Urokinase Overproduction Results in Increased Skeletal Metastasis by Prostate Cancer Cells in Vivo

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ABSTRACT

We previously reported that urokinase (uPA) is produced by the human prostate cancer cell line, PC-3, and could function as a growth factor for cells of the osteoblast phenotype. To examine the role of uPA in metastasis to the skeleton and to extraskeletal sites, we have developed a homologous model of uPA overexpression in a rat prostate cancer cell line. Full length cDNA encoding rat (r) uPA was isolated and subcloned as a homologous model of uPA overexpression in a rat prostate cancer cell line. Experimental clones expressing at least 5-fold higher (pYN-ruPA) or 3-fold lower (pYN-ruPA-AS) than controls were selected, and control and experimental cells were inoculated into the left ventricles of inbred male Copenhagen rats. Animals were sacrificed at timed intervals to examine the evolution of metastatic lesions. Control animals developed metastases to the lumbar vertebrae resulting in spinal cord compression and hind limb paralysis at 20–21 days postinoculation. Animals inoculated with cells overexpressing uPA developed hind limb paralysis significantly earlier (by day 14–15 postinoculation). Additionally, more widespread skeletal (ribs, scapula, and femora) metastases were seen. Serum from experimental animals showed a progressive elevation in alkaline phosphatase levels, and histological examination of lumbar metastases revealed markedly increased osteoblastic activity over that observed in control animals. In contrast to this, animals inoculated with cells underexpressing uPA developed hind limb paralysis significantly later (days 25–29 postinoculation) and displayed decreased tumor metastasis. These studies support a role for the catalytic domain of uPA in enhancing both skeletal and nonskeletal prostate cancer invasiveness and are consistent with a role for the growth factor domain of uPA in mediating an osteoblastic skeletal response.

INTRODUCTION

Adenocarcinoma of the prostate represents one of the leading causes of cancer death in males. This high rate of mortality is associated with widespread metastatic disease within the first year of diagnosis of stage D patients (1). Prostate cancer is unique among cancers due to its high association with osteoblastic rather than osteoclastic lesions (2). In previous studies, we have demonstrated the presence of a peptide mitogen with growth factor-like effects for cells of the osteoblast phenotype in the conditioned culture medium of the human prostate cancer cell line, PC-3, and identified it as the amino-terminal fragment of human uPA (3). These effects of uPA were localized within its receptor binding growth factor domain encompassing amino acids 4–43. Furthermore, within the human molecule, fucosylation at Thr18 was shown to be essential for these effects (4). uPA is a member of the serine protease family which also includes tPA. These proteins are products of 2 discrete genes and are expressed in several normal cell types (5, 6). The function of tPA is primarily related to intravascular thrombolysis, whereas uPA is thought to be involved in generation of pericellular proteolysis during cell migration and tissue remodeling. Plasminogen activators also play an important role in neoplastic processes and higher levels are found in malignant tumors (7). Of particular significance is the fact that in these cases the plasminogen activator produced is predominantly uPA, although tPA is secreted by a few malignant cell types as well (8, 9). In these cancers, uPA promotes tumor cell migration and invasion by converting the inactive zymogen plasminogen into the active serine protease, plasmin, which then cleaves extracellular matrix components including laminin, fibronectin, and collagen (10). It has been suggested that uPA-dependent proteolysis is contingent on uPA being bound to the specific cell surface receptor on these uPA-producing cells (11, 12). Stimulation of tumor cell growth may also occur and be due to the growth factor-like effects of uPA or due to its capacity to activate latent transforming growth factor-β (13).

In the present study, we have examined the role of uPA in metastases associated with prostate cancer. We have studied this in our recently developed model of rat prostate cancer in male Copenhagen rats (14, 15). In this model, intracardiac injection of rat prostate cancer cells (Mat LyLu) results in the development of a metastatic pattern which appears to mimic the spread of human prostatic cancer. We have used this paradigm and gene transfer methods to develop a homologous system of prostate cancer which overexpresses or underexpresses uPA to determine the influence of this molecule on the nature and extent of organ-specific colonization by prostate cancer.

MATERIALS AND METHODS

Plasmid Preparation. The expression vector pYN-ruPA was constructed by cloning the entire coding region of rat ruPA cDNA (amino acids 20–21 to +1412) into a 1.4-kilobase Xhol-BspHI fragment derived from the ruPA clone-3 into the poly linker region of puc-1318 (a puc derived vector) (16, 17). From there it was subcloned as a BamHI insert into the unique BamHI cloning site of the 9.9-kilobase Moloney murine leukemia retroviral vector pYN (18) (Fig. 1). In this construct, the insert expression is driven by the Moloney murine leukemia virus long terminal repeat promoter. The selectable marker, neo, is driven by an internal herpes simplex thymidine kinase promoter. Clones containing the insert in the sense and antisense orientation were selected to generate the experimental plasmid pYN-ruPA and pYN-ruPA-AS, respectively.

Cells and Cell Culture. The control (pYN) and experimental (pYN-ruPA and pYN-ruPA-AS) plasmids were each transfected into the ectopic packaging cell line, GF+E86 (19), by electroporation. Cells were selected in G418 (600 μg/ml) and supernatants from these cells were filtered and added to the rat prostate cancer cell line Mat LyLu in the presence of 4 μg/ml Polybrene. Cells were again selected in G418 for 10 days, and resistant colonies were isolated and allowed to grow as monoclonal cell populations. Clones express-
with pYN-ruPA (uPA) and pYN-ruPA-AS (uPA-AS) were electrophoresed on a 1.1% agarose/formaldehyde gel. Filters were probed with a 32P-labeled ruPA antisense ribo probe or with a 3P-labeled cyclophillin cDNA probe as described in "Materials and Methods." "pYN-uPA" indicates the position of ruPA transcribed from the vector, whereas "uPA" indicates the position of endogenous uPA.

The Dunning R3327 Mat LyLu cell line was obtained from Dr. J. T. Isaacs (The John Hopkins School of Medicine, Baltimore, MD). Cells were maintained in vitro in RPMI 1640 supplemented with 2 mM L-glutamine (MA Bioproducts), 5% fetal bovine serum, 100 units/ml of penicillin-streptomycin sulfate (Gibco), 250 nM dexamethasone, and 0.2% gentamicin (Sigma).

Animal Protocols. Intact male Copenhagen rats weighing 150–200 g were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Before inoculation, control and experimental Mat LyLu tumor cells grown in serum-containing medium were washed with Hanks' buffer and trypsinized for 5 min. Cells were then collected in Hanks' buffer and centrifuged at 1500 rpm for 5 min. Cell pellets (10–50 × 10^6 cells) were resuspended in 200 μl saline and injected using 1-ml insulin syringes into the left ventricle of rats anesthetized with ketamine/sodium pentobarbital (1:2). Animals were maintained in standard animal cages with food and water provided ad libitum. They were weighed every third day to monitor their growth and survival. When animals were sacrificed, they were weighed and their tumors were excised to determine their growth rates. Tumors were then weighed and measured to determine their volumes. The tumors were then fixed in 10% formalin and embedded in paraffin for histological examination.

Histological Examination of Metastases. Skeletal tissue (vertebrae, ribs, femora, scapulae, and sternum) which was removed from control and experimental animals was fixed in 4% formaldehyde in phosphate buffered saline for 48 h and decalcified in Cal-Ex decalciﬁcation solution (Fisher Scientiﬁc). After dehydration through graded concentrations of ethanol up to 100%, samples were cleared in xylene and embedded in paraffin. Sections (5 μm thick) were mounted on glass slides and stained with hematoxylin and eosin and examined under a Zeiss microscope. In addition, lumbar vertebrae (L1 to L5) removed from control and experimental animals were processed without decalcification after embedding in methylmethacrylate. Sections (8 μm thick) were stained by Goldner’s technique (20). Nonneural tissues (kidney, urinary bladder, liver, spleen, lymph nodes, and lungs) were removed at the time of sacriﬁce and were either snap frozen and stored at —80°C or ﬁxed in phosphate buffered formalin for later assessment.

RESULTS

Rat prostate cancer Mat LyLu cells were transfected with either the pYN retroviral vector alone or with the experimental plasmid pYN-ruPA and pYN-ruPA-AS. Growth of cells in medium containing G-418 allowed selection of drug-resistant colonies which had stably integrated the neo-resistant gene. These colonies were then screened by Northern analysis to detect those which produced the highest and lowest amounts of uPA, as determined by Northern blot analysis, for further studies. The Dunning R3327 Mat LyLu cell line was obtained from Dr. J. T. Isaacs (The John Hopkins School of Medicine, Baltimore, MD). Cells were maintained in vitro in RPMI 1640 supplemented with 2 mM L-glutamine (MA Bioproducts), 5% fetal bovine serum, 100 units/ml of penicillin-streptomycin sulfate (Gibco), 250 μM dexamethasone, and 0.2% gentamicin (Sigma).

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Northern Blot Analysis. Total cellular RNA was isolated from control and experimental Mat LyLu tumor cells by acid guanidium thiocyanate-phenol-chloroform extraction (21). Twenty μg of total cellular RNA were electrophoresed on a 1.1% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran) by capillary blotting. Hybridization was carried out with a 32P-labeled uPA antisense ribo probe. This was obtained by subcloning ruPA into a pBR322 plasmid (Invitrogen) at its unique XbaI site. Plasmid with insert cDNA in the sense orientation was linearized with HindIII and used for in vitro transcription of the antisense RNA probe with SP6 RNA polymerase and [32P]UTP. Following an 18-h incubation at 37°C, filters were washed 4 times for 30 min with 0.1X SSC (10X SSC is 1.5 M NaCl, 0.5 M sodium citrate, pH 7.0), 0.1% SDS at 37°C. Alternatively, filters containing Mat LyLu RNA were hybridized with ruPA cDNA or with an 800-base pair BamHI restriction fragment of rat cyclophilin cDNA as a control for the amount of RNA loaded (22). The filters were incubated at 42°C for 2 h, then successively washed in 1X SSC, 1% SDS for 15 min at room temperature; 0.5X SSC, 0.5% SDS for 15 min at room temperature; 0.1X SSC, 0.1% SDS twice for 15 min at room temperature; and then once for 30 min at 55°C. Autoradiography of filters was carried out at —70°C using XAR film (Eastman Kodak Co., Rochester, NY) with 2 intensifying screens. The level of uPA expression was quantiﬁed by densitometric scanning.

Immunological Assessment of uPA. Levels of uPA protein were determined by immunohistochemistry and by enzyme-linked immunosorbent assay using anti-human and anti-mouse uPA antibody (American Diagnostica, Greenwich, CT) according to supplier’s instructions (4).

Statistical Analysis. Results are expressed as the mean ± SE of at least triplicate determinations, and statistical comparisons are based on the Student’s t test or analysis of variance. A probability value of <0.05 was considered to be significant.

RESULTS

Rat prostate cancer Mat LyLu cells were transfected with either the pYN retroviral vector alone or with the experimental plasmid pYN-ruPA and pYN-ruPA-AS. Growth of cells in medium containing G-418 allowed selection of drug-resistant colonies which had stably integrated the neo-resistant gene. These colonies were then screened by Northern analysis to detect those which produced the highest and lowest amounts of uPA. uPA mRNA expressed in cells transfected with pYN-ruPA and pYN-ruPA-AS was compared with endogenous uPA mRNA expressed in control untransfected and in vector-only transfected cells. mRNA encoding uPA which had been transcribed from the vector was 4.5 kilobases and was easily distinguished from the endogenous 2.1-kilobase uPA mRNA (Fig. 1). Transfection of the expression vector alone did not alter the endogenous levels of uPA mRNA when compared to untransfected cells (Fig. 1). One colony transfected with the experimental plasmids produced considerably (at least 5-fold) higher levels of uPA mRNA, when compared to untransfected cells transfected with experimental plasmids produced considerably (at least 3–4-fold) increases and results (see below) with all 3 clones were analogous. Colonies transfected with antisense showed...
Typical histological findings of both osteoblastic and osteolytic (osteoclastic) lesions were seen in hematoxylin and eosin-stained sections of lumbar vertebrae in both control and experimental animals once metastases had occurred. However, animals given injections of cells overexpressing uPA had overall increased osteoblastic activity (relative to osteolytic activity), especially at their periosteal surfaces. This osteoblastic activity exceeded that due to normal bone turnover and was greater than that seen in animals injected with tumor cells transfected with vector alone (Fig. 3). Staining of undecalcified bone sections with Goldner’s stain provided further evidence of such increased new bone formation (Fig. 4).

Serum from control and experimental animals was also tested for biochemical evidence of increased bone turnover. Experimental animals receiving cells overexpressing uPA showed a significant increase in their serum alkaline phosphatase levels when compared to the control animals or animals receiving cells underexpressing uPA (Fig. 5).

**DISCUSSION**

Over the last several years, intense efforts have been made to understand the mechanisms of metastatic disease in prostate cancer, a neoplasm in which skeletal metastases are a common occurrence (1, 23). One of the major difficulties in this process has been the development of an animal model of prostate cancer which mimics the human syndrome. Most of the animal models available involve laborious inoculation techniques or result in the development of tumor metastases which are extraskeletal only and differ markedly from human prostate cancer (24—27). In the present study, we have utilized our previously developed *in vivo* model of rat prostate cancer in which Dunning R-3227 Mat LyLu cells are inoculated by the intracardiac route. This routinely results in tumor metastases to bone, thus mimicking the observed metastatic behavior of human prostatic carcinoma cells (15).

The molecule uPA, by virtue of its catalytic activity, has been identified as a factor capable of enhancing tumor invasiveness, including prostate cancer invasiveness. Binding of uPA to a surface receptor on uPA-producing cancer cells is believed to be critical to the focal proteolysis of the extracellular matrix which can be initiated by uPA, and which is important for its presumed action in tumor spread. However, most of the evidence to date implicating a role for uPA in tumor spread has been indirect. Thus, uPA has been reported to be produced in greater abundance by more highly aggressive human prostate cancers, including the relatively poorly differentiated human prostate cancer cell line PC-3, than by more well differentiated prostatic tumors or non-neoplastic tissue (28, 29). Additionally, allelic loss

**SITES OF TUMOR METASTASIS**

<table>
<thead>
<tr>
<th>Site</th>
<th>15 Days</th>
<th>21 Days</th>
<th>15 Days</th>
<th>27 Days</th>
</tr>
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<tbody>
<tr>
<td>Adrenals</td>
<td>-</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Kidneys</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>1</td>
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<tr>
<td>Lungs</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Vertebral Column</td>
<td>-</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Scapula</td>
<td>-</td>
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<td>2</td>
<td>-</td>
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<tr>
<td>Ribs</td>
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<td>Femur</td>
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*Fig. 2. Assessment of metastatic disease in control and experimental animals. Control animals receiving Mat LyLu cells transfected with pYN vector alone (Mat LyLu-pYN) and animals receiving Mat LyLu cells transfected with pYN-ruPA (Mat LyLu-pYN-ruPA) or pYN-ruPA-AS (Mat LyLu-pYN-ruPA-AS) were sacrificed at timed intervals as shown. Animals were examined for the development of macroscopic metastasis at various nonskeletal and skeletal sites as described in "Materials and Methods." The total number of metastatic foci involved as determined by macroscopic examination is indicated.*

**Table 1 Development of hind limb paralysis postinoculation**

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*Significantly different from control (P < 0.05).*

- Reduced (3-fold) mRNA encoding uPA (Fig. 1). Differences in the levels of uPA protein in control and experimental cells were determined by immunohistochemistry and by enzyme-linked immunosorbent assay, and paralleled the changes in mRNA levels.

- To determine the effect of uPA overproduction or underproduction on tumor metastasis, control and experimental cells (from the overexpressing or underexpressing colonies) were inoculated by intracardiac injection into the left ventricle. The control groups of animals which received either untransfected (Mat LyLu) tumor cells or tumor cells transfected with vector alone, reproducibly developed hind limb paralysis 20 to 21 days postinoculation (Table 1). In contrast, animals receiving cells overexpressing uPA developed hind limb paralysis at a much earlier time, that is, by day 14 or 15 postinoculation (Table 1). Animals receiving cells underexpressing uPA failed to develop paralysis by 27 days postinoculation (Table 1).

- At the time of development of hind limb paralysis, animals receiving either untransfected cells or vector-only transfected cells showed evidence of macroscopic metastases in the adrenal glands and occasionally in the kidney and lungs. In contrast, animals inoculated with cells overexpressing ruPA at the time of hind limb paralysis exhibited evidence of macroscopic metastases at multiple nonskeletal sites including the adrenals, kidneys, and lungs (Fig. 2). Soft tissue metastases in these experimental animals were highly aggressive, resulting in the development of large tumor masses (Fig. 2). The presence of these metastases was confirmed by histological examination. Metastatic tumor cells showed increased levels of uPA production, due to the expression of the pYN-ruPA plasmid.

- In the skeleton, in animals receiving vector-only transfected cells (or untransfected cells), no evidence of macroscopic or microscopic metastases was seen at day 15, and at day 21, metastases were only observed in lumbar vertebrae (Fig. 2). In contrast, in addition to the development of macroscopic metastases to the lumbar vertebrae, experimental animals with tumors overexpressing uPA also displayed metastases in multiple osseous sites such as the ribs, scapula, and femora. The presence of these metastases was also confirmed by microscopic examination.

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**Column-242 Scapula--2-Ribs--2-Femur--I-**

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of the uPA gene in prostatic tissue of patients with prostate cancer is associated with a better prognosis, thus implicating uPA as a negative prognostic factor (30). However, more direct evidence for a role of uPA in enhancing the aggressiveness of cancer generally has been sought. Thus, overexpression of human uPA in murine melanoma cells B16 F1 and in ras-transformed murine NIH 3T3 cells by gene transfer methods resulted in increased metastatic activity of these tumors in murine hosts in vivo (31, 32). However, in those heterologous studies, human uPA could not be bound by the murine cells due to an interspecies blockade in binding of human uPA to the murine uPA receptor. Therefore, those studies showed that secreted (non-surface bound) uPA promoted tumor invasion. Inasmuch as the surface binding of uPA is believed to be important in directing site-specific proteolysis, those studies were limited in scope (33). To overcome these limitations, a homologous system was developed in our study which allows interaction of transfected rat uPA with its endogenous receptor on rat prostate cancer cells. Thus, by overexpressing rat uPA in rat prostate cancer in syngeneic host rats, the effects of uPA on organ specific metastases could be studied in a model which more closely represents the characteristic of the in vivo situation.

Inoculating cells via the intracardiac route resulted in immediate access of all tissues of the body to tumor cells. Nevertheless, preferential growth of tumor cells overexpressing uPA occurred predominantly at various skeletal sites. This effect is even more remarkable when one considers that the skeleton receives only about 10% of the total blood supply from the left ventricle (34). Enhanced colonization of sites in the skeleton in this model may have involved uPA action in
one of several ways. Thus, the enzymatic activity of the carboxyl region of the molecule may have augmented tumor cell extravasation from the blood and increased the breakdown of skeletal matrix, facilitating tumor spread. Additionally, growth factor-like effects of the amino terminal domain of uPA in bone cells might give skeletal sites the advantage of entrapping high uPA-producing cells via uPA receptors located on the surface of these skeletal cells.

At skeletal sites, paracrine actions of uPA on cells of the osteoblast phenotype may contribute to their active proliferation, resulting in new bone formation. Such an osteoblastic response was seen much more abundantly in experimental animals harboring tumor cells overexpressing uPA than in control animals. This osteoblastic response was associated with some osteolytic lesions as well. Such lesions may have been due to the proteolytic actions of uPA and/or to the release of osteoclast-stimulating cytokines by the tumor cells, and/or to the release of osteoclast-stimulating cytokines by the activated osteoblasts. The increase in alkaline phosphatase levels observed in the serum of experimental animals by day 15 after tumor inoculation was also consistent with the increased osteoblastic response seen in histological sections of skeletal tissues.

These studies therefore provide direct experimental support for the role of uPA in the colonization processes involved in metastatic prostate cancer in extraskeletal sites, especially within the skeleton, and suggest that both the amino-terminal and carboxyl-terminal domains of the molecule may contribute to the particular characteristics of the skeletal metastases. Development of this homologous in vivo model of prostate cancer associated with uPA expression will now allow the design of therapeutic strategies to block prostatic metastases. Of particular significance in these strategies may be the use of recently described uPA specific inhibitors. These can now be evaluated for their capacity to block both uPA action and the development of tumor metastasis (35, 36).

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