The Expression of the Urokinase Plasminogen Activator System in Metastatic Murine Osteosarcoma: An in Vivo Mouse Model¹

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ABSTRACT

The role of urokinase plasminogen activator (uPA) in osteosarcoma is poorly understood. We examined the importance of uPA, its receptor, uPAR, and its inhibitor, PAI-1, in our in vivo model of metastatic osteosarcoma. Rodent osteosarcoma cells (UMR 106-01) were inoculated into the tibia of athymic mice. Animals were sacrificed and autopsied at 4 days to 5 weeks after inoculation. Tibiae and lungs were excised, fixed, and examined histologically and by in situ hybridization. Osteosarcoma development was associated with tibial swelling and lameness, and radiographic changes included osteolysis and new bone formation. Lung metastases developed spontaneously. In the tibial tumors, uPAR mRNA was expressed early (4 days), whereas uPA and PAI-1 mRNA increased as the tumor invaded the surrounding tissue (3 weeks). There was also an increase in the mRNA expression of the osteoblast-related genes, α(I) procollagen and osteopontin, but not matrix Gla protein. Lung metastases also expressed mRNA for the uPA system and the bone-related proteins. We have produced a model of metastatic osteosarcoma, which typifies the characteristics of the human tumor. Our results suggest that the uPA system plays a role in the local aggressiveness and metastasis of osteosarcoma and, in particular, indicates a possible therapeutic role for uPAR antagonists in the treatment of osteosarcoma.

INTRODUCTION

Osteosarcoma is the most common primary bone malignancy in the young (1). Although modern multimodality treatment has dramatically improved resectability and survival, local destruction and metastatic disease remain challenging problems.

Little is known about the interactions between host and tumor cells that govern growth and progression of osteosarcoma in vivo. For example, processes such as angiogenesis, tumor cell attachment, and invasion, which are known to be fundamental in carcinoma (2), have not been similarly explored in osteosarcoma. It is important to dissect the sequences of events that underlie the growth and spread of osteosarcoma if more efficacious treatment strategies are to be developed.

Local proteolysis is a pivotal step in the initiation of tumor invasion and spread, and the serine protease uPA³ system appears to be of major importance (3). uPA catalyzes the conversion of plasminogen to its active form plasmin, which directly degrades proteins such as fibrin, fibronectin, and laminin (4). In addition, plasmin activates latent forms of other proteases, such as the collagenases and stromelysins (5). In this regard, uPA may have a central role in initiating the proteolytic cascade that facilitates the invasion of blood vessels by tumor cells, their dissemination through the circulation, and their final deposition and growth at distant sites. uPA activity is regulated by binding to its specific cell surface receptor, uPAR, and may be further modulated by its specific inhibitors, PAI-1 and PAI-2. The value of uPA levels in breast carcinoma and other malignancies is now well-established (6, 7). Little is known, however, of the role of uPA in osteosarcoma.

We used an in vivo model of osteosarcoma, similar to that described by Berlin et al. (8), that used a well-characterized rodent osteosarcoma cell line, UMR 106-01. The UMR 106-01 cells are a subclone of the UMR 106 cells, which were originally derived from a rat transplantable osteogenic sarcoma with an osteoblastic phenotype (9). These cells are known to produce transforming growth factor-β (10) and parathyroid hormone-related protein (11) but not parathyroid hormone (12).

Using this model, we examined in detail the temporal sequence and spatial distribution of uPA, uPAR, and PAI-1 mRNA expression during tumor development. To this end, we have exploited in situ hybridization techniques to detect mRNA of the components of the uPA system and to study the patterns of their colocalization as our model of osteosarcoma grows, invades, and extends beyond its osseous confines. We also examined the pattern of expression of three bone-related genes, COL, OPN, and MGP that may have some relevance in tumor progression. This model allows us to map osteosarcoma development, which will significantly aid the development of strategies to minimize or inhibit tumor progression and metastasis.

³The abbreviations used are: uPA, urokinase plasminogen activator; uPAR, uPA receptor; PAI, plasminogen activator inhibitor; COL, type I α1 procollagen; OPN, osteopontin; MGP, matrix Gla protein.
MATERIALS AND METHODS

Animals and Animal Maintenance. Congenitally athymic female nude mice (BALB/c, nu/nu; Animal Resource Center, Perth, WA) were purchased germ-free at 2 to 3 weeks of age and housed in the Orthopaedic Department Sterile Facility. Animals were kept in sterile cages (maximum of five mice/cage) bedded with sterilized soft wood granulate and fed irradiated rat chow ad libitum with autoclaved and acidified (pH 2.5) tap water, with a 12-h light/12-h dark cycle. Animals were kept at least 1 week before experimental manipulation. All manipulations were performed in a laminar flow hood.

Anesthesia of Mice. Before tumor inoculation and radiographic examination, mice were anesthetized with an i.p. mixture of 50 mg/kg ketamine, 5 mg/kg xylazine, and 0.75 mg/kg acepromazine. For euthanasia, animals were given a lethal dose of ketamine, xylazine, and acepromazine, and the coronary artery was severed. The St. Vincent’s Hospital Animal Ethics Committee approved all experimental procedures, and animal care was in accordance with the National Health and Medical Research Council/Melbourne University animal ethics guidelines. Because osteosarcoma produces a soft tissue mass, in addition to bone destruction, we observed tumor size directly in these animals. In addition, we monitored the effect of tumor formation on the mice by looking for behavioral changes, such as inactivity and lameness, and by observing possible weight loss. All of the animals remained healthy and active during the course of the experiment.

Cell Culture. The rat osteosarcoma cell line UMR 106-01 (from Prof. T. J. Martin, St. Vincent’s Medical Research Institute, Fitzroy, Melbourne, Victoria, Australia) was maintained in α-MEM, supplemented with 10% fetal bovine serum at 37°C in 5% CO₂ in air. Cells in log phase growth were harvested by trypsinization, and medium containing 10% fetal bovine serum was added; the cells were then washed three times by trypsinization, and medium containing 10% fetal bovine serum was used. Right limbs were inoculated with medium alone. The needle was then reinserted through the cortex of the anterior tuberosity as aspirated into a 25-μl syringe; the syringe was flushed between inoculations. The needle was inserted 3–5 mm down the diaphysis of the tibia, and 10 μl of cell suspension were injected (2000 cells/inoculum). The needle was then reinserted from the bone, and the syringe was flushed between inoculations. Right limbs were inoculated with medium alone.

Radiographic Examination. Mice were anesthetized and transported to the St. Vincent’s Hospital Medical Imaging Department. Radiograms were taken with a mammography unit (Picker, LoRad), using Fuji computer radiography cassettes, and printed to hard-copy films. Radiograms were taken at 4-day, 1-week, and 2-week intervals.

Fixation and Decalcification. Mice were euthanized by anesthetic overdose at 4 days (n = 4) and 1 (n = 4), 2 (n = 5), 3 (n = 6), 4 (n = 6), and 5 (n = 6) weeks after inoculation. The hind limbs and the lungs were immediately dissected and fixed in 4% paraformaldehyde in PBS at 4°C for 24 h. After fixation, the limbs were transferred to a sterile decalcification solution (15% EDTA and 0.5% paraformaldehyde in PBS, pH 8.0). Decalcification was performed for 2 weeks, with the solution changed every second day. The samples were then paraffin embedded for in situ hybridization and histological study. All specimens were handled under sterile conditions to avoid RNA degradation.

Synthesis of Riboprobes. Digoxigenin-labeled RNA probes were prepared as described previously (13). Briefly, mouse uPA cDNA was linearized with either PstI or HindIII and transcribed with the SP6 promoter system or T7 polymerase to generate antisense or sense riboprobes, respectively. Full-length murine uPAR cDNA was either linearized with BamHI and transcribed with T3 polymerase to generate a sense riboprobe or linearized with HindIII and transcribed with T7 polymerase to generate a sense riboprobe. Similarly, rat PAI-1 cDNA was linearized and transcribed with either Hhal and T3 polymerase or HindIII and T7 polymerase to generate antisense and sense riboprobes, respectively.

COL cDNA was linearized using EcoRI or HindIII and transcribed with T7 RNA polymerase to generate antisense and sense strands, respectively. Human OPN cDNA was linearized with Xbal or XhoI and transcribed with T7 or T3 RNA polymerase to generate antisense and sense riboprobes, respectively. Rat MGP cDNA (a kind gift from Dr. P. Price, University of San Diego, La Jolla, CA), subcloned into the pBS plasmid, was linearized with HindIII and transcribed with T3 RNA polymerase to generate an antisense riboprobe or linearized with BamHI and T7 polymerase to produce a sense riboprobe.

In Situ Hybridization. In situ hybridization was performed on tibiae as described previously (13). Briefly, the 5-μm-thick paraffin sections, mounted on slides, were de-waxed with xylene and rehydrated in ethanol before rinsing in water. The sections were deproteinized with 0.2 M HCl, digested with 2 μg/ml proteinase K, and fixed in 4% paraformaldehyde in PBS. Prehybridization was performed at 65°C for 1 h in hybridization buffer, the riboprobe was then applied to each section, and the slides were incubated for 16–18 h at 65°C in a humidified chamber. Excess probe was removed by incubation with 25 μg/ml RNase A, and the slides were washed in decreasing concentrations of SSC at 37°C. Detection of hybridized probe was with the alkaline phosphatase-coupled antidigoxigenin antibody, used according to the manufacturer’s instructions. Negative control slides were pretreated with 150 μg/ml RNase A prior to hybridization or hybridized with sense and then treated as described above. All solutions were pretreated with pyrocarnonic acid diethyl ester. After in situ hybridization, tissue sections were counterstained with Nuclear Fast Red. Additional sections were stained with H&E to examine tumor histology. A semiquantitative system was used to describe the signal: −, no signal; +, weak signal; ++, moderate signal; ++++, intense signal.
RESULTS

Primary Tumor Formation

Radiology. There were no tibial tumors apparent at 4 days after inoculation radiographically, and the inoculated limbs looked similar to the control limbs. By 1 week, tibial tumors were observed in three of the four mice (Fig. 1a). From 2 weeks onward, tumors were observed in all of the tibiae inoculated (Fig. 1, b and c). The lesions showed radiographic features that were typical of human primary osteosarcoma, including metaphyseal osteolysis and peristeal elevation, with new bone formation and extension into the surrounding soft tissue.

Macroscopic Appearance. There were no tibial tumors observed macroscopically during the first 2 weeks after inoculation. However, by 3 weeks, all of the animals inoculated had developed palpable tibial tumors, which increased in size over the following weeks.

Microscopic Appearance. Isolated tumor nests had established in the metaphysis of the injected limb in two of the four tibiae at 4 days after inoculation and in three of the four mice after 1 week (Fig. 1d; Table 1). Within 2 weeks, tumor nests had invaded the medullary cavity in all of the inoculated tibiae, and tumor cells had penetrated the periosteum (three of five mice) and invaded the growth plate (two of five mice; Fig. 1e).

After 3 weeks, well-established tibial tumors, which had escaped the periosteum (five of six tibiae) and invaded the growth plate (two of six tibiae), were observed. There were small areas of necrosis, as evidenced by loss of nucleus and cell integrity, toward the center of the tumors, and areas of new bone formation associated with tumor cells on the periphery. By 4 weeks, the tumors had increased further in size, and all had escaped through the periosteum, invading the surrounding soft tissue as well as the growth plate. There were large areas of necrosis as well as areas of new bone formation (Fig. 1f). After 5 weeks, all of the inoculated mice had the large tibial tumors that had almost completely destroyed the remaining cortical bone and were surrounded by soft tissue. The growth plate was totally overgrown by tumor cells. Large areas of necrosis were

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Fig. 1 Radiographs and matching histological sections of induced tibial osteosarcomas. Representative radiographs of osteosarcoma at 1 week (a), 2 weeks (b), 4 weeks (c), and matching H&E stained sections at 1 week (d), 2 weeks (e), and 4 weeks (f). In a–c, arrows show areas that are representative of tumor progress in the tibia metaphysis with increasing destructiveness and a growing soft tissue compartment. In D, the arrow represents intramedullary tumor. Bars, 200 μm.
evident, as was new bone formation. In additional mice, the inoculated limbs were immediately amputated, dramatically reducing the formation of pulmonary metastases (results not shown), as described previously by Berlin et al. (8).

**Gene Expression.** We confirmed the ability of the digoxigenin-labeled riboprobes to bind rodent RNA of interest by Northern analysis (results not shown). As shown previously by others (14), UMR 106-01 cells produced low levels of uPAR and PAI-1 mRNA, which was up-regulated by transforming growth factor-β. We have also confirmed the presence of mRNA for COL, OPN, and MGP in UMR 106-01 cells (results not shown), as reported previously (Table 2; Refs. 15 and 16).

During the first 2 weeks after inoculation, only isolated tumor cells expressed a weak signal for uPA mRNA. However, after 3 weeks, low levels of staining for uPA mRNA was localized to the tumor cells, particularly those on the periphery of the tumor and associated with bone fragments (Fig. 2, a and b) and the growth plate. This expression increased further after 4 weeks, and a moderate signal for uPA mRNA was localized to the tumor cells at the periphery of the tibial tumors, especially in areas associated with bone and soft tissue. By 5 weeks, the signal had decreased, and the tumor cells expressed only low levels of uPA mRNA.

In contrast, uPAR mRNA was present throughout the tumor. Low levels of signal were observed at 4 days and 1 week after inoculation in the tumor cells, which increased to moderate at 2, 3, and 4 weeks and subsequently decreased to low levels by 5 weeks. From 3 weeks, the signal was primarily localized to the periphery of the tumor mass, particularly areas associated with bone fragments (Fig. 2c), the growth plate, and surrounding soft tissue.

There was no signal observed for PAI-1 mRNA during the first week after inoculation. By 2 weeks, low levels of PAI-1 mRNA were localized predominantly to the tumor cells on the periphery of the tumor mass, and this was maintained throughout the remainder of the time course (Fig. 2d). The tumor cells expressed low levels of COL mRNA during the first 2 weeks after inoculation. At 3 weeks, the signal was moderate, particularly in the tumor cells at the periphery, and this expression pattern was maintained throughout the remainder of the experiment (Fig. 2e). OPN mRNA expression was low during the first 3 weeks and then increased to moderate at 4 weeks and high at 5 weeks after inoculation (Fig. 2, f and g) and was greater at the periphery of the tumor. Tumor cells expressed moderate levels of MGP mRNA throughout the time course (Fig. 2h), which was only marginally higher in the cells on the periphery of the tumor after 3 weeks, than in the cells toward the center. The corresponding sense probes and RNase pretreated sections for uPA, uPAR, PAI-1, COL, OPN, and MGP showed no hybridization signal.

**Pulmonary Metastases Formation**

**Macroscopic Appearance.** No lung metastases were observed macroscopically at 4 days, 1 week, or 2 weeks. However, macroscopically visible lung metastases were observed at 3 (two of six mice), 4 (three of six mice), and 5 (three of six mice) weeks after inoculation.

**Microscopic Appearance.** There were no pulmonary metastases observed macroscopically in any of the lungs at 4 days or 1 week (Table 1). After 2 weeks, four of the five inoculated mice had developed small lung metastases. Small to moderately sized lung metastases were evident macroscopically in four of the six animals at 3 and 4 weeks after inoculation (Fig. 2i). By 5 weeks, numerous large metastases were observed in five of the six inoculated mice.

**Gene Expression.** At 2 weeks after inoculation, the tumor cells in the pulmonary metastases expressed moderate levels of uPA mRNA (Table 3). This expression had decreased by 3 weeks (Fig. 2j), and the metastatic tumor cells displayed low signal for uPA mRNA for the remainder of the time course. The levels of uPAR mRNA were consistently moderate during this period (Fig. 2, k and l), whereas the levels of PAI-1 mRNA remained low in these tumor cells.

The pulmonary metastatic cells expressed moderate levels of COL mRNA, which was distributed evenly throughout the metastases. In contrast, signal for OPN mRNA was low when the metastatic cells first developed at 2 weeks and then peaked at 4 weeks at moderate levels. This increase was short-lived, however, and by 5 weeks, the signal for OPN mRNA in the metastatic tumor cells was low. At 2 weeks after inoculation, the metastatic tumor cells in the lung exhibited low signal for MGP mRNA, and they remained at these levels throughout the experiment.

No signal was detected in the RNase pretreated sections or in the corresponding sections incubated with sense probe for uPA, uPAR, PAI-1, COL, OPN, or MGP.

### Table 1 Transplantability and spontaneous metastasis of UMR 106-01 osteosarcoma cells orthotopically implanted into nude mice

<table>
<thead>
<tr>
<th>Time course</th>
<th>Tumor take rate at inoculation site</th>
<th>Mice with microscopically visible lung metastases</th>
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<tr>
<td>4 days</td>
<td>2/4</td>
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<td>1 week</td>
<td>3/4</td>
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<td>4 weeks</td>
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<td>4/6</td>
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<tr>
<td>5 weeks</td>
<td>6/6</td>
<td>5/6</td>
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### Table 2 In situ hybridization results for uPA, uPAR, PAI-1, COL, OPN, and MGP mRNA of orthotopically induced tibial tumors

<table>
<thead>
<tr>
<th>Time course</th>
<th>uPA</th>
<th>uPAR</th>
<th>PAI-1</th>
<th>COL</th>
<th>OPN</th>
<th>MGP</th>
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<tr>
<td>4 days</td>
<td>+1</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>1 week</td>
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<tr>
<td>2 weeks</td>
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<td>3 weeks</td>
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<tr>
<td>4 weeks</td>
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<td>5 weeks</td>
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* - no signal; +, weak signal; +, distinct signal; +++, intense signal.
DISCUSSION

The uPA system has an important role in the tumor progression of many cancers. Although not demonstrated previously, the gross local destruction and high risk of metastasis associated with osteosarcoma strongly implicates the uPA system in this process. The importance of the uPA system in osteosarcoma has been alluded to by De Bart et al. (17), who reported that interleukin 1α increased uPA production in the human osteosarcoma cell line, MG63, which resulted in increased matrix degradation in vitro. Furthermore, increased uPA levels have been shown to correlate with tumor invasiveness in surgical specimens of osteosarcoma (18). However, the in vivo expression of the components of the uPA system and their relationship to the growth and destruction caused by the osteosarcoma remain unknown.

To examine the mRNA expression of uPA, uPAR, and PAI-1 during osteosarcoma development, we developed a model of osteosarcoma in congenitally athymic nude mice using the well-characterized rodent osteosarcoma cell line UMR 106-01 (9, 15). This model of osteosarcoma produced invasive tumors that were radiographically and histologically similar to human osteosarcoma and which spontaneously produced pulmonary metastases.

Fig. 2 In situ hybridization of tibial tumors and pulmonary metastases from osteosarcoma. a–h: tibial tumors. a, low uPA mRNA expression localized to the tumor cells at the tumor/bone interface at 3 weeks (arrow); b, corresponding uPA RNase control; c, moderate uPAR mRNA expression in the tumor cells adjacent to bone at 3 weeks (arrow); d, low PAI-1 mRNA expression at 3 weeks (arrow); e, moderate COL mRNA expression at 3 weeks (arrow); f, high OPN mRNA expression at 5 weeks (arrow); g, OPN RNase control; h, moderate MGP mRNA expression at 1 week (arrow); i–l, metastases. i, H&E of lung metastases (arrow) at 3 weeks after inoculation; j, low signal for uPA mRNA (arrow); k, moderate uPAR mRNA expression (arrow); l, corresponding uPAR RNase control. Bars, 200 μm.
Importantly, we were able to demonstrate for the first time that UMR 106-01 cells are capable of establishing distant metastases when implanted orthotopically. Extensive studies using s.c., tail-vein, and intracardiac injections have failed previously to demonstrate any metastatic lesions (19). Our current results highlight the potential importance of the bone environment in priming the osteosarcoma cells for metastasis. Similar observations were reported after orthotopic inoculation in breast (20) and prostate (21) carcinoma cells.

Our study demonstrated that uPAR mRNA was expressed very early during tumor development (4 days) by osteosarcoma cells and paralleled the development of the tumor. Several researchers have suggested previously that uPAR plays a pivotal role in triggering the proteolysis associated with the uPA system in tumor invasion and metastasis. Costantini et al. (22) demonstrated significant differences in uPAR levels between human invasive breast carcinomas, which had higher levels, than other breast tumors. Furthermore, Kariko et al. (23) examined the role of the uPA system in matrix degradation in a human osteosarcoma cell line (HOS), which was stably transfected with uPAR. They reported a 4-fold increase in matrix degradation in the uPAR-expressing HOS cells, compared with the parental cell line.

In contrast to uPAR, uPA had a later appearance and was only consistently expressed at 3 weeks, once the tumor was well-established. The expression of this mRNA increased noticeably as the tumor extended through the cortex and reached a peak when the osteosarcoma invaded the extraosseous soft tissue, thus implicating uPA as a major factor in the local extension of osteosarcoma and corroborating earlier in vitro data (23, 24).

Unlike uPA, PAI-1 mRNA was only expressed at low levels throughout the development of the osteosarcoma. In this regard, Hakel et al. (18) reported that high levels of uPA protein correlated with low PAI-1 production in human osteosarcoma specimens. Despite its role as an inhibitor of uPA, PAI-1 expression has been shown to correlate with metastatic potential, high uPA activity, and poor prognosis in several tumor types (4, 6). Those authors proposed that PAI-1 may act to reduce excessive uPA activity, thereby stabilizing cell matrix attachment, thus increasing invasiveness.

In the current study, there was a preferential colocalization of uPA, uPAR, and PAI-1 mRNA to the advancing front of the tibial tumors. Using 125I-labeled plasminogen binding studies, Campbell et al. (25) found that plasminogen bound to the cell surface of the human osteosarcoma cell line MG63 and was activated to plasmin by uPA. Our model supports these findings, suggesting that surface-bound uPA may be concentrated at particular sites, allowing tumor cells to selectively degrade the extracellular matrix and migrate into surrounding tissues.

The relationship between uPA-uPAR and integrins also has important implications for cell adhesion and motility associated with tumor development and spread (26). In this regard, we found that OPN was coexpressed at the advancing edge of the tumor, and its expression closely followed uPAR expression. The importance of this has been raised by other studies that demonstrated increased OPN expression in a number of transformed cell lines (27, 28) and human tumors, including breast and lung (29). In addition, uPAR and OPN have been shown to form complexes that are important for cell migration (30, 31), and because mRNA for both of these molecules was expressed throughout the stages of tumor development in our model, we cannot ignore their role in the invasive nature of our osteosarcoma model.

MGP mRNA was expressed throughout the development of the tibial tumor. The significance of this is unclear, but a role in tumor invasion cannot be excluded in the light of the study by Loeser and Wallin (32), which reported that the U2-OS human osteosarcoma cells adhered to and spread on MGP-coated surfaces. This adhesion was completely inhibited by an RGD-containing peptide. Because MGP is found in many tissues that are recognized sites of osteosarcoma spread such as bone, cartilage, and lung (32, 33), it may have some significance in targeting these tissues for local invasion or metastasis.

Type I collagen is the major component of the mineralized matrix of bone, although it is also present in many other tissues. We and others have shown it to be important in invasive breast carcinoma (13, 34). In the current study, we have localized COL mRNA expression to the tibial tumors, which was particularly prominent at the bone tumor interface. This suggests an affinity of the tumor cells for bone, or alternatively, it may simply reflect tumor cell origin or degradation of the extracellular matrix.

Lung metastases, which exhibited a similar pattern of mRNA expression as the tibial tumors, were first observed at 2 weeks in our model, further supporting the notion that they arose spontaneously, as reported previously by Berlin et al. (8). It is tempting to conclude that the expression of uPA and its receptor may have importance not only in the local invasion and growth of the primary tumor but also for the invasion and implantation into distant sites such as the lung. Certainly, increased uPA activity has been shown to correlate with metastatic potential in a clonal cell lines (35), and we have demonstrated recently the up-regulation of uPA, uPAR, and PAI-1 mRNA in osseous metastases from breast carcinoma (13).

Our model of osteosarcoma provides a simple and reproducible method of producing invasive tumors that are radiographically and histologically similar to human osteosarcoma and that spontaneously give rise to pulmonary metastases. The model may prove useful in examining the steps in invasion and metastasis and may pave the way for developing strategies to minimize the local growth and spread of osteosarcoma. Our results suggest that the uPA system plays an important role in the local aggressiveness of osteosarcoma and possibly in its...
metastasis to lung. In this respect, the manipulation of the tumor by genetic and therapeutic strategies to target the uPA system may have significant implications for treating local and distant spread of the disease.

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