino acid(s); %ID/g, the percentage of injected dose/g tissue.
Escherichia coli and used this recombinant protein to generate a polyclonal antirat uPAR antibody (ruPAR IgG). This antibody, which was previously shown to block rat smooth muscle cell migration, cell shape change, and cytoskeletal reorganization (19, 20), was evaluated for its ability to block tumor cell invasion and uPAR binding capacity in rat prostate (Mat Ly Lu) and breast (Mat B-III-uPAR) cancer cells in vitro. We also evaluated the ability of 125I-labeled ruPAR IgG to detect microscopic occult tumor metastases and examined whether uPAR IgG can block growth and metastasis of Mat B-III-uPAR cells in vivo.

MATERIALS AND METHODS

Cell and Cell Culture. The Dunning R3227 Mat Ly Lu rat prostate cancer cell line was obtained from Dr. T. J. Isaacs (Johns Hopkins School of Medicine, Baltimore, MD). Rat breast cancer cell line Mat B-III was obtained from American Type Culture Collection (Manassas, VA). Mat B-III-uPAR cells overexpressing uPAR were developed and maintained in culture as described previously (9, 16).

Antirat uPAR Antibody. Full-length cDNA encoding ruPAR was isolated from a rat osteoblast cDNA library (11), and a 271-bp cDNA fragment encoding aa 25–114 of rat uPAR was subcloned into the expression vector pTrcHis A (Invitrogen, San Diego, CA). Recombinant ruPAR protein was expressed and purified according to the manufacturer’s instructions. The amino acid sequence of recombinant ruPAR was confirmed using a PI 2090E Integrated microsequencing system (Beckman Instruments, Mississauga, Ontario, Canada) at Sheldon Biotechnology Center, McGill University. Rabbids were immunized s.c. at 4-week intervals with ruPAR at multiple (8–11) sites using Freund’s incomplete adjuvant (Sigma Chemical Co., St. Louis, MO) and bled 10 days after each immunization. The antisera used was obtained after the third boost. Immunoglobulin fraction (IgG) was purified from antisera using a CNBr-activated Sepharose 4B (Sigma Chemical Co.) column immobilized with rat uPAR (aa 25–114) protein according to the manufacturer’s instructions.

Indirect Immunofluorescence. The ability of this species-specific ruPAR IgG to recognize surface expression of ruPAR was examined in Mat Ly Lu and in Mat B-III-uPAR cancer cells using a protocol described previously (16). Sequentially, cells were incubated with primary rabbit IgG to ruPAR and goat anti-rabbit IgG conjugated to FITC. Pictures were taken at ×25 magnification using a Zeiss MC-63 microscope (16). In some experiments, Mat B-III-uPAR cells were treated with ruPAR IgG alone or in the presence of recombinant rat uPA (American Diagnostica, Greenwich, CT) or phosphatidylinositol phospholipase C (Roche Molecular Biochemicals, Laval, Quebec, Canada), according to previously described methods (16).

Western Immunoblot Analysis. Cellular protein extracts (Mat B-III-uPAR cells) and tissue protein extract (normal lung tissue) were obtained using a Tris-buffered saline-based lysis buffer (Tris-buffered saline, 20 mM EDTA, and 0.1% Triton X-100). Membranes were incubated with ruPAR IgG (1:1,000) diluted in Western antibody buffer, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:20,000). Enhanced chemiluminescence was used for detection according to the manufacturer’s specifications (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec, Canada) and exposed on XAR film (Eastman Kodak, Rochester, NY).

Receptor Binding Assay. Preimmune or ruPAR IgG (100 μg) was labeled with 1 μCi of 125I using the chloramine T method, yielding a specific activity of 0.8–1.0 μCi/μg protein (8). The free 125I was separated from the labeled IgGs on a Sephadex G-25 gel filtration column (Amersham Pharmacia Biotech) that was equilibrated and eluted with PBS containing 0.1% BSA.

Mat Ly Lu and Mat B-III-uPAR cells were plated in 24-well plates. After mild acid treatment (50 mM glucose and 10 mM NaCl [pH 3.0]), cells were incubated at 37°C in a final volume of 300 μl containing serum-free medium, 1 mg/ml BSA, 20 mM HEPES (pH 7.4), and 125I-labeled ruPAR IgG (100,000 cpm) in the presence or absence of increasing concentrations of competitor (ruPAR protein), as described previously (8, 16).

Matrigel Invasion Assay. The capacity of ruPAR IgG to block the invasiveness of Mat Ly Lu and Mat B-III-uPAR cells was tested by two-compartment Boyden chambers (Corning Costar Corp, Cambridge, MA) and basement membrane Matrigel (Becton Dickinson, Oakville, Ontario, Canada) according to previously established methods (16). Ten fields under ×100 magnification were randomly selected, and the mean cell number was calculated.

Animal Protocols. Inbred female Fischer rats weighing 200–250 g (Charles River, Inc. St. Constant, Quebec, Canada) were inoculated with Mat B-III-uPAR tumor cells as described previously (16). On day 10 after tumor cell inoculation, animals were injected with 125I-labeled preimmune or ruPAR IgG (25 μg, 25 μCi) via tail vein injection. Animals were sacrificed 0.5–96 h after injection, and the primary tumors were removed and evaluated for radioactivity uptake. The amount of radioactivity retained as compared with the amount injected was calculated and then used to determine the biodistribution of 125I-labeled ruPAR IgG, which is expressed as %ID/g of 125I-labeled ruPAR IgG or 125I-labeled preimmune IgG (21). Alternatively, 12 h after the injection of radiolabeled preimmune or ruPAR IgG, primary tumors and various organs (heart, liver, lung, spleen, kidneys, adrenals, muscle, bone, and lymph nodes) were removed, and total radioactivity uptake was monitored using a gamma counter. As a control, non-tumor-bearing animals were injected with 125I-labeled preimmune or ruPAR IgG.

To examine the effect of ruPAR IgG on tumor-bearing animals, ruPAR IgG (50–100 μg/day) was injected s.c. into the mammary fat pad of experimental animals (inoculated with Mat B-III-uPAR cells) from day 1 to day 7 after tumor cell inoculation. Control groups of tumor-bearing animals received 50–100 μg/day preimmune rabbit IgG as control.

All animals were monitored for the development of tumors for 2–3 weeks after tumor cell inoculation, and the tumor volume in control and experimental animals was calculated (16). At day 15 after tumor cell inoculation, animals were sacrificed, examined, and scored for the development of macroscopic metastases in various tissues. This evaluation was carried out by at least two independent scientists who were blinded to the study protocol. This allowed us to achieve maximum reliability despite the limited sensitivity of this methodology.

RNA Extraction and PCR Analysis. Total cellular RNA was isolated from normal and tumor-bearing female Fischer rat tissues by guanidinium thiocyanate-phenol-chloroform extraction (22), and 2 μg of total RNA were used for reverse transcription (11). A 358-bp portion of ruPAR was amplified using PCR primers corresponding to nucleotides 550–568 and 881–908 of the sequence. Glycerinaldehyde-3-phosphate dehydrogenase primers were used as a control. Results were quantified by densitometric scanning using the Gel Doc system (Bio-Rad, Mississauga, Ontario, Canada).

Histology. The analysis of tumor sections for tumor necrosis and apoptosis was performed using previously described techniques for H&E and TUNEL staining (23). All histological examinations were carried out by light and fluorescence microscopy using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set (Chromotechnology Corp., Brattleboro, VT). For quantitation of TUNEL-positive cells, three sections from each tumor were analyzed using NIH Image version 1.61 and expressed as integrated density/field of examination. All slides were interpreted by two independent investigators.

Statistical Analysis. Statistical analysis was done by one-way ANOVA or Student’s t test.

RESULTS

Characterization of ruPAR IgG. The ability of ruPAR IgG to recognize the cell surface receptor for uPA, abundantly expressed by Mat B-III-uPAR cells, was examined by immunofluorescence. Preimmune rabbit IgG failed to show any binding to ruPAR-expressing cells, as assessed by immunofluorescence (Fig. 1A). In contrast, incubation with ruPAR IgG (10 μg/ml) showed fluorescence reaction in Mat B-III-uPAR cells (Fig. 1B). Coincubation of Mat B-III-uPAR cells with ruPAR IgG in the presence of recombinant rat uPA or phosphatidylinositol phospholipase C, which can dissociate uPAR from the tumor cell surface, resulted in a significant reduction in fluorescence reaction as compared with cells incubated with ruPAR IgG alone. This further demonstrated the specificity of ruPAR IgG (data not shown). This antibody-receptor complex was seen on the cell surface, where uPAR is reported to be expressed. Similar results were obtained with ruPAR IgG in Mat Ly Lu cells (data not shown).
Western blot analysis showed the ability of anti-ruPAR IgG to detect the glycosylated and nonglycosylated forms of ruPAR in Mat B-III-uPAR breast cancer cells and in normal lung tissue (Fig. 1C). Coincubation of Mat B-III-uPAR tumor cell extracts in the presence of the purified rat uPAR protein used to generate the antibody neutralized the blotting of rat uPAR, demonstrating the specificity of this antibody (Fig. 1C). Western blot analysis done with preimmune IgG failed to show any bands relating to the uPAR protein (data not shown).

Receptor Binding of 125I-labeled ruPAR IgG. The capacity of our species-specific ruPAR IgG to interfere with the functional ability of uPAR was evaluated in a receptor binding assay. Total binding of 125I-labeled ruPAR IgG to Mat Ly Lu and Mat B-III-uPAR cells was determined. The addition of different concentrations (0.1–2.0 μg/ml) of unlabeled recombinant rat uPAR inhibited the binding of 125I-labeled ruPAR IgG in a dose-dependent manner in both Mat Ly Lu (Fig. 2A) and Mat B-III-uPAR (Fig. 2B) cells. A similar dose-dependent decrease in 125I-labeled ruPAR IgG binding was seen after the addition of different concentrations of unlabeled ruPAR IgG as compared with preimmune rabbit IgG (data not shown). ruPAR IgG was also able to block the binding of 125I-labeled rat uPA to both Mat Ly Lu and Mat B-III-uPAR cells in a dose-dependent manner (data not shown).

Effect of ruPAR IgG on Tumor Cell Invasion in Vitro. The effect on tumor cell invasion of ruPAR IgG binding to ruPAR was examined in Mat Ly Lu and in Mat B-III-uPAR cells using Matrigel invasion assay. After a 24-h incubation, both Mat Ly Lu and Mat B-III-uPAR cells were able to penetrate the basement membrane. Incubation of these cells in the presence of 100 μg/ml preimmune rabbit IgG did not cause any significant inhibition in the invasive capacity of these rat prostate and breast cancer cells (Fig. 3). In contrast, the number of cells invading through the basement membrane significantly decreased when these cells were treated with anti-ruPAR IgG (Fig. 3). These effects were found to be dose dependent: 50 and 100 μg/ml IgG inhibited Mat B-III-uPAR cell invasion by 40% and 85%, respectively. This decrease in tumor cell invasion occurs because the anti-ruPAR IgG is able to block the binding of uPA to its receptor (uPAR), resulting in reduced invasive capacity due to the inability of uPA to localize within the tumor cell milieu.

Receptor Binding of 125I-labeled ruPAR IgG in Vivo. To examine the specificity and time course of 125I-labeled ruPAR IgG uptake by primary and disseminated tumors in vivo, ruPAR IgG and preimmune IgG were labeled with 125I and injected via the tail vein into Mat B-III-uPAR tumor-bearing female Fischer rats on day 10 after tumor cell inoculation. Control and experimental animals were sacrificed at timed intervals (0.5–96 h), and the primary tumors were removed and evaluated for 125I uptake. The %ID/g of ruPAR IgG was highest in tumor-bearing animals 12 h after injection of 125I-labeled ruPAR IgG, after which the %ID/g declined for up to 96 h (Fig. 4).

We then examined the %ID/g of 125I-labeled ruPAR IgG in blood, primary tumors, tissues that are a common site of tumor metastases after s.c. inoculation of Mat B-III-uPAR cells (liver, spleen, lungs, and lymph nodes), and tissues that do not have metastatic involvement in this model (adrenal glands, bone, kidney, heart, and muscle). For these studies, 125I-labeled preimmune IgG was used as a control to determine the %ID/g of 125I in various organs in normal and in Mat B-III-uPAR tumor-bearing animals (Fig. 5). Minimal amounts of radioactivity were seen in adrenal glands, bone, and muscle. The
levels of $^{125}$I were slightly higher in the heart due to the presence of blood and in the kidneys due to the clearance of these labeled preimmune IgGs in the urine, which also showed high amounts of radioactivity (data not shown). These low and comparable levels of $^{125}$I preimmune IgG uptake in normal and tumor-bearing animals serve as a control for these studies. In contrast, significantly higher levels of %ID/g of ruPAR IgG were seen in the liver, spleen, lungs, and lymph nodes and in the primary tumor as compared with preimmune IgG (Fig. 5). Macroscopic tumor metastases were not observed in any organ when experimental animals were sacrificed on day 10 after tumor cell inoculation. However, higher $^{125}$I uptake was observed in lymph nodes, lungs, liver, and spleen at day 10, and these organ sites developed macroscopic metastases by day 15 after tumor cell inoculation. Thus the antirat uPAR IgG was able to detect occult metastases in liver, spleen, lungs, and lymph nodes that later progressed to macroscopic tumor metastases.

To evaluate the specificity and selectivity of this ruPAR IgG to recognize endogenous uPAR expressed in different tissues, $^{125}$I-labeled ruPAR IgG was injected via the tail vein into normal, non-tumor-bearing animals. No significant difference in $^{125}$I uptake as compared with tumor-bearing animals was seen in muscle, bone, adrenal glands, heart, and kidney (Fig. 5). In contrast, significantly lower levels of $^{125}$I uptake were observed in the liver, spleen, lungs, and lymph nodes of normal animals as compared with tumor-bearing animals (Fig. 5). Basal levels of $^{125}$I uptake, established by injecting $^{125}$I-labeled preimmune IgG into non-tumor-bearing animals, showed no significant difference from levels obtained by injecting $^{125}$I-labeled preimmune IgG into experimental, tumor-bearing animals (data not shown).

To further characterize and quantify endogenous uPAR expression as compared with that observed in tumor-bearing animals, tissues were removed from normal and experimental animals, and the expression levels of uPAR were determined by reverse transcription-PCR (Fig. 6). uPAR levels in liver, lung, and spleen were barely detectable in normal animals; however, due to the likely presence of microscopic tumor metastases at these sites by day 10 in tumor-bearing animals, uPAR mRNA expression was significantly higher. uPAR expression was even more prominent in the primary tumor and at the auxiliary and retroperitoneal lymph nodes (Fig. 6). Similar results were obtained by Northern blot analysis (data not shown).

**Effect of ruPAR IgG on Tumor Growth and Metastasis in Vivo.** Implantation of Mat B-III-uPAR rat mammary adenocarcinoma cells into the mammary fat pad of syngeneic female Fischer rats results in the development of tumors by day 9 after tumor cell inoculation. These mammary tumors show a linear increase in tumor volume for up to 3 weeks. Injection of different concentrations of preimmune rabbit IgG (50–100 μg/ml/day) into the mammary fat pad of tumor-bearing animals for 7 days did not result in any significant difference in tumor growth throughout the course of this study. In contrast, injection of 100 μg/day ruPAR IgG from day 1 to day 7 after tumor cell inoculation resulted in a significant decrease in tumor volume in these experimental animals throughout the course of this study (Fig. 7). This decrease in tumor volume was more pronounced in the later stages (day 15–day 21), when animals receiving ruPAR IgG experienced not only a decrease in tumor growth but also experienced tumor regression (Fig. 7). A lower dose (50 mg/day) of ruPAR IgG showed a similar but less pronounced decrease in tumor volume as compared with control animals (data not shown). To determine the effects of ruPAR IgG on tumor metastasis, control tumor-bearing animals injected with preimmune rabbit IgG and experimental animals receiving ruPAR IgG were sacrificed at day 15 after tumor cell inoculation. Control animals reproducibly developed large macroscopic tumor metastases to auxiliary, retroperitoneal, and mesenteric lymph nodes. Evidence of macroscopic tumor metastasis was also seen in lungs, liver, and spleen (Table 1). In contrast, tumor-bearing animals receiving ruPAR IgG showed significantly smaller metastatic foci at retro-
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DISCUSSION

The role of receptor-bound uPA in promoting tumor cell invasion and metastasis has been well established in a number of experimental studies (12, 13). In these malignancies, uPAR plays a key role not only in localizing uPA activity but also in mediating various signaling events essential for the differentiation and migration of cells within the tumor environment (16, 24). The proteolytic actions of uPA can be blocked by natural and synthetic inhibitors of uPA catalytic activity as well as by anti-uPA antibodies, leading to inhibition of tumor progression (25, 26). Similar effects were observed when uPA was displaced from its receptor or when its proteolytic active site was blocked by chemical antagonists. The promising results obtained in these studies have prompted efforts to develop novel anticancer therapeutic strategies directed against uPA-uPAR interactions. These strategies would have critical impact on both the proteolytic cascade initiated by uPA and on various signaling pathways mediated by uPAR. Targeting of the uPAR has the added advantage of not only blocking uPA localization but also interfering with its ability to induce a variety of downstream signaling events as well as interactions with integrins, vitronectin, and PAI-1.

Prostate cancer and breast cancer are the two most common human cancers that result in a high degree of morbidity and mortality, mainly due to metastases of these tumors to various distal organs and particularly to bone (27, 28). A major problem in the treatment of these malignancies is the inability to detect and treat occult metastatic foci. These distal lesions are often refractory to conventional therapies and continue to grow, resulting in the high rates of mortality associated with metastatic prostate and breast cancer. We have previously demonstrated the importance of uPAR in the progression of metastatic breast disease using a syngeneic rat model of breast cancer (16). Similar results have recently been demonstrated in a human gastric peritoneal and mesenteric lymph nodes, without any evidence of tumor metastases in lungs, liver, or spleen (Table 1).

**Effect of ruPAR IgG on Tumor Necrosis and Apoptosis.** After a progressive increase in tumor growth, most tumors, including Mat B-III-uPAR, undergo spontaneous tumor necrosis. Control tumors exhibited tumor necrosis in their core zones as determined by H&E staining (Fig. 8A, i). However, despite the significantly smaller volume of experimental tumors, this zone of tumor necrosis was significantly higher in animals receiving ruPAR IgG (Fig. 8A, ii). TUNEL staining of sections from tumors of control animals receiving preimmune IgG exhibited few TUNEL-positive cells (Fig. 8A, iii), whereas tumors from experimental animals receiving ruPAR IgG showed extensive tumor cell apoptosis (Fig. 8A, iv). This suggests that the decrease in tumor volume and the observed necrosis of the primary tumor were due to tumor cell death. Quantification of TUNEL-positive cells showed a significant increase in experimental animals receiving ruPAR IgG (Fig. 8B).

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*Ax., auxiliary; R.P., retroperitoneal; Mes., mesenteric.*

Table 1 Effect of antirat uPAR on tumor metastases

Control tumor-bearing animals injected with preimmune rabbit IgG and experimental animals receiving 100 μg/ml ruPAR IgG were assessed. All control and experimental animals were sacrificed at day 15 after tumor cell inoculation and examined for the presence of macroscopic tumor metastases. The total number of metastatic foci was counted and compared. ND, tumor metastases not detected. Results represent at least three animals in each group in three different experiments.

Fig. 6. mRNA uPAR and glyceraldehyde-3-phosphate dehydrogenase expression levels in normal animals as compared with Mat B-III-uPAR tumor-bearing animals. mRNA extraction and PCR analysis were done as described in "Materials and Methods." mRNA expression levels in non-tumor-bearing animal tissues (liver, lung, and spleen) were compared with those observed in experimental animals (liver, lung, spleen, tumor, auxiliary lymph nodes, and retroperitoneal lymph nodes). Gels were quantified by densitometric scanning (bottom panel). Results are the mean ± SE of six animals in each group, in three different experiments. Significant difference in uPAR expression is represented by an asterisk (*, P < 0.05).

Fig. 7. Effect of ruPAR IgG on Mat B-III-uPAR tumor volume. Tumor volume was determined at timed intervals as described in "Materials and Methods." Tumor volume of experimental animals after treatment with 100 μg/day ruPAR IgG was compared with the tumor volume of control animals receiving 100 μg/day preimmune rabbit IgG. Results represent the mean ± SE of six starting animals in each group in four different experiments. Significant differences from control tumor-bearing animals at each time point are represented by asterisks (*, P < 0.05).
cancer, in which uPAR has been shown to be a marker of the metastatic phenotype (29). In the present study, we evaluated the ability of a neutralizing polyclonal anti-uPAR antibody to inhibit invasion of rat prostate (Mat Ly Lu) and breast (Mat B-III-uPAR) cancer cell lines in vitro and its capability to decrease Mat B-III-uPAR tumor growth and metastasis in vivo. Mat B-III-uPAR cells have a distinct advantage over wild-type cells because they closely mimic the subpopulation of patients in whom uPAR is overexpressed, leading to poor prognosis (30). For these studies, a species-specific, antirat uPAR IgG was developed that interacts with tumor cell surface uPAR and inhibits the binding of rat uPA to this receptor. In addition, the antibody was developed against a fragment of rat uPAR that spans both domains 1 and 2 and thus has the potential to block uPAR binding by steric hindrances or by interfering with other activities of uPAR that are downstream of uPA binding, such as its interaction with matrix or integrins (31, 32). Due to these interactions, we and others have observed greater than expected levels of nonspecific binding of 125I-labeled uPAR IgG (32). The net result of dissociation of uPA/uPAR by this antibody is a potent inhibition of tumor cell invasion in vitro due to its ability to prevent uPA localization to the tumor cell surface and inhibit the activation of uPAR-dependent signaling pathways requiring uPA-uPAR interaction. Due to the polyclonal nature of this antibody, we were unable to segregate the activities to specific epitopes of uPAR. However, the observed effects are most likely to be mediated via the uPAR because preimmune IgG from the same animals failed to have any effect on Mat Ly Lu or Mat B-III-uPAR invasion.

In addition to inhibiting tumor cell invasion in vitro, we used the ruPAR IgG to demonstrate that only tumor-bearing tissue localized this antibody. These findings confirmed our previous studies demonstrating that uPAR expression was specific to tumors at primary and metastatic sites. Biodistribution studies with 125I-labeled uPAR IgG in primary tumors were time dependent, with a maximal uptake occurring 12 h after infusion of 125I-labeled ruPAR IgG, which allowed ample time to occupy uPAR sites located on tumor cell surface and block uPA/uPAR-mediated events. Of the various organs examined for the presence of 125I-labeled ruPAR IgG, localization was observed only in organs known to be metastatic sites in this model of breast cancer. However, careful inspection of these organs failed to demonstrate any evidence of macroscopic metastases. Development of mac-
scopic metastases at these sites at later time points not only confirmed these organs as preferred sites of tumor metastases in this model but also demonstrated the ability of the 125I-labeled ruPAR IgG to localize to occult metastatic foci already present in these organs. Detection of these metastatic sites was not otherwise possible in whole animals because it would involve intentionally tagging the tumor cells, a strategy that is not possible in the clinical situation. Increased expression of uPAR at the metastatic sites could be due to injury or neovascularization in the presence of tumor cells, events that are closely followed by the development of tumors. Results from these studies demonstrate the potential clinical application of uPAR-based strategy for detection of occult metastatic foci in the diagnosis and follow-up of patients with malignancies.

Due to the close interaction of different components of this PA system, the components can serve as independent markers of disease progression and can also present as attractive alternative therapeutic targets due to the ability of uPA and PAI-1 to affect tumor angiogenesis. The significance of this system in tumor progression is further supported by a series of studies carried out in uPA PAI-deficient mice that have clearly demonstrated that production of host uPA produced by stromal cells and PAI-1 plays an important role in tumor cell invasion, growth, and vascularization (33, 34). Whereas uPA promotes tumor progression due to its ability to breakdown ECM, uPAR can not only localize these effects within tumor cell environment but can also interact with integrins to enhance tumor cell adhesion and activate intracellular signaling pathways. All of these events can collectively enhance the invasive and metastatic potential of cancer cells (35, 36).

In studies related to evaluation of the capacity of uPAR to block tumor progression, ruPAR IgG decreased tumor growth in a dose-dependent manner. Failure of the preimmune IgG to alter tumor growth under identical experimental conditions provided compelling evidence that the observed effects are due to targeting of uPAR by our antibody. These effects were due to the ability of ruPAR IgG to interfere with uPA-uPAR interaction in the early stage of tumor development and resulted in the sustained decrease in tumor volume at later time points. Additionally, these effects can be mediated through immune system activation, coating of tumor cells with ruPAR IgG, T cells, natural killer cells, or macrophages in host animals. Furthermore, other pathways that could be involved include the blocking of the antiapoptotic and angiogenic effects of uPA, which will lead to bimodal effects of ruPAR IgG in reducing tumor volume, as seen in this study. This was also observed in our previous studies, where an 8-mer uPA-derived peptide decreased breast cancer growth by blocking tumor angiogenesis and promoting tumor cell apoptosis in vivo (23). For these in vitro studies, we used a high concentration of ruPAR IgG to overcome low bioavailability, dissociation, permeability, and local catalysis of this antibody at various organs that can reduce the amount of bioavailable ruPAR IgG. This treatment strategy was preventative in nature, and additional studies in other tumor models will allow us to determine the ability of these agents to detect and eradicate tumor cells once they have established growth at primary and metastatic sites. Examination of various organs in this study also demonstrated the ability of the ruPAR IgG to delay the development of tumor metastasis to liver, spleen, kidney, and lymph nodes. These findings are particularly significant because previous studies using an anti-uPA IgG inhibited spontaneous but not experimental metastasis (25). Additional studies aimed at comparing this strategy with NH2-terminus, terminal fragment toxins, peptide antagonists, and antisense technology directed at the uPAuPAR system will directly evaluate the clinical benefits of this approach.

Our studies clearly demonstrate the usefulness of uPAR as a suitable cancer target for both therapeutic and diagnostic applications. In addition, we have specifically demonstrated the significance of uPAR in the progression of metastatic disease. Future studies using epitope-specific monoclonal antibodies in xenograft models of human cancers should further validate uPAR as both a marker of the metastatic phenotype and a therapeutic target for the treatment of primary and metastatic disease.

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