# UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN A HUMAN SARCOMA CELLULAR MODEL FOR METASTASIS IN ATHYMIC MICE

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ABSTRACT

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Urokinase-type plasminogen activator in a human sarcoma cellular model for metastasis in athymic mice

Raya Mandler, Doctor of Philosophy, 1990

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Metastasis, the secondary spread of tumors, accounts for most fatalities in cancer patients, because current treatments do not effectively eradicate the metastatic cells. Focusing on properties that enable metastatic cells to disseminate and colonize may lead to more effective treatment strategies. In search for such property, this work focused on the hypothesis that metastatic competence may depend, in part, on elevated expression of urokinase-type plasminogen activator (uPA), particularly when the enzyme is bound to the surface

membrane of the tumor cells. In this configuration, uPA can mediate local matrix proteolysis, leading to tissue degradation, and facilitating invasion and metastasis. To test this hypothesis, a human sarcoma model was developed, which was adequate for quantitative studies of metastasis in-vivo, in athymic mice. Immortalized human osteosarcoma (HOS) cells were transformed with either EJ-ras or v-Ki-ras oncogenes. Several of the transformant sublines could form grossly visible pulmonary tumors when implanted intravenously in athymic mice. The metastatic potential of these sublines correlated with their endogenous uPA activity ($r=0.93$, $p<0.01$). This correlation involved mainly the surface-bound uPA rather than the secreted enzyme. Of the transformant sublines, the one designated KRIB was the most efficient in forming experimental lung metastases (75%-88% tumor-bearing mice, median number of 7-10 visible tumors/animal within 35 days post-inoculation). KRIB cells also expressed the highest uPA activity (~20 Plough units/ $10^6$ cells), whereas several other HOS transformants with lower metastatic potential exhibited lower uPA activity (between 4-9 Plough units/$10^6$ cells). Furthermore, uPA was found to play a functional role in metastasis. Inhibition of uPA activity on KRIB cells' surface by specific monoclonal antibodies before inoculation (98% inhibition) caused a significant reduction in the metastatic yield (57% tumor-bearing mice, median number- 1 tumor/animal, $p<0.01$). Such an effect was not observed when non-neutralizing anti-uPA antibodies were used. These results

imply that endogenous uPA activity of tumor cells enhances their metastatic potential and suggest that uPA could potentially serve as a diagnostic marker to predict metastatic risk, and as a target for therapeutic intervention.

# Urokinase-type plasminogen activator in a human sarcoma cellular model for metastasis in athymic mice

by
Raya Mandler

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of the Department of Physiology
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## LIST OF ABBREVIATIONS

ATCC- American tissue Culture collection

B16- A murine melanoma line with metastatic ability in syngeneic and athymic mice

DMEM- Dulbecco modified essential medium, used for tissue culture

DMSO- Dimemthyl sulfoxide, an inhibitor of uPA production

E-ACA- Epsilon aminocaproic acid, an inhibitor of uPA and other serine proteases activity

EGF- Epidermal growth factor

EJ-ras- Mutated Harvey-ras oncogene, which was isolated from the established human bladder carcinoma EJ/T4 line

FACS- Fluorescence activated cell sorter, used in combination with immunofluorescent staining, for analysing cellular surface determinants

FCS- Fetal calf serum, used routinely as a supplement in tissue culture medium

FITC- Fluorescein isothianate conjugates (usually, labeled antibodies)

H-2- The murine histocompatibility gene complex

Ha-ras- Harvey-ras proto-oncogene

HEp-3- An established human larynx carcinoma line with metastatic capability in the chick embryo model

HLA- The human histocompatibility gene complex

HOS- An established human osteosarcoma line, which is tumorigenic but non-metatstatic in athymic mice. HOS was used in this work as the parental line for the cellular model system

LB- Luria broth, used as culture medium for bacteria (10 mg/ml Bacto-Tryptone, 5 mg/ml NaCl, pH7.5)

MuSV- Murine sarcoma virus. The Ki-MuSV strain carries the viral Ki-ras oncogene

PAI-1- Plasminogen activator inhibitor type 1. A 50KDa protein produced by endothelial cells, with strong anticatalytic activity on uPA and tPA

PBS- Phosphate-buffered saline (130 mM Nacl, 10 mM $NaHPO_4$, 5 mM $Na_2PO_4$)

RSV- Rous sarcoma virus, a RNA virus with transforming action on avian fibroblasts

TE buffer- A buffer used for washes and storage of RNA and DNA (10mM TRIS, 1mM EDTA, pH 8.0)

TGA buffer- A buffer used for gel electrophoresis of small-scale RNA preparations and Southern blot separation of digested DNA (4mM TRIS, 2mM NaOAC, 2mM EDTA pH 8.0)

3T3- An immortalized mouse fibroblast line, which is neither tumorigenic nor metastatic in mice

tPA- Tissue plasminogen activator. A 70KDa protein produced by endothelial cells, which is thought to be a major regulator of plasma fluidity

v-Ki-ras- The viral oncogene present in Kirsten murine sarcoma virus, homologous to the Ki-ras proto-oncogene.

# INTRODUCTION

Metastasis, the secondary spread of tumor cells from the primary site to distant locations, is the most severe complication of cancer. Secondary tumors account for the majority of deaths in cancer patients, because the various modalities of treatment (surgery, chemotherapy, radiotherapy etc.) do not effectively eradicate the metastatic cells. In spite of extensive research during the past 30 years, our understanding of the metastatic process is still unsatisfactory. Although studies in-vitro have elucidated various isolated characteristics of metastatic cells, crucial aspects of the dissemination and colonization processes are still not well understood (Poste, 1986 and Mareel, 1983). This is due, in part, to alack of appropriate model systems for in-vivo studies (DCT Board,1985). Therefore, the development of adequate model(s) that can be used to evaluate novel approaches for prevention and control of metastasis is of utmost importance.

Metastatic cells exhibit the most aggressive phenotype of neoplastic cells. They arise as tumor cell variants by gradual evolution and selection pressure. These processes, as a whole, are termed tumor progression and their manifestations are briefly described below.

## A. TUMOR PROGRESSION

Neoplastic transformation is driven by genetic and epigenetic cellular alterations and is a gradual and progressive process. Therefore, while the hallmark of cancer cells is their escape from normal regulatory mechanisms and the acquisition of autonomous growth, their deviation from the normal phenotype varies. Tumor cells may be identified at stages ranging from the "benign" to the extremely aggressive, metastatic phenotype. In early stages of neoplastic transformation, the cells may appear normal but they are more prone to become cancerous due to subtle genotypic changes. Upon exposure to the appropriate conditions (i.e., physical or chemical carcinogens, mitogens or hormones), such cells become overtly neoplastic (Berenblum, 1941 and Castagna, 1987). They gradually exhibit more aberrant morphology and loss of differentiation markers. Concomitantly, they become more aggressive in their interaction with the host environment and more refractory to normal regulatory mechanisms. The most advanced stage in tumor progression is the metastatic phenotype. Metastatic cells can detach from the primary tumor, invade blood or lymphatics vessels and recolonize in distant locations (Weiss and Ward, 1983 and Nicolson and Poste, 1983).

## B. THE METASTATIC PROCESS

The metastatic spread, which involves complex interactions between tumor cells and the host environment, includes several discrete sequential events.

At early stages, the primary tumor cells invade the surrounding tissues, causing physical damage and local degradation of the stroma (Barsky et al., 1983). Those that are more motile and invasive can migrate and degrade components of small blood vessel walls (Weiss and Ward, 1983) and eventually penetrate into the circulation. This process is called intravasation.

In the circulation, the metastatic cells have to survive various obstacles, such as immune reactions, clotting processes, engulfing by fibrin and physical shearing forces (Eccles, 1982). Tumor cells which are released into the circulation in small emboli seem to be better protected and thus more successful in surviving these obstacles. The small tumor clusters eventually lodge in the capillary bed of distant organs and adhere to the endothelial layer (Crissman et al., 1988). It takes approximately 24 hours for the tumor cells to circulate and arrest in the capillary bed. Thus, the stage in which tumor cells are borne in the circulation is relatively short.

The arrested metastatic cells displace the endothelial

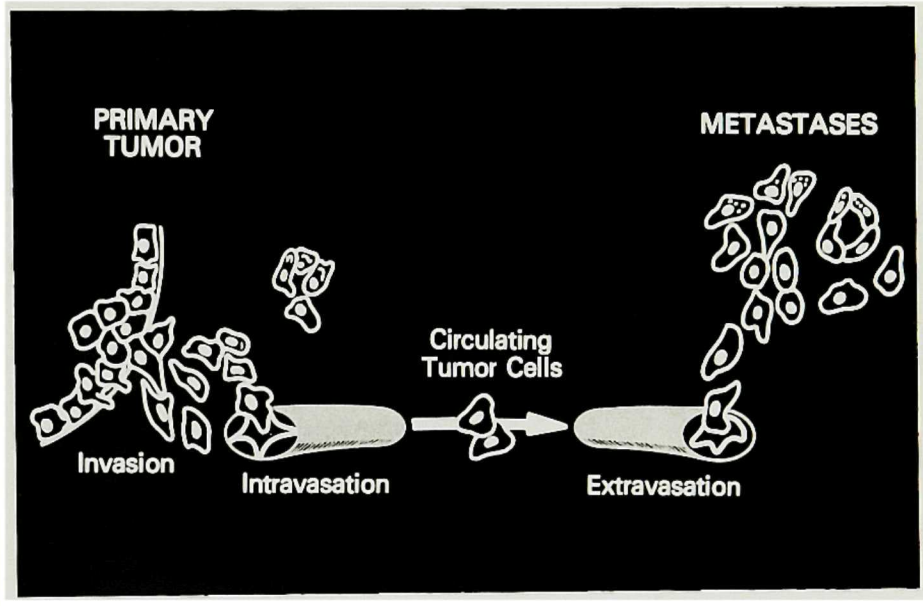
layer by physical damage and by biochemical degradation (Crissman et al., 1988 and Liotta et al., 1983). The process of degradation continues as the cells gain access to the subendothelial connective tissue, causing basal membrane dissolution. This, in turn, facilitates the exit of the cells from the capillary wall, a process called extravasation.

Metastatic cells can remain quiescent in their new location for a prolonged period. However, in certain cases they undergo mitosis within 24-48 hours after extravasation. Subsequent to their continuous replication, they form small neoplastic foci in their new location. Colonization continues as the new tumor induces sprouting of new blood capillaries, a process called angiogenesis. While the new vasculature itself is not malignant, it ensures blood supply to the developing foci and supports further growth of the tumor (Schor and Schor, 1983). Figure 1 is a schematic representation of the metastatic process.

As evident from this description, metastasis is a multifactorial process which involves complex cellular functions. While some isolated functions can be examined in-vitro, such studies do not cover all aspects of metastasis and their results do not always correlate with the tumor behavior in-vivo. Conclusive studies of metastasis depend, ultimately, on the use of in-vivo models.

Figure 1

Schematic representation of the metastatic process




At early stages, tumor cells invade their surrounding tissue. More aggressive and motile cells within the tumor population may, eventually, penetrate blood vessels and later re-colonize distant locations.

## C. CONSIDERATIONS AND CURRENT PROBLEMS IN CHOOSING ADEQUATE MODELS FOR in-vivo STUDIES OF METASTASIS

Cellular models for in-vivo studies of metastasis in animals should fulfil three main criteria. First, it is preferrable to work with tumor cells which produce grossly visible, rather than microscopic, metastatic lesions. Such metastases are more biologically relevant since their development is closer to the pathological picture seen in the clinical setting. They also allow for more accurate quantitative analysis, and thus better evaluation of the cells' metastatic potential. Second, it is important to assure that the metastatic behavior of the cells is an inherent, stable property. Cellular properties change sometimes with long term maintenance in culture, a phenomenon called phenotypic drift (Nicolson and Poste, 1983). This can negate reproducibility, and thus complicate the interpretation of the experimental results.

The third consideration is that the neoplastic cellular model should be of human origin. "Many clinical investigators believe that murine tumor models are not relevant to the human cancer problem because claims of anticancer activity for drugs in experimental tumor systems often were not verified when these drugs were used in human cancer patients [..and there is..] poor correlation between

animal tumor model determinations of drug activity and clinical efficacy". This citation, from NCI report (DCT Board Committee, 1985), indicates the growing awareness that animal and human malignancies differ considerably in their developmental pattern, metastatic behavior and drug sensitivity (also stated by Poste, 1986; Eccles, 1982; Graham et al., 1978 and Kyriazis et al., 1978) and reflects the difficulties in extrapolating data from animal tumor studies to human neoplasia. Since 1985, there has been an increasing emphasis on identifying adequate human model systems and on focusing attention on their unique characteristics.

Human tumor cells rarely metastasize efficiently in experimental animals, including many which are known to be aggressive and highly metastatic in patients (Nicolson and Poste, 1983 and Giovanella and Fogh, 1985). In one study, 106 different human tumors were tested for their metastatic ability in 1046 immunodeficient mice. Metastases were found in only 14 animals, out of which only 2 mice had grossly visible nodules, while in the other 12 cases the metastatic foci were microscopic (Sharkey and Fogh, 1979). The tumors in that study were obtained directly from biopsies. Equally discouraging results were obtained in studies using established human tumor cells. A few such lines have been reported, among them is the HEp3 larynx carcinoma, which is tumorigenic and metastatic in athymic mice and in an avian model system (chick embryo). In these model systems, the human

tumor cells form only microscopic metastases, and their development in mice is very slow (Ossowski et al., 1988 and 1987). Another established human cell line, A375 amelanolytic melanoma, gives rise to macroscopically visible lung nodules in immunodeficient mice (Fidler, 1986; Kozlowski et al., 1984 and Wilson et al,1988). However, A375 cells' metastatic yield in mice is poor and their phenotype is relatively unstable. In addition, to date, no human sarcoma with metastatic capability in animals has been described, although metastasis of sarcomas is a frequent complication seen in human patients.

Aiming to establish a cellular human model adequate for metastasis studies in animals, the use of the ras oncogenes was considered. These oncogenes can initiate neoplastic transformation and accelerate tumor progression. Two of these oncogenes- v-Ki-ras and mutated Ha-ras- have been shown to induce metastatic competence in rodent cells in gene transfer experiments (Egan et al., 1987 and Barbacid, 1987). ras oncogenes are briefly described below.

## D. ras ONCOGENES

The ras genes- Harvey-ras-1, Kirsten-ras-2 and N-ras- are ubiquitous to all eukaryotic species and are thought to

play a critical role in control of normal growth and differentiation (Barbacid, 1987; Mulcahy et al., 1985 and Bar-Sagi and Feramisco, 1985). These genes encode for closely related 21KDa proteins (p21) which resemble the G proteins in structure and functions. p21 proteins are thought to play a regulatory role in transducing mitogenic signals across cellular membranes (McCormick, 1989 and Barbacid, 1987).

Abnormalities in ras gene expression, either qualitative or quantitative, are associated with neoplastic transformation (Chang et al., 1982; McKay at al., 1986; Eadie et al., 1984, Loechler et al., 1984 and Tainsky et al., 1987). Modified ras alleles were identified in 10% of the most common human tumors (Marshall, 1985; Barbacid, 1985; Nardeux et al., 1987; Yanez et al., 1987 and Slamon et al., 1984) and were shown to induce neoplastic transformation <u>in-vitor</u> in mammalian cells (Shih and Weinberg, 1982 and Rhim et al., 1975). These alleles are, therefore, designated "ras oncogenes" and the normal counterparts are called ras "proto-oncogenes". The ras oncogenes encode for more stable or over-produced p21, as a consequence of gene alterations, such as missense mutations (e.g., substitution of $Gly^{12}$), linkage to a strong promotor (e.g., retroviral promotor located on the long terminal repeat), gene rearrangements due to chromosomal translocation or modifications which accompany gene transduction by retroviruses (Bishop, 1987). The latter gave rise to the retroviral ras oncogenes, which are of the

strongest transforming oncogenes known to date. In fact, ras-containing retroviruses were discovered about 15 years prior to the cellular counterparts due to their transforming capability (Harvey, 1964 and Kirsten and Mayer, 1967).

Two of the ras oncogenes- the viral Kirsten-ras (v-Ki-ras) and the mutated cellular Harvey-ras (EJ-ras)- are of particular interest for this work. v-Ki-ras was originally identified in the sarcoma retrovirus Ki-MuSV (Kirsten and Mayer, 1964). It is highly homologous to the cellular Kirsten-ras-2 gene, with two missense mutations (codons 12 and 59) and a truncated 5' exon (Duesberg, 1987). Its expression is driven by the viral strong promotor.

v-Ki-ras induces neoplastic transformation in a variety of immortalized mammalian cell lines, including human cells (Barbacid, 1987 and Rhim et al., 1975). Constitutive v-Ki-ras expression is required for sustaining the transformed state and tumorigenicity. However, it is not obligatory for the progression into metastatic phenotype. The cellular Ki-ras oncogene, which is closely related to v-Ki-ras, is found in spontaneous human cancers of different histological backgrounds. Its expression is reduced, however, in the metastatic tumors compared with the primary counterparts (Gallick et al., 1985; Ohuchi et al., 1986; Spandidos and Kerr, 1984 and Yanez et al., 1987).

EJ-ras is an allele of the cellular Ha-ras-1, carrying a mutation in codon 12. It was first identified in the EJ/T4

human bladder carcinoma cell line (Shih and Weinberg, 1982 and Reddy et al., 1982). EJ-ras is highly oncogenic and can transform even diploid, mammalian cells in primary cultures. In addition to tumorigenicity, this oncogene is capable of inducing metastatic potential in benign, immortalized murine cells (Barbacid, 1987; Duesberg, 1987 and Egan et al., 1987).

In the research studies presented here, ras oncogenes were used to transform human osteosarcoma cells, which were originally non-tumorigenic in athymic mice. Several of the transformant lines became metastatic as well. Characterization of these human cell lines, and in particular their metastatic pattern, were the focus of the first part of this research project.

Since the cellular system was originally derived from a human osteosarcoma tumor, this disease is briefly described below.

## E. HUMAN OSTEOSARCOMA

Osteosarcomas are malignancies arising from stem mesenchymal cells which, under normal developmental schedule, differentiate towards fibrous, cartilage or bone tissues. Accordingly, they are subdivided into fibroblastic, chondroblastic and osteoblastic osteosarcomas (Link and

Eilber, 1987 and Malawer et al., 1985). Histologically, the tumors are characterized by the presence of osteoid material and a mixture of large, atypical, proliferating bone spindle cells. The cells have irregular nuclei and abnormal mitotic figures.

Osteosarcoma is the third most frequent malignancy in adolescents and young adults, exceeded only by leukemias and lymphomas, yet is the most aggressive of the three (Link and Eilber, 1987; Dahlin and Coventry, 1967; Goorin et al., 1985 and Taylor et al., 1985). Incidence is slightly higher in males and epidimiology surveys suggest genetic predisposition as well, especially in patients with hereditary retinoblastoma (Fosdtad et al., 1986 and Friend et al., 1986). Ionizing radiation has been indicated as an enviromental agent inducing osteosarcoma, but for the most part the etiology of this malignancy is unknown (Dahlin and Coventry, 1967 and Malawer et al., 1985).

Treatment of the primary bone tumors by surgery is usually successful, in that recurrence in areas adjacent to the primary site is rare. However, approximately 80% of the patients develop secondary lung tumors and most of them die within 2 years after the first diagnosis of metastasis (Dahlin and Unni, 1977; Carter, 1984 and Calkins and Ramming, 1987). Several recent studies reported an improvement in prognosis following combined treatment of surgery and adjuvant chemotherapy (Link et al., 1986; Calkins and Ramming, 1987 and

Belli et al., 1989). This encouraging information has been disclaimed by other reports, noting that adjuvant chemotherapy altered the pattern of metastasis, with higher frequency of bone recurrence, rather than successful eradication of micrometastases (Jaffe et al., 1983; Goorin et al., 1985; Link et al., 1986 and Carter, 1984).

Osteosarcomas metastasize via hematogenous route only, since the bone does not have lymphatics drainage, and the target organ is almost exclusively the lungs (Jaffe et al., 1978). As can be inferred from the natural history and pathology of osteosarcoma, micrometastases are most probably already present in the lungs at the time the primary tumor is diagnosed. Early detection of these metastases, however, has not been very successful and awaits further characterization of determinants unique to the secondary tumors that would enable to locate and eradicate them effectively.

In that respect, it may be of interest to note that employing radio-labeled antibodies against specific antigens of metastatic cells combined with sensitive scanning techniques, has been considered as a promising approach for early detection of micrometastases (Armitage et al., 1986 and Baldwin et al., 1984). The studies presented here suggest that the expression of urokinase-type plasminogen activator could be relevant for such an approach. The reasons for focusing on the expression of uPA are discussed next.

## F. PUTATIVE ROLE OF UROKINASE-TYPE PLASMINOGEN ACTIVATOR IN METASTASIS

### 1. uPA structure and functions

The first discovery of fibrinolytic activity in the urine, due to plasminogen activation, was in 1951 (Williams, 1951). The new enzyme was, therefore, called urokinase. That uPA at a low concentration exists also in plasma was demonstrated approximately 25 years later (Astedt et al., 1978). The complete amino acid sequence of uPA was reported in 1982 (Gunzler et al., 1982 and Steffens et al., 1982) and the human genomic DNA sequence was isolated in 1984 (Verde et al., 1984).

The human uPA gene is located on chromose 10. It is comprised of 11 exons, 6.4 kb in length, which give rise to 2.5 kb mature mRNA (Tripputi et al., 1985; Gunzler et al., 1982; Riccio et al., 1986 and Rajput et al., 1985). uPA is translated as a single-chain 50KDa polypetide, which is subsequently glycosylated and secreted. The single-chain glycoprotein is inactive and, thus, is referred to as uPA pro-enzyme (pro-uPA) (Kline and Reddy, 1980; Dano et al., 1985 and Blasi et., 1986). Limited proteolysis (which may occur by spontanous hydrolysis or by plasmin) of pro-uPA activates it

and changes its configuration to two chains, held together by a disulfide bond (Figure 2).

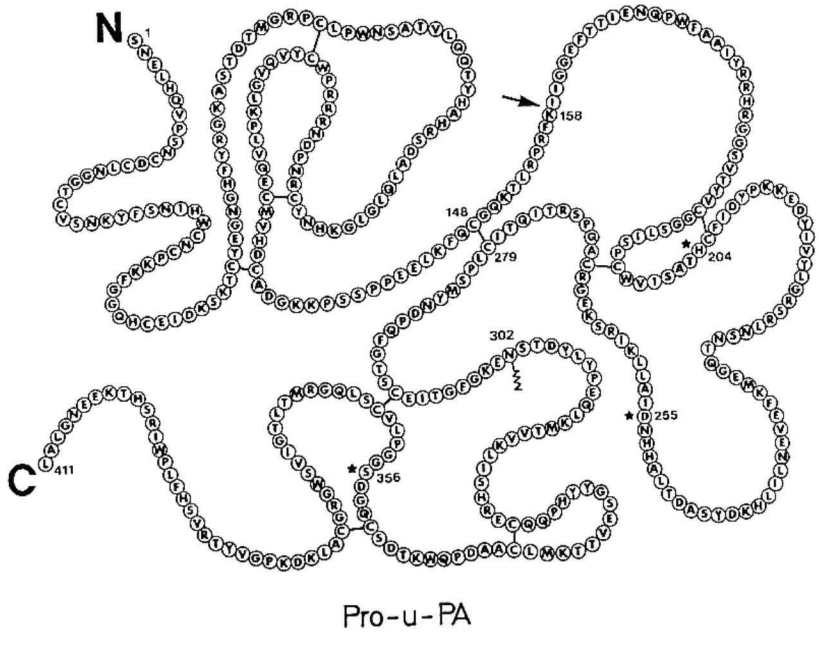
Upon cleavage of the disulfide bond, the activity is retained on the C-terminal chain, called the B chain. Thus, active uPA may exist in either the high molecular weight form (HMW uPA) comprised of two chains, or the low molecular weight form (LMW uPA) which is the B chain only (30 KDa) (Steffens et al., 1982 and Kline and Reddy, 1980). The N-terminal chain, called the A chain, is not involved in the hydrolytic activity of the enzyme, but is responsible for binding to specific uPA membrane receptors and may exert mitogenic responses, since it contains a domain homologous to epidermal growth factor (EGF) (Patthy et al., 1985 and Kircheimer et al., 1989).

uPA is a trypsin-like serine protease. While its substrate specificity is determined by the secondary and tertiary structure of the B chain, the catalytic pocket is comprised of $His^{204}$, $Asp^{255}$ and $Ser^{356}$ amino residues, common to all the proteases of this group. In neutral pH, the aspartic acid is negatively charged, having a stronger affinity for basic amino acids, which is the reason for the preferential activity on Arg-Val or Arg-Lys dipeptide bonds. Hydrolysis of the dipeptide bond itself is the result of nucleophilic attack by the hydroxyl group of the $Ser^{356}$ residue (Stroud et al., 1975).

The substrate for uPA activity is plasminogen, a zymogen abundant in plasma (its concentration is $2x10^{-6}M$) and

Figure 2

Structure of pro-uPA




Pro-uPA is a single-chain 50KDa glycoprotein. It is activated by cleavage of the dipeptide bond between $Lys^{158}$ and $Ile^{159}$ (arrow). The active site is located on the C-terminal chain, called the B-chain, and is comprised of $His^{204}$, $Asp^{255}$ and $Ser^{356}$ amino residues (*). The N-terminal chain, called the A-chain, contains a kringle homologous to EGF, which is the receptor binding site of the enzyme.

in the extracellular fluid compartment. uPA converts plasminogen to its active form, plasmin, by cleaving a specific Arg-Val dipeptide bond (Kline and Reddy, 1980). Both the affinity and the activity are highly specific, with a marked decrease observed in cross-species reactions (Wohl et al., 1983). The reaction product, plasmin, is a potent serine protease itself, with a variety of protein substrates. Its best characterized functions are related to hemostasis, since plasmin degrades fibrin clots. In addition, plasmin plays a role in various physiological and pathological processes by degrading extracellular matrix components such as laminin and fibronectin. It can also initiate hydrolysis of collagen type I and type IV by activating their respective latent collagenases (Kline and Reddy, 1980; Blasi et al., 1987; Liotta et al., 1981 and Dano et al., 1985). The biological significance and potential relevance of plasmin generation to metastasis is discussed later.

## 2. uPA inhibitors

uPA activity is blocked by serine protease inhibitors, such as E-aminocaproic acid (E-ACA), phenylmethanesulfonyl fluoride, diisopropyl-fluorophosphate, alpha tocopherol, metal ions (especially $Zn^{++}$) and $Ca^{++}$ chelators (Stroud et al.,

1975). In addition, the drug amiloride was found recently to inhibit uPA, but its mode of action is not clear (Vassalli and Belin, 1987). Apart from these blockers, there are two naturally-occurring inhibitors, called serpins (serine protease inhibitors)- PAI-1 and PAI-2 - which are produced by endothelial cells and by macrophages, respectively (Sprengers and Kluft, 1987; Blasi et al., 1987; Andreasen et al., 1986a and Cubellis et al., 1989). PAI-1 is thought to be the major regulator of uPA activity in-vivo because its ihibition, by binding to the active uPA form, is several orders of magnitude more rapid than the other major protease inhibitors in plasma (Blasi et a1., 1987 and Andreasen et al., 1986a). PAI-1 was reported to be more effective when bound to cell membrane (Pollanen et a1., 1987 and Sakata et a1., 1988). The secreted inhibitor is 90% inactive due, in part, to its interaction with specific PAI-1 binding protein, also found in plasma (Declerck et a1., 1988). However, in conditioned medium from cell cultures, PAI-1 can be activated by denaturants, such as detergents and mercapthoethanol (Declerck et a1., 1988 and Andreasen et a1., 1986 a,b).

## 3. uPA receptors

Cells which produce and secrete uPA usually bind it to

their outer membrane via specific, high-affinity receptors (Kd-$10^{-10}$M) (Nielsen et al., 1988; Blasi et al., 1986 and Vassalli et al., 1985). The receptor-binding domain resides on the amino terminal of the A chain, the so called "growth factor" region of the enzyme. Receptor-bound uPA is not internalized and its turn-over rate is slow ($t_{1/2}$- 5 hours) (Blasi et al., 1987). The presence of membrane uPA receptors allows cells to acquire surface-bound plasmin generating ability. This results in focalization of tissue degradation activity in the pericellular enviroment, which appears as an optimal configuration for effective matrix degradation and subsequent cell migration (Pollanen et al., 1987).

## 4. Plasminogen activators in biological processes

Plasminogen is activated, either directly or indirectly, by several other enzymes as well, such as tissue plasminogen activator (tPA), kallikreins, thrombin and streptokinase (Kline and Reddy, 1980 and Dano et al., 1985). Of these, only tPA is physiologically relevant since the activation rate by blood coagulation factors is much slower than the clearance rate of plasminogen and streptokinase is a bacterial product not synthesized by eukaryotic cells. tPA is a 70 KDa protein, encoded by a gene unrelated to uPA (Dano

and Reich, 1978 and Rajput et al., 1985). This enzyme is produced by endothelial cells, its level in plasma rises sharply upon venous occlusion and its activation is accelarated markedly by binding to fibrin (Dano et al., 1985). On the other hand, uPA is produced in large quantities in various physiological processes which involve tissue degradation (as in the case of mammary involution [Ossowski et al., 1979]), cell migration and invasion (as seen in embryo implantation [Strickland et al., 1976] and in the inflammatory reaction following tissue injury [Berman et al., 1980]), and angiogenesis (Goldfarb et al., 1986). For these reasons, tPA is thought to have a preferential role in the maintenance of hemostasis, while uPA is implicated in the physiology and pathology of tissue remodeling and cellular migration and invasion.

## 5. uPA putative role in metastasis

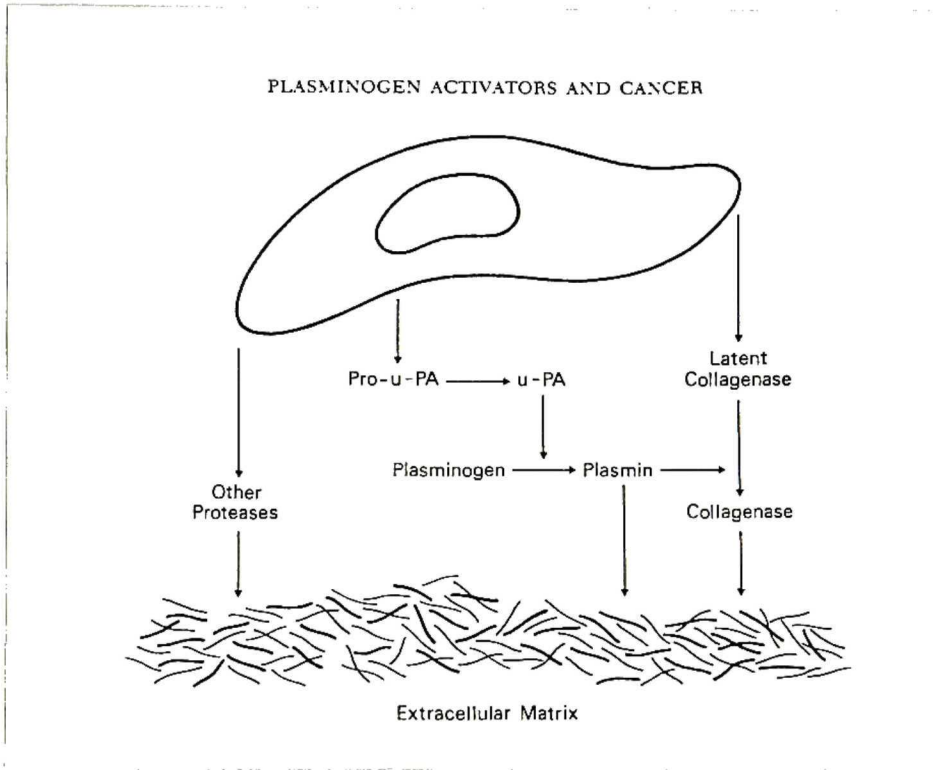
Metastatic cells have to be equipped for invasion and degradation of tissues and connective matrix components. One possible mechanism for such processes is the utilization of proteolytic enzymes. Indeed, metastatic cells have been shown to elaborate various types of proteases, some exhibit broad spectrum of activity (stromelysin or trypsin [Galloway et al.,

1983 and Murphy et al., 1981]), while others are more substrate-specific (collagenases type IV and type I or elastases type I, II and III [Goldfarb, 1982; Quigley, 1979; Dano et al., 1985 and Zetter, 1990]). Of these proteases, urokinase-type plasminogen activator seems to be the most important quantitatively due to the unique features of both its substrate and its product (Reich et al., 1988; Liotta et al., 1981 and Dano and Reich, 1978). uPA generates plasmin, which acts directly on extracellular matrix components, such as fibronectin and laminin (Liotta et al., 1981 and Dano et al., 1985). In addition, plasmin is capable of activating latent proteases, such as collagenases type I and type IV which, in turn, degrade the scaffold structure of the extracellular stroma and basal lamina, respectively. In that way, uPA activity can initiate a proteolytic cascade leading to dissolution of virtually all matrix components (Liotta et al., 1983 and Mignatti et al., 1986). Since the zymogen plasminogen is abundant in the plasma and in the interstitium, the rate limiting factor for initiation of the proteolytic cascade is the conversion of plasminogen into the active form, plasmin. uPA may, therefore, play a central regulatory role in initiating tissue degradation via the direct and indirect actions of plasmin (Mignatti et al., 1986 and Ossowski, 1988). Figure 3 depicts the putative role of uPA in the initiation of tissue degradation and metastasis.

Indeed, uPA was found to be over-produced by various

Figure 3

The putative role of uPA in regulating proteolytic events in metastasis




Some cancer cells produce uPA, which generates plasmin. According to the hypothesis depicted here, plasmin degrades certain matrix components directly (i.e., fibronectin and laminin) and promotes hydrolysis of other components as well, by activating latent proteases, such as collagenases type I and type IV.

tumors, such as human breast and colon carcinomas (DeBruin et al., 1987 and Ossowski et al., 1979), both spontaneous and induced murine mammary tumors (Dano et al., 1985; Colombi et al., 1986 and Pereya-Alfonso and Bal de Kier, 1986), and established neoplastic human lines (Quax et al., 1990). Moreover, in human colon tumors, a positive correlation was found between the aggressiveness of the tumor cells, their invasive nature and an increase in their uPA production (Cajot et al., 1986 and DeBruin et al., 1987). In addition, neoplastic transformation by RNA viruses had been associated with increased production of uPA. For instance, RSV-infected chicken fibroblasts were shown to produce uPA, which was undetected in their normal couterparts. Moreover, in cultures infected with the temperture-sensitive virus, uPA activity appeared only at the permissive temperature. Conversely, anti-uPA antibodies reversed the morphology of these fibroblasts to the less transformed phenotype (Sullivan and Quigley, 1986). In another study, immunohistochemical staining of metastatic Lewis lung tumor showed the highest intensity in areas with invasive growth and degradation of normal parenchyma (Skriver et al., 1984).

However, uPA involvement in malignancy is still a controversial issue in light of other reports showing no evidence for a correlation between uPA and malignancy (Dano et al., 1985). In addition, since increased uPA activity was also associated with rapid cellular proliferation in normal

tissues (such as gastrointestinal tract epithelium and uterine endometrium [Larsson et al., 1984]), the higher expression of this enzyme in neoplastic cells was suggested to be a non-specific phenomenon, associated with the general enhanced mitotic activity of malignant cells (Mira-Y-Lopez and Ossowski, 1987).

In light of these findings, it appears that while the hypothesis of uPA involvement in metastasis seems logical and attractive, conclusive evidence for it is still unsatisfactory. The research studies described here were conducted in order to re-examine this hypothesis and to elucidate the extent of uPA contribution to secondary colonization of human malignant cells.

## RATIONALE FOR THE STUDIES AND EXPERIMENTAL APPROACHES

The fact that the high mortality rate in cancer patients is, in most cases, the outcome of tumor cells' metastasis stresses the need to develop alternative and novel methods for early diagnosis and therapy of this condition. Improvemnet of current treatment modalities could be achieved with identification of properties unique to the metastatic cells. Since metastatic cells have to migrate into and invade naturally-impermeable biological barriers, efficient proteolysis could, possibly, be one of their distinctive characteristics. Indeed, a variety of proteases have been described in association with malignancies. uPA is quantitatively the most important of them. However, evidence for a correlation between uPA expression and metastatic capability or for a functional role of this enzyme in tumor metastasis, is still inconclusive. Therefore, the goal of this research project was to examine the involvement of uPA in secondary tumor colonization and to evaluate its role in conferring metastatic ability to neoplastic cells.

Several considerations had to be addressed in designing the appropriate experimental studies. Metastatic cells represent only a minute fraction of the primary tumor cells and are unique in that they can successfully complete all stages of the metastatic process. For this reason, it was

important to demonstrate that uPA expression is a characteristic associated with the metastatic and not the non-metastatic tumor cells, as opposed to comparing them with normal cells. Accordingly, the experiments had to include both metastatic and non-metastatic tumor cell variants, preferably from the same origin. It was also crucial to examine the metastatic cells' behavior in-vivo, in addition to performing in-vitro assays. In-vitro experiments focus on isolated cellular properties and, thus, do not address fully the complexity of the metastatic process. In addition, human cellular models are more desirable for such studies, as data accumulated so far indicates discrepancies between human and animal malignancies, making studies in cellular human cancer models potentially more applicable in the clinical setting.

In light of these considerations, the first steps of this research project involved the development of a human cellular model adequate for in-vivo studies of metastasis in athymic mice. Since human cancer cells do not efficiently colonize secondary organs in animals, transformation by ras oncogenes was considered. ras oncogenes were demonstrated to induce tumorigenicity and even metastatic potential in established animal cell lines, raising the possibility that this approach could be successful in human cells as well.

In examining the involvement of uPA, the experiments were based on tests of the catalytic activity of the enzyme, in addition to the demonstration of its biosynthesis and level

of transcription. The latter parameters do not always correlate with the net biological activity of uPA, due to post-translational modulations (e.g., inhibitor presence or low activation rate of pro-uPA). In addition, as evident from the discussion above, subcellular localization of uPA may be an important parameter affecting uPA stability and the effectiveness of its action. Therefore, the experiments were designed to test and compare surface-bound uPA activity versus the secreted form.

Finally, in order to determine whether uPA has a functional role in metastasis, the enzyme activity of the metastatic cells was blocked and the effect of this inhibition on the dissemination and colonization of the malignant cells was examined. In this set of experiments, appropriate controls were used to exclude the possibility of non-specific effects due to the antibodies binding on the cells' surface.

# MATERIALS AND METHODS

## A. REAGENTS

### 1. Tissue culture

Dulbecco's modified essential medium (DMEM), Eagle medium (EMEM) and Noble agar (used in ancorage-dependent growth test) were purchased from S&S Media Inc., Rockville, MD. Fetal calf serum (FCS) was from Gibco, Grand Inland, NY. Phosphate-buffered saline (PBS, 10mM $NaP0_4$/145mM NaCl pH 7.4) and trypsin buffer without $Ca^{++}$ and $Mg^{++}$ were routinely made by the central Pathology department facilities. Bovine serum albumin (BSA) was from Sigma, St. Louis, MO.

### 2. Antibodies

Murine monoclonal antibodies against human uPA were from American Diagnostica Inc., New-York, NY. One of them (# 394) is inhibitory, while the other one (# 3922) binds to the B chain but without a neutralizing effect. Anti-HLA class 1 monoclonal antibodies were a generous gift from Dr. Stephen Shaw at the NINCDS, Bethesda, MD. The stock solution was conditioned medium of the hybridoma cells producing the

antibodies. Goat anti-mouse antibodies, conjugated to fluorescein isothionate (FITC-goat anti mouse) was purchased from Cappel laboratories, Cochranville, PA.

3. Motility and chemotaxis assays

The modified Boyden chambers and the polycarbonate filters (8 uM pores, 25x80 mm with no PVP coating) were obtained from Nuclepore, Pleasanton, CA. Basement-membrane Matrigel coating, consisting of laminin, collagen type IV, heparan sulfate and entactin, was purchaed in powdered form from Collaborative Resaerch Inc., Bedford, MA. Giemsa stain was purchased from Sigma.

4. RNA preparation and Northern blots

Nonidet P-40 (NP-40), NaCl, ethylenediaminetetra acetic acid (EDTA), isoamyl alcohol, ethidium bromide, glycerol and (3-[N-morpholino])propanesulfonic acid (MOPS) were purchased from Sigma, St. Louis, MO. Sodium dodecyl sulfate (SDS) and bromophenol blue were purchased from Bio-Rad, Richmond CA. Chloroform was from Mallinckrodt, Paris,

Kentucky. Phenol, agarose and formamide were from BRL, Gaithersburg, MD. Ethanol- from Midwest Grain Products, Pekin, Il. Sodium acetate and formaldehyde (37% w/w) were purchased from Fisher Scientific Company, Fair Lawn, NJ. Nitrocellulose membranes were from Schleicher & Schuell, Keene, NH. Polaroid films were purchased from Polaroid, Cambridge, MA. XAR5 X-ray film- from Kodak, Rochester, NY, and $^{32}$P-dCTP was from NEN (DuPont), Boston, MA.

## 5. Plasmid amplification and DNA transfection

Bacto-Tryptone, yeast extract and agar were purchased from DIFCO, Detroit, MI. Ampicillin, glucose, $NH_4OAc$, Triton X-100 and RNAse A were from Sigma. Lysosyme (from hen egg white) and proteinase K- from Boehringer Mannheim. Restriction enzymes were obtained from different companies, including BRL, Beohringer Mannheim and Biolab New-England. Lipofectin was purchased from BRL and geneticin (G418)- from GIBCO.

## 6. DNA probes

The probe for human histocompatibility class 1 was a

4kb HindIII/ECoRI fragment of the cDNA HLA A3 gene and was generously provided by Dr. Cowan (Cowan et al., 1985). For mouse histocompatibility class 1, a 550bp PstI fragment of the H-2L cDNA was obtained from Dr. G. Jay (Tanaka et al., 1983). v-Ki-ras 6.2kb EcoRI fragment and Ha-ras cDNA were purchased from Oncor, Gaithersburg, MD. The probe for the a-1 chain of human type 1 collagen was a 1.8kb cDNA fragment and was a gift from Dr. Ramirez (Chu et al., 1982). Porcine uPA cDNA was a generous gift from Dr. Nagamine (Nagamine et al., 1984) and was amplified and cut in our laboratory (Xbal, 2.5kb fragment). Human uPA cDNA (Verde et al., 1984) was purchased from ATCC, amplified and cut in our laboratory (PvuII-NdeI, 0.8kb). The probe for PAI-1 was a 2.0kb BamHI fragment of the pcD-X plasmid, which was a gift from Dr. P. Andreasen (Andreasen et al., 1986b). Details of the amplification and isolation of the DNA plasmids are provided in the Methods section.

## 7. DNA plasmids

pEJ-ras (Shih and Weinberg, 1982) and pSV2neo (Southern and Berg, 1982) were made available by Dr. C. Laughlin from the Pathology department at USUHS. pCD-X, which contains the complete human PAI-1 cDNA linked to the Okayama-

Berg expression vector was a gift from Dr. P. Andreasen (Andreasen et al., 1986b and Okayama and Berg, 1983).

8. Fibronectin degradation assay

$^{125}$I- fibronectin was purchased from ICN, Irvine, CA. Human plasminogen and porcine plasmin were from Sigma.

## B. EXPERIMENTAL METHODS

## in-vitro ASSAYS FOR DETERMINATION OF CELLULAR PROPERTIES

### 1. Tissue culture conditions

The TE-85 human osteosarcoma cell line, designated HOS, and its v-Ki-ras transformant line, KHOS/NP (R-970-5), were obtained from ATCC, Rockville, MD. They were maintained in DMEM, supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics (DMEM/FCS). The cells were routinely grown to sub-confluency and passed once a week to fresh

medium, up to 16 passages. Cultures used for experiments were at the logarithmic phase of growth, unless specified otherwise.

To determine the appropriate conditions for harvesting the cultures at the logarithmic phase, a growth curve was established prior to the experiments. The cells were seeded at a concentration of $10^6$ cells/ 75 $cm^2$ tissue culture flask in total of 14 flasks, and were counted (details for cells' detachment and counting methods are given below) each day thereafter, for 7 days, in duplicates. It was found that between days 2 and 5, the cultures were at the logarithmic phase. This was confirmed by the general appearance of the cultures, as at that phase they were not confluent and the cells covered 50%-75% of the flask area. Therefore, for the experiments described below, the cells were usually seeded 48 hours earlier.

## 2. Soft-agar assay for determination of cellular transformation

The growth of cells in soft agar (i.e., anchorage-independent growth) is one of the *in-vitro* characteristics of neoplastic cells. In this assay, $1\text{-}5x10^4$ cells/ml were suspended in EMEM/FCS containing 0.35% agar and 2 ml of the

suspension were then poured on pre-layered, solidified 0.9% agar/ EMEM/ FCS, in 60 mm plates. The soft-agar layer containing the cells was allowed to solidify at room temperature, and the plates were then incubated at $37^0$C, 5% $CO_2$. Cells growing on the soft agar formed colonies, which could be detected within 10 days, and were usually counted 2 weeks after plating.

3. Motility and chemotaxis

Increased motility, response to autocrine chemo-attractants, and the capability to invade the basement-membrane are associated with the development of malignant phenotype. Both spontaneous and directed migration of the cells through a basement membrane-like layer were measured in these assays, using the modified Boyden chamber.

The chemoattractants used in the assays were serum-free conditioned media from 24-hour cultures of HOS, KRIB or 3T3 cells. The conditioned media were collected, centrifuged for 20 minutes at 1100 Xg to remove dead cells and cell debri. The supernatants were transferred to clean tubes, and were used immediately in the assay, as described next, or were stored at $-20^0$C. Frozen conditioned media could be stored for 30 days without apparent loss of activity.

The artificial basement-membrane was prepared by coating a porous polycarbonate filter (25X80 mm) with 600 ul fresh Matrigel solution made in 4$^0$C $H_2O$, at a concentration of 0.1 mg/ml, and allowing the Matrigel to solidify at room temperature. The filter was then placed with the Matrigel coating facing up, on top of the bottom wells (total of 24 wells) of the modified Boyden chamber. These wells contained 40 ul of either chemoattractants (for test of directed motility) or DMEM alone (test of spontanous motility).

The cells to be tested were detached from the plates by a 3 minute treatment with 1.5 ml 0.5% trypsin solution in PBS at room temperature, and were pelleted by centrifugation at 1100 xg for 10 minutes at room temperature. The pellets were resuspended in DMEM, at final concentration of 0.5x10$^6$ cells/ ml, and 50 ul aliquots were placed in the upper wells of the Boyden chamber, so that the cells would have to invade through the Matrigel layer as they move towards the chemoattractant. After a 6 hour incubation at 37$^0$C in a 5% $CO_2$ incubator, the Boyden chamber was disassembled and the filter was removed, dipped in 5 ml methanol for 2 minutes and air-dried in order to fix the cells. Staining was carried out in 5 ml 4% Giemsa solution for 15 minutes. The cells on the upper face of the filter (those which did not migrate through) were removed by gentle scraping with a wet cotton swab. The number of cells which crossed the filter was determined microscopically.

## HANDLING OF THE MICE AND in-vivo PROCEDURES

### 1. Experimental metastasis

Athymic female mice, 4 weeks old, were received from the Division of Cancer Treatment, NCI Animal Program, Frederick Cancer Research Facility. They were kept in semi-sterile conditions, with food and water ad lib.

The cells to be tested for their metastatic potential, were detached from the plate, as described above, and were incubated for 1 hour in 10 ml DMEM/FCS at room temperature to allow recovery of surface membrane components. The viability of the cells was usually >95%, as determined by the trypan blue exclusion test. Prior to injection, the cells were washed twice with DMEM by centrifugation (see details above) and were resuspended to a final concentration of $10^6$ cells/ml.

Inoculation into the tail vein of the recipient mice was carried out under sterile conditions, using 1 ml disposable syringes and $25G^3/_4$ hypodermic needles. In order to make the procedure easier and less stressful, slight vasodilatation was induced by keeping the mice under a 100 watt lamp for 15 minutes prior to the injection. The mice were then placed in a harness, with the tails exposed, and 0.1 ml of the cell suspension ($10^5$ cells per mouse) was injected into

the lateral vein.

In the in-vivo blocking experiments, the cells were treated with anti-uPA monoclonal antibodies after the 1 hour recovery period with DMEM/FCS. Two types of antibodies were used: one blocks 98% of uPA activity at a concentration of 0.1 mg/ml (# 394), whereas the other binds to the B chain, on which the active site is located, but with no neutralizing effect (# 3922). The antibodies were dissolved in PBS and were added at a final concentration of 0.1 mg/ml, to $10^6$ cells in 100 ul DMEM/ 1% bovine serum albumin (BSA). Control cells were treated with PBS/BSA alone. After incubation for 40 min at room temperature, the cells were washed twice with DMEM, brought to final concentration of $10^6$ cells/ml, and injected I.V. as described above.

2. Surgical procedures

Metastatic colonies were usually counted 5 weeks after the I.V. transplantation. For that purpose, the animals were sacrificed by cervical dislocation, which is a swift and painless method, and the rib cage was opened using sterile scissors. The lungs were removed, washed briefly with sterile PBS, and examined for the presence of visible nodules on the surface. In several experiments, a few such isolated nodules

were excised and placed in culture dishes, in order to re-culture the cells from the metastatic lesions. The cells were partially dissociated by gentle teasing and mincing of the tissue with sterile scissors, and were allowed to grow in tissue culture conditions, as described above.

For enumeration of the total pulmonary colonies, the lungs were fixed for 24 hours in Bouin's solution. In the fixed organs, the tumors appeared as white nodules against the diffused gray background of the normal tissue, which enabled better detection, especially of the smaller-size nodules. After counting the tumor colonies, samples of the fixed lungs were processed for histological analysis as well (see details in the section describing histological analysis). Other organs, such as heart, liver, kidneys, and spleen were screened in the same fashion in order to establish the pattern of the metastatic spread.

3. Subcutanous transplantation of cells

The cells to be transplanted subcutanously were prepared in the same manner as for the I.V. injection. However, they were brought to a final concentration of $5x10^6$ cells/ml, so that $0.5x10^6$ cells were introduced into each subcutanous site. Local implantations were carried out using

the same syringes and needles as for the I.V. transplantation, and the cells were injected under the skin, close to the superficial epidermis, usually at the shoulder level (2 sites per animal). KHOS cells gave rise to detectable tumors within 10-14 days and AD cells- after 50 days. Therefore, cell lines which did not produce tumors within 120 days post-inoculation were considered non-tumorigenic.

The mice were sacrificed, usually one week after the first detection of subcutanous growth, when the tumors were still relatively small, and when the animals appeared otherwise healthy without signs of emaciation. The tumors were excised, fixed, and processed for histological analysis (see below). In order to evaluate the cell population composition in the subcutanous growth, tumors were excised under sterile conditionds and re-cultured.

## HISTOLOGY AND ELECTRON MICROSCOPY

The organs (lungs, hearts, spleens etc.) or subcutanous tumors were fixed in Bouin's solution (picric acid, formalin, glacial acetic acid at 15:5:1 vol/vol) for 24 hours, were embedded in paraffin at $56^0$C, then sectioned and stained for routine analysis with hematoxylin-eosin. Some sections were stained instead with Alizarin red for analysis

of $Ca^{++}$ deposits or for keratin presence. In these staining procedures, embryonic bone tissue was used in parallel as a positive control.

For electron microscopy, the tissues were fixed in 1% glutaraldehyde/ 4% formaldehyde in 0.1M phosphate buffer, pH 7.2 and were sectioned, stained, and photographed by the electron microscopy facilities at USUHS.

## MOLECULAR BIOLOGY ASSAYS

### 1. Purification of cytoplasmic RNA

Cells (usually $3\text{-}4x10^7$) were harvested and washed twice with cold PBS according to the protocol detailed above. The cell pellet was lysed on ice in 200 ul of 1% NP-40/ 10mM NaCl/ 1.5mM $MgCl_2$ for 30 minutes, and then centrifuged at 47K xg to pellet the nuclei fraction. The supernatant was transferred to clean tubes (all equipment for RNA isolation was sterile and autoclaved, to ensure RNAse-free conditions), and treated with 500 ul SDS buffer (0.5% SDS/ 50mM NaCl/ 10mM EDTA/ 200mM TRIS-HCl, pH 8.0) to dissolve cytoplasmic proteins. Alkaline phenol (prepared by adding 1 part of 0.5M TRIS/10mM NaCl/10mM EDTA/0.5% SDS to 9 parts of water-

saturated phenol) and 4% chloroform in isoamyl alcohol were then added at equal volumes (usually 350 ul each), and mixed gently with the cell lysate. After centrifugation at 47K xg for 5 minutes, the upper layer was transferred to clean tubes, and treated with 700 ul of chloroform/isoamyl alcohol. The RNA, which was extracted in the upper, aqeuous layer, was precipitated by adding 70 ul of 3M sodium acetate (NaOAc) and 2 ml ethanol, and incubating it for at least 2 hours at $-20^0$C. (The samples were usually stored in the ethanol solution at $-20^0$C until used). The precipitate was pelleted at 47K Xg, at $4^0$C for 10 minutes, the supernatant discarded, and all traces of the ethanol were allowed to evaporate. The pellet was then resuspended in 1mM EDTA/ 10mM TRIS-HCl, pH 8.0. (TE buffer).

2. Small-scale analysis of RNA (and DNA) samples

RNA preparations were examined for composition, size, and purity by small-scale gel electrophoresis, prior to performing Northern blot assays. Thin 0.6% agarose gel was prepared by boiling the agarose in 15 ml of 4mM TRIS/ 2mM sodium acetate/ 2mM EDTA pH 8.0 (TGA buffer). Ethidium bromide was added (12 ul of 1 mg/ml solution) as the mixture cooled down, just before pouring. The RNA (or DNA) samples were mixed with 1/10 volume of loading buffer (50% glycerol/ 1mM EDTA/

0.4% bromophenol blue) and 5-10 ul of samples (approximately 1 ug total RNA in each) were loaded on the gel, after it had solidified. Markers' sample of 1Kb ladder DNA was included. Electrophoresis was carried out in TGA buffer, at 40-60 volts for 1 hour. The gel was then photographed on a U.V. light box, using a polaroid film.

3. Northern blot transfer (for mRNA analysis)

Total cytoplasmic RNA samples were denatured at $55^0$C for 15 minutes, in buffer containing 6.5% formaldehyde/ 50% formamide/ 20mM MOPS/ 5mM sodium acetate/ 0.5mM EDTA. Usually, 20 ug of each sample was loaded on the gel, and mixed with 1/10 volume of loading buffer (see above). 1% agarose-formaldehyde gel was prepared by boiling the agarose in water, and adding 4% MOPS/ 10mM sodium acetate/ 1mM EDTA (running buffer) and 6.3% formaldehyde. Ethidium bromide was added at a final concentration of 0.4 ug/ml just before pouring. Separation of the RNA molecules was carried out in running buffer, at 30-40 volts for 16-18 hours. After electrophoresis, the gel was neutralized by soaking in 3M NaCl/ 0.3M sodium citrate pH 7.0 for 20 minutes, and was placed on absorbing paper, soaked in 1.5M NaCl/ 150mM sodium citrate.

Transfer of the RNA from the agarose gel onto a

nitrocellulose membrane was carried out by blotting with 1.5M NaCl/ 150mM sodium citrate. The wet nitrocellulose membrane was placed on the gel, and a thick absorbing paper layer was pressed on top of it for 10 hours. When all the RNA had been transferred, the membrane was heat-dried in vacuum, and was stored covered, at room temperature.

4. Radioactive-labeling of DNA probes

Quantitative and qualitative analysis of mRNA of specific genes was carried out by hybridizing the RNA on the nitrocellulose membrane with a $^{32}$P-labeled DNA probe of the corresponding gene. Radioactive labeling of DNA probes was performed using reagents from a random-labeling kit, and following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Briefly, 20-50 ng DNA probe were brought to a final volume of 10 ul in $H_2O$, and denatured by 5 minutes boiling, followed by quick cooling on ice. A mixture of dATP, dGTP and dTTP at 25 uM each was added, together with 50 uCi $^{32}$P-dCTP. The reaction was initiated by adding buffer containing hexanucleotides and 2 units of DNA polymerase (Klenow enzyme). The reaction was stopped after 30 minutes at 37$^0$C, by adding 100 ul TE buffer. The labeled DNA was separated from free nucleotides on a sephadex column with TE

buffer containing 140mM NaCl.

5. Hybridization of radiolabeled DNA probes with immobilized cellular RNA

Cellular RNA, transferred onto nitrocellulose membrane as described above, was pre-treated with low molecular weight salmon DNA, in order to block non-specific binding sites by incubation at 42$^0$C in buffer containing 50% formamide/ 0.75M NaCl/ 7.5mM sodium citrate/ 10mM TRIS/ 0.1% SDS /5x Denharts solution. The $^{32}$P-labeled DNA probe, denatured by 5 minutes boiling, was added to the mixture 2 hours later, and was allowed to hybridize with the RNA for 18 hours at 42$^0$C. Low affinity hybridization forms were dissociated by washing the membrane with 0.3M NaCl/ 30mM sodium citrate/ 0.1% SDS, for 1 hour at room temperature, followed by another 1 hour wash at 50$^0$C with only 1/20 the concentration of the salts. The membrane was then dried and autoradiographed on XAR-5 Kodak film, at -70$^0$C.

6. Plasmid transfer into E. Coli (bacterial transformation)

DNA plasmids can be produced in large quantities (several milligrams) by introducing the plasmid into bacteria, where it can be replicated many fold. This procedure, called plasmid amplification, requires that the plasmid contains a bacterial expression vector and a selective marker gene, which confers drug resistance to the bacteria (usually to ampicilin). The pCD-X (Human PAI-1 gene-containing plasmid) was received as pure DNA and had to be introduced into bacteria first, in order to obtain large quantities. All other plasmids used in these studies were received already inserted in transformed E. Coli, and were amplified as described below.

pCD-X was inserted into E. Coli cells which were made permeable to macromolecules by stripping their cell wall (DH5 alpha strain, purchased from BRL). Fifty ul of a freshly-thawed bacteria suspension were mixed with ~20 ng plasmid DNA, incubated on ice for 30 minutes, and then transferred to a $42^0$C water bath for 45 seconds to permeabilize the bacteria and to allow penetration of the plasmid. The heat-shock was followed by brief cooling, and the bacteria were then incubated in 0.5 ml growth broth (LB medium, 10 mg/ml Bacto-Tryptone, 5 mg/ml Bacto-yeast extract, 5 mg/ml NaCl, pH 7.5) at $37^0$C for 1 hour to promote replication. Aliquots of 0.1 ml of the bacteria suspension were then seeded on solidified agar

plates containing LB and 100 ug/ml ampicillin. Because of the ampicillin presence, only transformed bacteria could grow on that medium. Several such single-cell colonies were transferred from the LB-agar plate (using a sterile bacteriological needle) to clean tubes, grown in 5 ml LB broth at $37^0$C for 18 hours, and examined for the presence of pCD-X DNA.

7. Small-scale preparation of plasmids from transformed bacteria

DH5-alpha cells from a 3 ml culture suspension were pelleted by centrifugation at 8K xg for 5 minutes at $4^0$C. The supernatant was discarded and the pellet was lysed in 100 ul of 50mM glucose/ 10mM EDTA/ 25mM Tris, pH 8.0 containing 200 ug lysosyme. After 30 minutes incubation at $4^0$C, 200 ul of 0.2N NaOH/ 1% SDS were added, followed by 150 ul of 3M NaOAc. The lysate was centrifuged for 10 minutes at 47K xg to pellet proteins and bacterial DNA. The plasmid DNA was precipitated by mixing the supernatant with 112 ul isopropanol and incubating this fraction at $-20^0$C for 1 hour. To remove RNA and protein contaminations, the pellet was treated with 0.1 ug/ml RNAse A in 200 ul $H_2O$ for 30 minutes at room temperature, followed by phenol-chloroform extraction (details

of phenol-chloroform extraction are given in the section desscribing isolation of RNA). The DNA was precipitated again, washed from the salt with cold 70% ethanol, and resuspended in 50 ul TE buffer. Approximately 5 ul aliquots were used in restriction digest analysis, and the digested samples were separated on a small-scale electrophoresis gel, as described above.

8. Amplification of DNA plasmids

Bacteria carrying the plasmid were seeded in 500 ml LB containing 100 ug/ml ampicillin, and were allowed to replicate on a shaking plate at $37^0$C. After 18 hours, the bacteria were centrifuged, as described previously, and lysed in 35 ml of 8% glucose/ 5% Triton x-100/ 50mM EDTA/ 50mM Tris, pH 8.0 (STET solution), to which 25 mg lysosyme were added. The lysate was heated in a boiling water-bath until the mixture became viscous, and was then cooled on ice. The denatured proteins were pelleted by centrifugation at 47K xg for 40 minutes at $4^0$C, and the supernatant was transferred to clean tubes containing 20 ml cold isopropanol. The DNA was precipitated by 10 minutes incubation at $-20^0$C, followed by 15 minutes spin at 47K Xg. The pellet was dried at room temperature, with the tubes inverted, and was then resuspended

in 6 ml $H_2O$. To remove RNA and protein contaminations, this solution was treated with 60 ug RNAse A for 30 minutes at $37^0C$, followed by phenol-chloroform extraction, as described previously. The DNA was precipitated with NaOAc and ethanol and was washed with cold 70% ethanol to remove the salt (details for precipitation and washes of DNA are given above). This fraction contained both chromosomal (bacterial) and plasmid DNA. The two DNA species were separated according to their size by a pZ523 column, following the manufacturer's instructions (5 Prime-3 Prime, Pooli, PA). At low ionic strength this column binds DNA molecules of various sizes but with increased salt concentrations only larger DNA molecules bind while the smaller-size molecules are washed through. The DNA fraction obtained from the transformed E. Coli was passed through the column in 1M NaCl/ TE, which allowed binding of the bacteria chromosomal DNA only, whereas the plasmid DNA was eluted. The plasmid was precipitated in ethanol, washed from the salt, as described above, and subjected to restriction digest analysis, as described previously, in order to confirm its characteristic sequence.

9. Gene transfer into human cells (transfection by DNA plasmids)

Two methods of plasmid transfection were tested on the HOS cells- the $Ca^{++}$ phosphate percipitation and lipofection. The efficiency of the latter proved higher. The transfected plasmids were pEJras and pCD-X (PAI-1 cDNA), and each was co-transfected with pSV2neo, as a marker for DNA uptake (successfully-transfected cells can subsequently grow in selective medium containing the drug geneticin [G418]).

Four ug of pEJras or pCD-X and 1 ug pSV2neo were mixed with 10 ug lipofectin in water, at a final volume of 33 ul. Controls included pSV2neo alone. After 20 minutes incubation at room temperature, the mixtures were added dropwise to HOS cells, which were plated 24 hours earlier in 35 mm dishes and were ~50% confluent at the time of transfection.

After 24 hours incubation at $37^0$C, the cells were detached from the plates with trypsin solution, as described above, and were re-plated in 100 mm plates (2 for each original dish). G418 was added the next day, at a final concentration of 350 ug/ml. Single-cell colonies, which grew in the selective medium for 10-14 days, were transferred to 100 mm plates, and when they were almost confluent, the cells were harvested and screened for the expression of the transfected gene by Northern blot analysis. In some cases,

the incorporation of the gene into the cellular genome was examined by Southern blot analysis as well.

## 10. Isolation of chromosomal DNA from human cells- the salting-out procedure

The cell cultures were harvested at confluency, and $30x10^6$ cells were washed and resuspended in 9 ml of 0.5M Tris/ 20mM EDTA/ 10mM NaCl, pH 9.0. They were lysed by adding 1 ml of 10% SDS and the proteins were digested by incubation with proteinase K (0.5 mg/ml final concentration) at $48^0$C for 5 hours. Two and a half ml of saturated NaCl solution were then added, and the proteins were pelleted by 30 minutes centrifugation at 8K xg. The supernants were transferred to clean tubes containing 26 ml ethanol at room temperature, and the chromosomal DNA was allowed to float and gather at the top. The DNA strands were collected using hook-tipped Pasteur pipets, rinsed by dipping the pipet in cold 70% ethanol, air-dried, and dissolved in 400 ul TE buffer. In some cases, larger volume and incubation at $37^0$C were required in order to dissolve completely the DNA. The samples were further cleaned from protein traces by phenol-chloroform extraction, were precipitated with 1M $NH_4OAc$/ 80% ethanol, and adjusted to 1 ug/ul concentration, according to their $OD_{260}$ absorbance

reading. The DNA was digested with ECoRI (BRL), according to the manufacturer's instructions, and after the restriction digest was completed, the DNA samples were separated by large-scale agarose gel electrophoresis.

## 11. Separation of HMW DNA by agarose gel electrophoresis and Southern blot transfer

The presence of foreign DNA in the cellular genome can be detected in samples of restriction-digested chromosomal DNA by virtue of forming different digestion pattern. Twenty ug chromosomal DNA samples were isolated from the cells before and after gene transfer, as described, were mixed with loading buffer, and separated by electrophoresis on 150 ml 1% agarose/ TGA gel containing ethidium bromide. The gel was then rinsed and irradiated by a UV transilluminator for 5 minutes twice in order to nick the DNA molecules, so that they could be transferred more readily onto the nitrocellulose membrane. This was followed by soaking the gel in 300 ml 0.5M NaOH/ 1.5M NaCl for 30 minutes to denature the DNA and remove traces of RNA. Before transferring to nitrocellulose, the pH of the gel was adjusted by a 1 hour wash with 300 ml 1M Tris/ 1.5M NaCl, pH 7.5.

Transfer to a nitrocellulose membrane and

hybridization with DNA probes were carried out the same way as for Northern blots.

## uPA ASSAYS

### 1. The fibronectin degradation assay for uPA activity

In this assay, uPA activity was determined indirectly by initiating the conversion of plasminogen to plasmin and measuring the consequent, direct proteolytic activity of plasmin on radiolabeled fibronectin. Since plasmin activity is very rapid, the rate-limiting step in this chain reaction is the generation of plasmin by uPA. Thus, uPA activity assumes an apparent first-order kinetics. The assay sensitivity range was determined by dose-response experiments, using known amounts of uPA for the standard curve (details are given below). It was found to be sensitive for detecting as little as 20 milliunits of uPA. This assay was used for detection of catalytically-active uPA in both the secreted and the surface-bound forms.

In preparation for the assay, $^{125}$I-fibronectin was immobilized on the surface of a flat-bottom 96 multiwell plate by dispensing 70 nCi in 50 ul PBS to each well, and allowing

the fibronectin to adhere to the plastic for 24 hours at $4^0$C. The excess, unbound $^{125}$I-fibronectin was then washed by cold DMEM. In the case of measuring surface-bound uPA, the cells to be tested were prepared by trypsinization and recovery in DMEM/FCS, as described above (see preparation for I.V. injection). They were then washed twice in DMEM, resuspended to $4x10^6$ cells/ml, and 200 ul aliquots were added to each fibronectin-coated well. Human plasminogen in 50 ul DMEM (1 milliunit/ ul) were added to each well to initiate the chain-reaction. Control wells for plasminogen-independent background activity contained no plasminogen. Total degradation was measured in separate wells to which plasmin alone had been added (50 milliunits/well) without cells. The plate was incubated at $37^0$C for 1 hour in order to allow degradation of the labeled fibronectin and the release of small, soluble radioactive fragments into the medium. Aliquots of 50 ul medium from the wells were then collected and read in a gamma counter. For measurement of secreted uPA activity, conditioned medium was collected from exponentially-growing cultures after maintaining the cells without serum for 24 hours. Aliquots of 200 ul conditioned medium were added to the wells, with and without plasminogen, as in the case of the cells' suspension. In some cases, the conditioned medium was concentrated 5-8 fold, to increase detection sensitivity.

A standard curve with known amounts of uPA was run in parallel. In these wells, the concentration range for uPA was

usually 0.5-50 Plough units/ ml, and they were prepared in duplicate, with or without plasminogen. This concentration range was chosen since the cells tested in these assays produced activity between 1 and 20 Plough units. However, prior to the experiments with cells, the sensitivity of the assay was established by testing it for a range of 1 milliunit to 100 units. The dose-response curve was linear in that range.

2. Immunofluorescence staining of surface-bound uPA

This assay was used to confirm the localization of uPA on the outer aspect of the cells and to compare the levels of bound uPA on HOS and KRIB cells. The cells were harvested and washed once with PBS, as described previously, and brought to a concentration of $10^6$ cells in 0.1 ml PBS/ 1% BSA. Anti-human uPA antibodies were added at a final concentration of 0.1 mg/ml, and allowed to interact with the surface-bound uPA for 40 minutes at room temperature. Controls for non-specific binding were run in parallel, using an IgG preparation from MOPC culture (Litton Bionetics, Rockville, MD) at the same concentration. (MOPC is a murine B-lymphoma line, producing high levels of IgG). To remove excess antibodies at the end of the incubation, the cells were washed twice in 1 ml PBS/

1% BSA by a 1 minute centrifugation at 47K xg, and the pellets were resuspended in 50 ul of FITC-goat anti mouse antibodies, diluted 1:50 from the stock solution provided by the manufacturer (Cappel, PA). Incubation was on ice, in the dark, for 30 minutes, and was followed by two washes with 1 ml PBS/ 1% BSA/ 0.1% $Na_3N$ (1 minute at 47 Xg). Treatment with $Na_3N$ minimizes antigen capping after antibody binding. To fix the cells, one additional wash was performed under the same conditions, using 2% formaldehyde in the wash buffer. The pellets were resuspended in cold 0.2 ml formaldehyde buffer, and subjected to FACS analysis 2-4 hours later. Flow cytometry was carried out in a Becton-Dickinson FACS 440, at NCI Bethesda, MD, using a 300 milliwatt argon ion laser beam, at a 488 nm excitation wavelength. It was supervised with the kind assistance of Dr. J.E. Cupp.

# EXPERIMENTAL RESULTS

# A. DEVELOPMENT OF A HUMAN CELLULAR MODEL FOR EXPERIMENTAL METASTASIS IN MICE

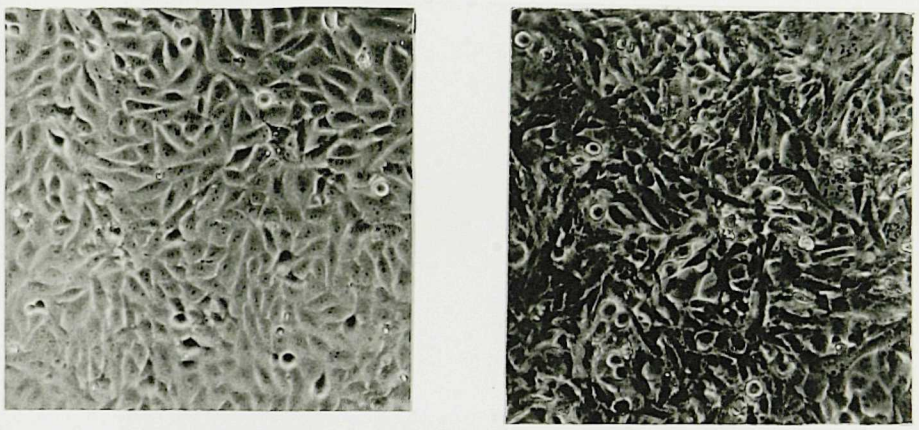
## 1. Transformation of human osteosarcoma cells by ras oncogenes

The cellular model system chosen for this study consisted of a human osteosarcoma cell line (HOS) and its subclones transformed by ras oncogenes. HOS is an immortalized, established line, originally obtained from a tumor of the distal femur (McAllister et al., 1971). In culture, HOS cells appeared as flat cells of irregular shape, and were contact-inhibited upon reaching confluency (Figure 4). They grew very poorly in soft agar (plating efficiency <0.5%), as characteristic of immortalized, non-transformed cells. HOS cells were non-tumorigenic in athymic mice.

Transformation of HOS by ras oncogenes was carried out by two different methods: infection with Ki-MuSV and transfection by pEJras. Following Ki-MuSV infection, a subline was obtained - KHOS - which had typical features of neoplastic morphology in culture. The cells were more elongated than HOS, they formed a criss-cross pattern and when confluent, they piled up and formed foci (Figure 4). Their growth was found to be anchorage-independent by the soft-agar test. KHOS cells were tumorigenic in immunodeficient mice (Rhim et al., 1975).

Figure 4

Morphology of HOS and KHOS cells in culture



HOS cells are flat, irregular cells which are contact-inhibited when confluent. In contrast, the v-Ki-ras transformed KHOS cells are spindle-like, they form criss-cross pattern and pile up in foci.

In the studies described here, these cells gave rise to detectable subcutanous tumors within 14 days after inoculation.

Transformation with EJ-ras was carried out by transfection of HOS cells with the EJras-containing plasmid, pEJras. In this case, the cells were co-transfected with the G418-resistance gene as a marker for DNA uptake and were subsequently transplanted subcutanously in order to select for tumorigenic subpopulations. One of the tumorigenic lines, designated AD, was chosen for further studies. AD cells formed subcutanous tumors which were encapsuled in a thick layer of fibrin and were first detected 50 days after transplantation. Thus, this cell line appeared to be less aggressive than KHOS. Control sublines were obtained by transfection with the G418-resistance gene in combination with carrier DNA. They did not become transformed, as judged by their flat morphology and contact-inhibited growth and by their failure to form subcutanous tumors within 120 days post-transplantation.

## 2. Isolation of ras-transformed human osteosarcoma subclones with metastatic ability in athymic mice

KHOS cells were not only tumorigenic, but metastatic as well. When injected I.V into athymic mice, they formed

visible nodules primarily in the lungs, which were detected and counted 5 weeks after transplantation. Tumors developed in the mediastinum as well and invaded the ribs. Other organs (liver, kidney, spleen, heart) had neither macroscopic nor microscopic foci.

Cells from isolated nodules on the lungs and the surface of the ribs were cultured for 7 days and, when re-injected into mice, were proven to maintain their metastatic ability (Samid and Mandler,1989). One of these lines, designated KRIB, which had the highest tumor yield, was chosen for further studies.

KRIB cells formed lung tumors in 88% of recipient mice (84 out of 96 treated mice in repeated experiments). In 50% of the examined animals, the tumor yield was over 10 nodules per mouse. The lung nodules were grossly visible as early as 19 days after inoculation, when they reached a size of 0.5mm in diameter (Figure 6). In most experiments they were counted after 5 weeks.

Another subclone of KHOS was isolated in-vitro and was designated KHOS-5. This subclone was metastatic but less aggressive than KRIB; KHOS-5 formed tumors in 50% of recipient mice, with a median number of 6 lesions per animal as determined five weeks after inoculation.

From AD, the EJ-ras transformant, two single-cell clonal lines were isolated in-vitro: AD15 and AD110. While both were tumorigenic and grew well in soft-agar, only the

AD15 line was metastatic. However, AD15 was less aggressive than KRIB cells, as judged by the size and number of the pulmonary metastases and the period required for the lung nodules to become visible.

The HOS-derived cellular system is comprised, therefore, of closely-related cell lines which represent different stages of tumor progression. The phenotypic changes were acquired following ras transformation. Figure 5 is a flow chart of the sequential steps in the isolation of these subclones. The phenotypic variations of the HOS subclones are summarized in Tables I and II.

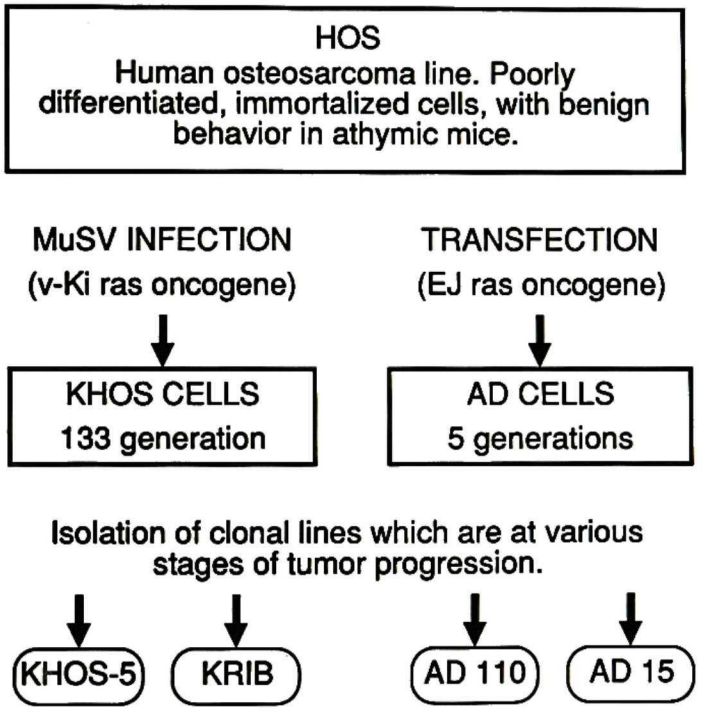
## B. CHARACTERIZATION OF THE ras-TRANSFORMANT HOS SUBCLONES

### 1. Histology and electron microscopy

Histological examination of the pulmonary metastases revealed highly invasive tumors, composed of poorly differentiated neoplastic cells with scattered inclusions which are typical of osteosarcomas (Figures 7 and 8). Keratin staining was negative, as expected of sarcoma cells, and there was no evidence for calcium deposits, which is in line with the poor differentiation state of the cells. Electron

Figure 5

Sequential steps in the development of the HOS model system




HOS, the parental cell line, was established from a human osteosarcoma tumor in the distal femur. KHOS and AD were derived from HOS by ras oncogene transformation (v-Ki-ras and EJ-ras, respectively). From KHOS, two subclones were isolated: KHOS-5 (a single-cell clonal line, isolated in-vitro) and KRIB (established from an individual pulmonary metastase). From AD, two additional single-cell clonal lines were obtained: AD15 and AD110. While KRIB, AD15 and KHOS-5 were metastatic in athymic mice, AD110 was only tumorigenic, and the parental HOS cell line was neither tumorigenic nor metastatic.

Table I

TUMORIGENIC PHENOTYPES OF THE HOS-DERIVED CELL LINES

| CELL LINE | TUMORIGENICITY | |
|---|---|---|
| | tumor-bearing mice | time when first detected (days) |
| HOS | 0/4 | * |
| AD | 3/4 (75%) | 50 |
| AD110 | 3/4 (75%) | 15 |
| AD15 | 3/4 (75%) | 15 |
| KRIB | 4/4 (100%) | 7 |

In the tumorigenicity assay, $5X10^5$ cells were injected subcutanously. The time when tumors were first visibly detectable is indicated.

* No subcutanous tumors were detected within 120 days post-inoculation

Table II



METASTATIC PHENOTYPES OF THE HOS-DERIVED CELL LINES

| CELL LINE | METASTATIC YIELD | | TIME WHEN* FIRST DETECTED |
|---|---|---|---|
| | tumor-bearing mice | tumors/animal median (range) | (days) |
| HOS | 0/5 (0) | 0 | |
| AD | 0/7 (0) | 0 | |
| AD110 | 0/7 (0) | 0 | |
| AD15 | 9/13 (70%) | 2(0-6) | 70 |
| KHOS-5 | 6/12 (50%) | 6(0-80) | n.d. |
| KRIB | 9/12 (75%) | 9(0-100) | 19 |

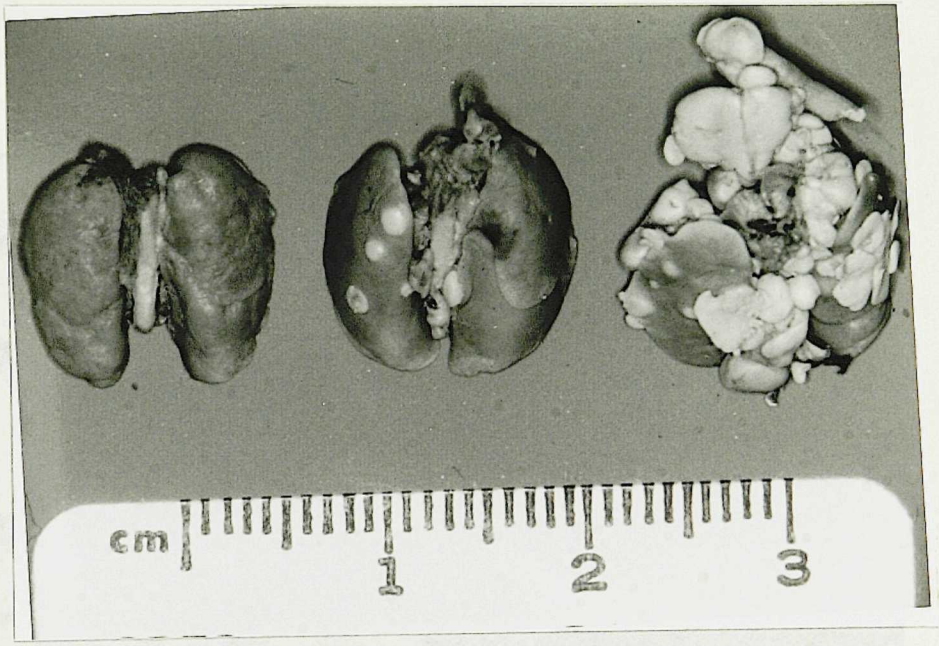
In the metastatic assay, $10^5$ cells were inoculated I.V. Lung tumors were counted after 35 days (KRIB and KHOS-5). By day 35, AD15 lesions were still undetectable, thus the values for AD15 are from 70 days post-inoculation. HOS, AD, and AD110 were negative even after 90 days.

* Pulmonary metastases were first grossly visible when they reached a size of 0.5 mm in diameter.

n.d. Not determined.

Figure 6

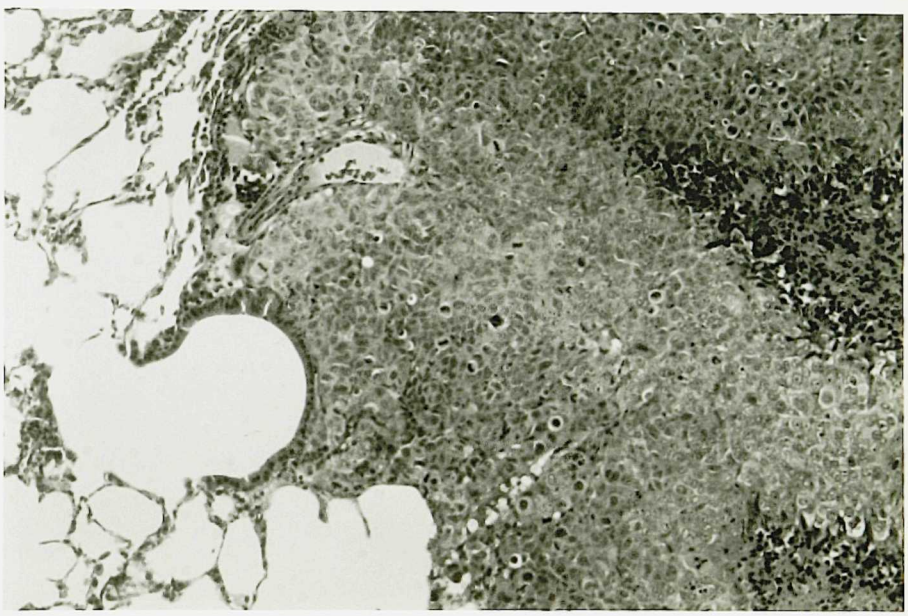
Gross appearance of the pulmonary sarcoma tumors in athymic mice




Athymic mice were challenged with $10^5$ cells of either HOS, AD15 or KRIB by I.V injection. Pulmonary tumors, which appeared as soft, white nodules on the lung surface, were counted 5 weeks later. The lungs were fixed in Bouin's solution to improve detection of small nodules. **Left,** HOS cells did not form detectable tumors. **Center**, few isolated metastases were detected after AD15 inoculation. **Right**, over 80 tumors developed in the lung after KRIB cells' inoculation. They were large, sometimes fused together and displaced the lung tissue.

Figure 7

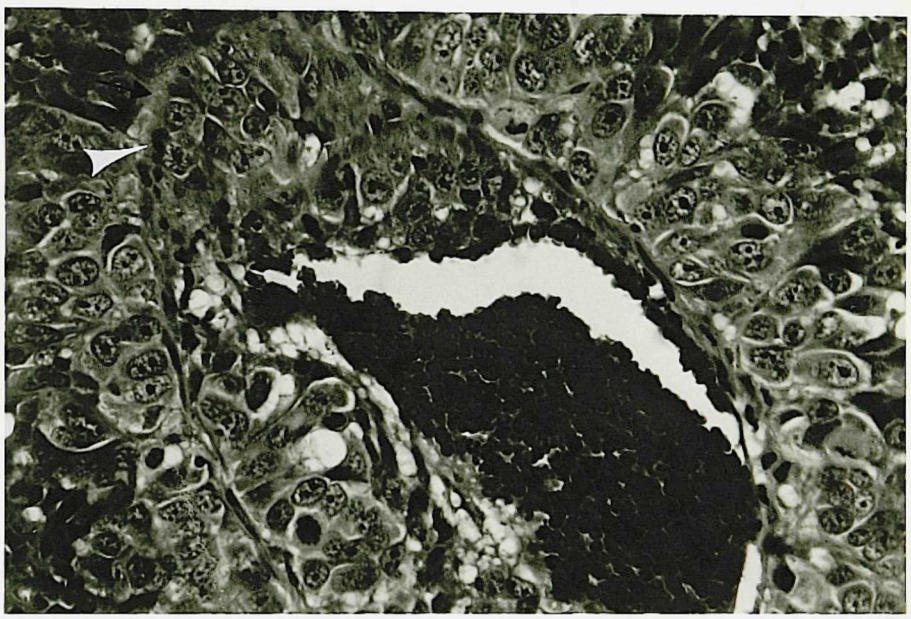
Microscopic section of a pulmonary tumor



Low magnification (125X) photograph of a pulmonary lesion section, stained with hematoxylin-eosin, showing enlarged, poorly-differentiated neoplastic cells with a high ratio of mitotic figures. On the left side, an unaffected lung area. Note the necrotic area on the upper right side.

Figure 8

Invasion of pulmonary tumor cells through a blood vessel wall



KRIB tumor cells are shown invading through the endothelial layer of a pulmonary blood vessel (312X, H&E staining). At the site of invasion, endothelial cells have been displaced by the tumor cells (arrow), while the structure of the vessel wall is preserved in other areas. The lumen is indicated by the presence of a blood clot.

photomicrograph depicted rapidly-dividing cells with enlarged, aberrant nuclear morphology and two prominent nucleoli. It also showed deposition of collagen and no evidence of tight junctions, which are characteristics of sarcomas (i.e., mesodermally-derived tumors) (Figures 9 and 10).

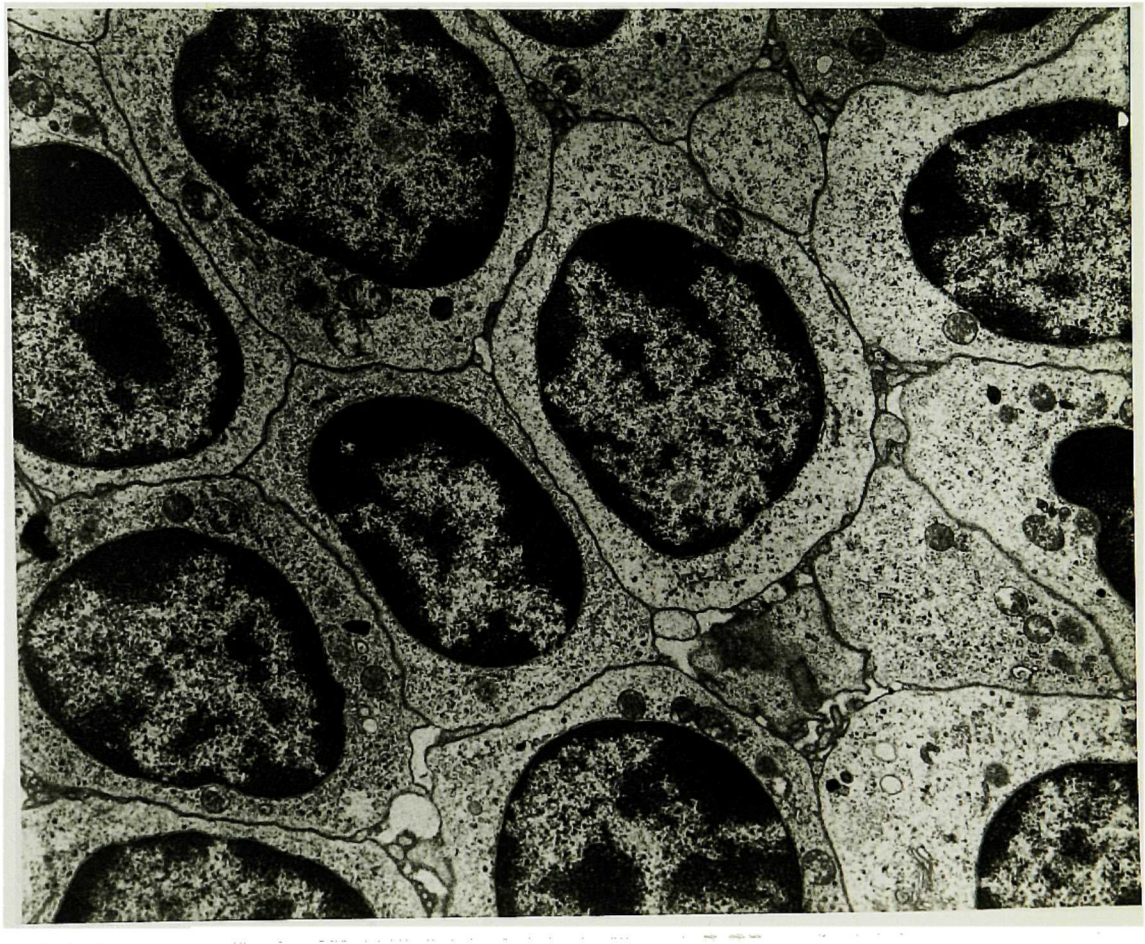
Production of collagen type I was confirmed by Northern blot analysis as well. As seen in figure 11, high transcript levels were detected in the parental, "benign" HOS cells and was lower in the metastatic subclones KHOS-5 and KRIB.

2. Species origin

Northern blot analysis, using DNA probes for both human HLA gene (A3 class I) and mouse H-2L gene revealed that prior to transplantation in mice, HOS-derived cells expressed the human histocompatibility gene but not the corresponding murine gene, as expected (Figure 12, the lanes for HOS and KHOS. The mouse cell lines, 3T3 and B16, are shown for comparison). The same pattern was observed in cells collected from tumors which developed in the recipient mice (both subcutanous and pulmonary), indicating that these tumors were indeed comprised of human, and not mouse, cells. (Figure 12-AD, AD15 and AD110 lanes are of cells from subcutanous tumors,

Figure 9

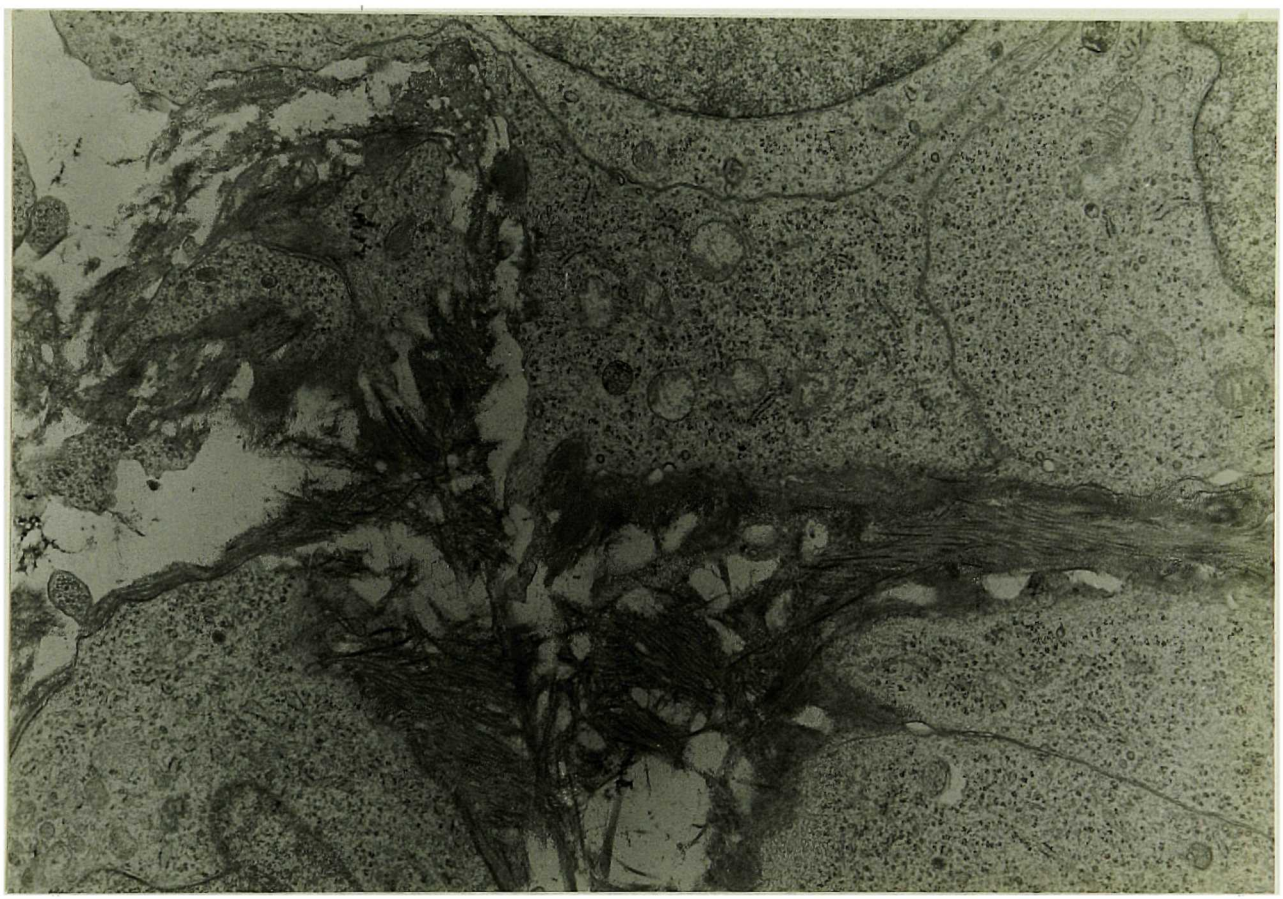
Electron photomicrograph of KRIB tumor cells in the lung



The cells are poorly-differentiated, with high nucleus: cytoplasm ratio and puffy chromatin. There is no evidence for tight junctions between the cells, which is characteristic of mesodermally-derived tissue. (7250X).

Figure 10

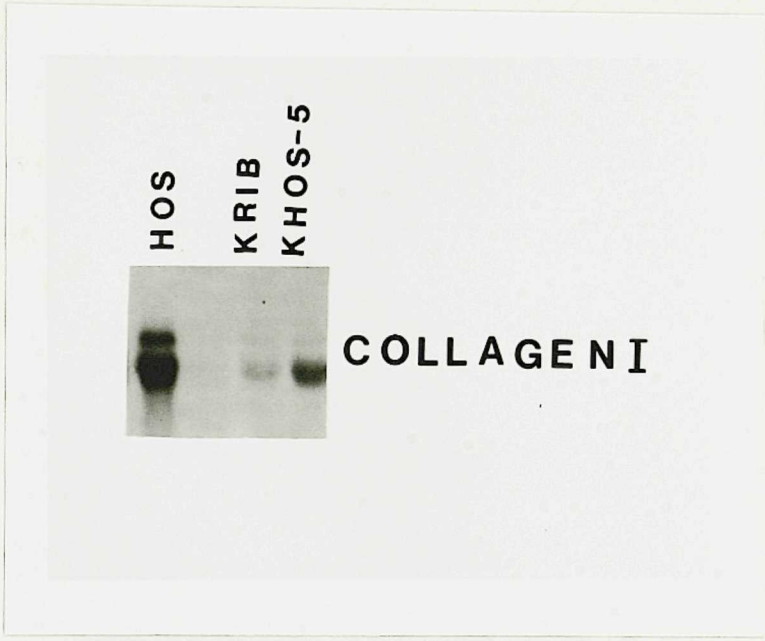
Electron photomicrograph of an area in lung tumor section with collagen deposits



Collagen production is a marker of the soft-tissue origin of the cells. This area is from a KRIB lung tumor (100,000X), showing fibrous structures, typical of collagen deposits, between the tumor cells.

Figure 11

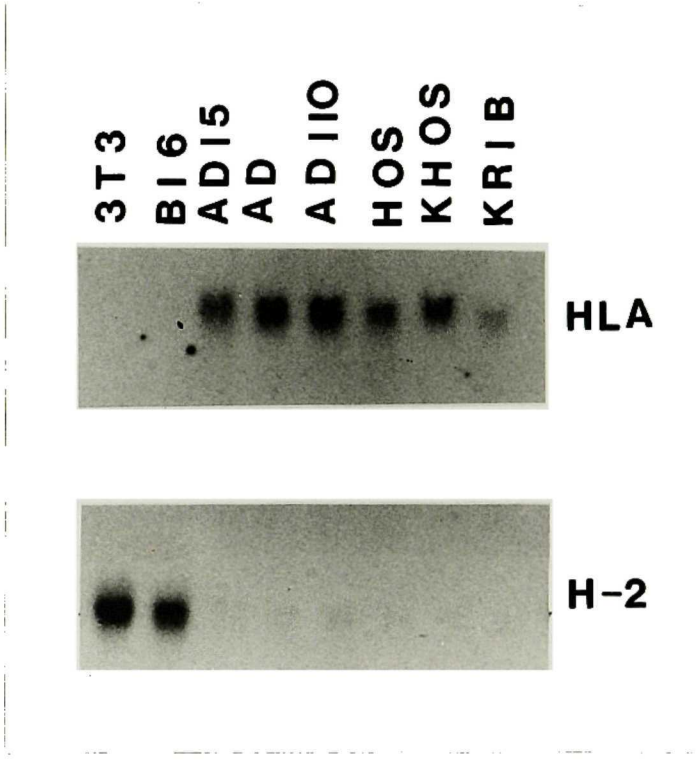
Expression of collagen type I by HOS-derived cell lines (Northern blot)




Twenty ug of cytoplasmic RNA from each cell line were separated by gel electrophoresis, transferred onto a nitrocellulose membrane and hybridized with a DNA probe for collagen type I. As can be seen, cells from either tissue culture (HOS, on the left lane) or from lung tumors (KRIB and KHOS-5, on center and right lanes), showed detectable levels of collagen. Note that transcript levels of the metastatic lines, KRIB and KHOS-5, were lower as compared with HOS, the parental, benign cell line.

Figure 12

Expression of human and mouse histocompatibility genes by HOS-derived cell lines (Northern blot)




Cytoplasmic RNA was isolated from cells derived from subcutanous tumors (AD, AD15 and AD110), from cells in culture (HOS and KHOS) or from cells derived from a lung tumor (KRIB). In all these samples, the human-specific HLA gene was expressed but not the mouse-specific H-2 gene. Samples from mouse fibroblasts (3T3) and melanoma (B16) lines are shown on the left lanes for comparison.

which developed following implantation of the corresponding cell lines. KRIB lane is of cells from a pulmonary nodule which developed after I.V injection of KRIB cells). These results were further confirmed by immunofluorescent staining for the HLA class 1 surface antigen which showed the presence of the human tissue marker on the cells' surface.

3. Expression of ras oncogenes

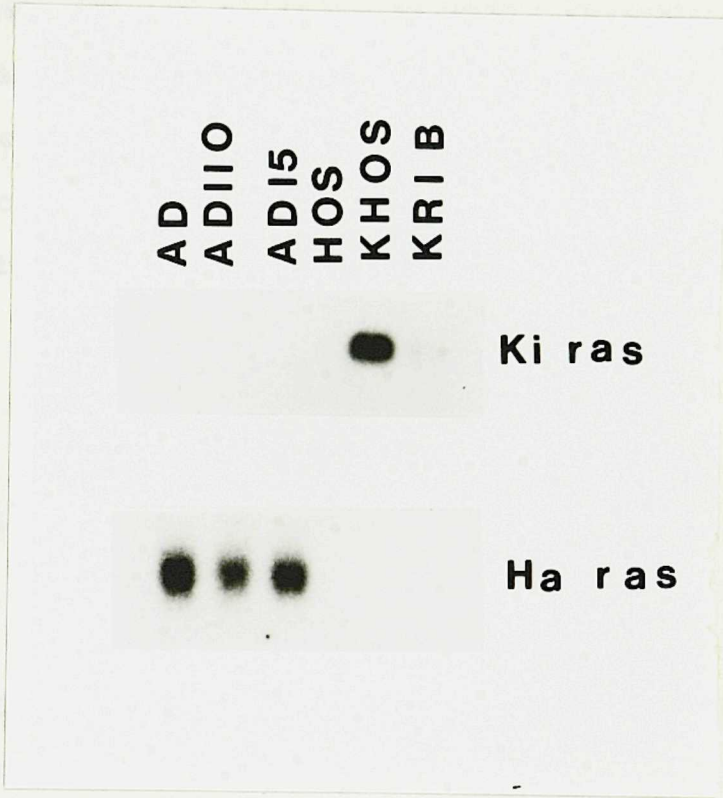
The KHOS-derived subclones expressed the Ki-ras oncogene, while subclones obtained from AD expressed high levels of the Ha-ras, as expected. Northern blots hybridized with the respective oncogenes are depicted in figure 13. In KRIB cells the transcript level of Ki-ras was lower than in KHOS. Therefore, ras expression could be used as an additional marker for the origin of the lung tumors, since it was found in cells collected from lung tumors in much higher levels than are normally detected in this organ.

4. Phenotypic stability

KRIB cells maintained their metastatic potential with

Figure 13

Expression of ras oncogenes by HOS-derived cell lines (Northern blot)




Twenty ug cytoplasmic RNA of each cell line were separated by gel electrophoresis, transferred onto a nitrocellulose membrane and hybridized with DNA probes for Ki-ras and Ha-ras genes. AD and its subclones, AD15 and AD110 (lanes on the left), had high transcript levels of Ha-ras, but undetectable levels of Ki-ras. On the other hand, KHOS and KRIB cells (lanes on the right) expressed the Ki-ras but not the Ha-ras gene. Note that transcript levels in KRIB were lower than in KHOS. HOS, the parental line, did not express detectable levels of either ras gene (center lane).

no apparent alterations in tumor yield or pattern of dissemination after 2, 4, 9, and 13 weeks of continuous growth in culture (Samid and Mandler, 1989). Thus, this line can be considered stable with respect to its metastatic phenotype. In the EJ-ras lineage, AD15 cells showed a slight increase in biological aggressiveness with time (2 weeks vs. 15 weeks in culture), but were consistently less metastatic than KRIB. AD110 cells did not become metastatic even after 12 passages in culture.

5. Motility and chemotaxis

HOS and KRIB, the cell lines representing the two extreme phenotypes in the cellular model system, were tested for their migration through a basement membrane-like layer (matrigel-coated filter) in the modified Boyden chamber. In the absence of chemoattractants (i.e., test for spontanous motility), KRIB cells showed slightly higher motility, with 1%-4% of the cells crossing the matrigel layer within 6 hours, as opposed to <1% in HOS. On the other hand, these two cell lines showed no differences in their chemotactic response to conditioned media from either their own, or from murine 3T3, cell cultures. The results were not always reproducible, and were strongly influenced by the phase of the cells' growth

(cells from subconfluent cultures were much more motile), the concentration of the matrigel layer and the incubation time. In addition, when cells with intermediate malignant phenotypes were tested (KHOS-5 and AD), no differences were observed in either their spontaneous or directed motility.

## C. uPA IN THE HOS CELLULAR MODEL FOR EXPERIMENTAL METASTASIS

### 1. The correlation between metastatic potential and uPA activity

uPA expression in the various HOS lines was evaluated with respect to its biological activity, cellular localization and transcription levels. The biological activity was tested by the plasminogen-dependent fibronectin degradation assay. Under the experimental conditions of the assay, 98% of the measured activity could be inhibited by anti-uPA monoclonal antibodies, indicating that the activity measured was primarily of uPA, rather than other plasminogen activators.

uPA activity of the osteosarcoma cell lines correlated with their metastatic potential. Of the three metastatic sublines, the one with the highest metastatic potential (KRIB) also had the highest uPA activity (~20 Plough units/$10^6$

cells), and the lines with moderate metastatic behavior (AD15 and KHOS-5) showed lower levels of uPA activity (~8 Plough units/$10^6$ cells). Of the non-metastatic, tumorigenic sublines, AD110 uPA activity was considerably and consistenly lower than in the metastatic lines, and AD cells had barely detectable activity (~4 and 2 Plough units/$10^6$ cells, respectively). The lowest activity (<1 Plough units/$10^6$ cells) was measured in the parental, benign HOS cells.

In order to evaluate semiquantitatively the nature of this correlation, each cell line was graded with respect to its metastatic potential. The metastatic potential is proportional to the percentage of tumor-bearing mice, and the number of lung tumors per animal; it is inversely correlated with the length of time required for the development of the pulmonary tumors. Accordingly, metastatic index values were calculated for each cell line, by the following equation:

$$\text{Metastatic index} \propto \frac{\text{\% tumor-bearing mice} \times \text{median no. of lesions/mouse}}{\text{tumor development period (days after inoculation)}}$$

The numerical values used in this equation were

derived from the data in table II. A strong correlation was found between uPA activity and the metastatic index values, as shown in figure 14 ($r=0.93$, $p<0.01$ as obtained by linear regression analysis). Best-fitted curve analysis predicted that when uPA activity is 3.5 Plough units/$10^6$ cells (or lower), the metastatic index is 0.

Most of the uPA activity measured in these experiments appeared to be bound to the cell surface, as pre-treatment of the intact cells with anti-uPA antibodies (followed by removal of excess antibody prior to the assay) blocked most of the uPA activity. Since the antibodies could interact with outer membrane components only, this inhibition indicated the localization of the enzyme on the cells' surface. This was confirmed by immunofluorescent staining of intact, viable cells in suspension. The fluorescence intensity was measured by a fluorescence-activated cell sorter (FACS). It was high on the KRIB cells' surface and very low on the non-metastatic, parental HOS as well as on AD cells (Figure 15).

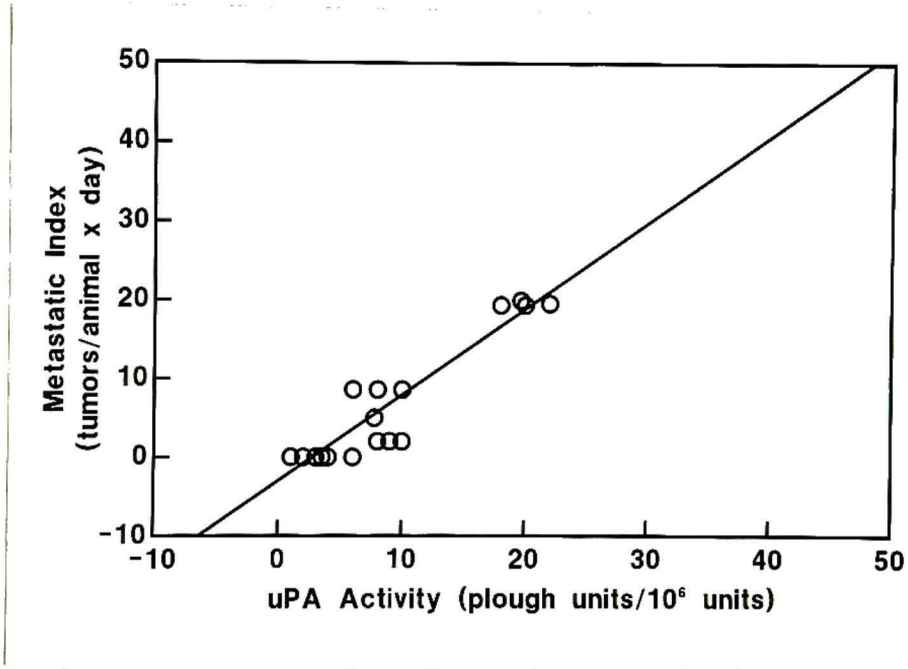
uPA activity was also detected in the conditioned media collected from the cell cultures. KRIB, AD110 and AD15 secreted approximately 20 Plough units per $10^6$ cells, while the other cell lines had much lower, and comparable, activity (about 10% of that secreted by KRIB or AD110).

Plasminogen-independent activity was detected in the assay as well. It constituted approximately 20% of maximal fibronectin degrading activity and was similar in all the cell

Figure 14



Correlation between uPA activity and metastatic potential of HOS-derived cell lines

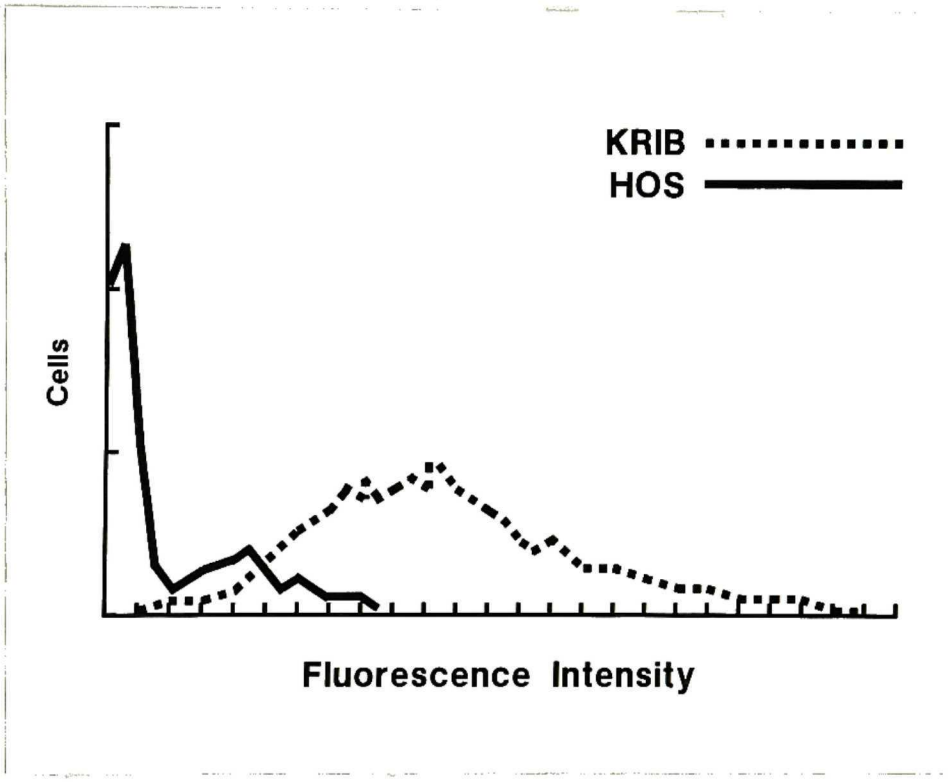



Cells were injected I.V. ($10^5$ per mouse), and their metastatic index was calculated as described in the text (a function of the percentage of tumor-bearing mice, the median number of lung nodules per mouse and the growth period of their development).

uPA activity was determined by the fibronectin degradation assay, and is expressed as Plough units per $10^6$ cells. The correlation between the uPA activity and the metastatic index was assessed by linear regression analysis ($r=0.93$, $p<0.01$)

Figure 15

FACS analysis of surface-bound uPA on HOS and KRIB cells




Viable HOS and KRIB cells were treated with monoclonal anti-human uPA antibodies, washed and incubated with FITC-goat anti mouse antibodies. After fixation with 2% formaldehyde, samples of $10^4$ cells were analyzed by fluorescence activated cell sorter (FACS). As can be seen, fluorescence intensity on the surface of KRIB cells was higher than on HOS cells, indicating a higher concentration of surface-bound uPA on these cells.

lines.

2. neutralization of surface-bound uPA reduced the metastatic potential of KRIB cells

The correlation between the metastatic behavior of the osteosarcoma cells and the levels of their surface-bound uPA activity suggested that uPA might be required for efficient metastasis. This hypothesis was tested by in-vivo blocking experiments, in which monoclonal anti-uPA antibodies were employed to block surface-bound uPA of KRIB cells prior to the inoculation into mice.

As mentioned above, these antibodies inhibited most of the KRIB uPA activity. They had no effect, however, on cell viability, morphology or plating efficiency, neither did they induce capping of membrane components. In the in-vivo experiments, two controls were included: one group of mice received cells which were pre-treated with non-neutralizing monoclonal antibodies to uPA (These antibodies were shown to bind to the cells' surface by immunofluorescence staining prior to the in-vivo experiments), the other group received cells with no prior treatment.

The results, summarized in table III, showed a statistically significant decrease in the metastatic

competence of KRIB cells subsequent to the inhibition of their uPA activity. Treatment with the neutralizing antibodies resulted in a lower incidence of tumor-bearing mice and a significant reduction in the number and size of the lung tumors ($p< 0.01$). In contrast, the non-neutralizing antibodies had no significant effect on the metastatic pattern, indicating that the reduction in the metastatic yield was not a consequence of non-specific antibodies' effects.

Note, however, that the anti-uPA antibodies did not abolish the cells' metastatic potential completely. This could be due, in part, to the transient nature of the antibody inhibition. In fact, in the fibronectin degradation assay, a decrease in the antibodies effect with time was observed and 90% of the uPA activity was usually restored within 2 hours.

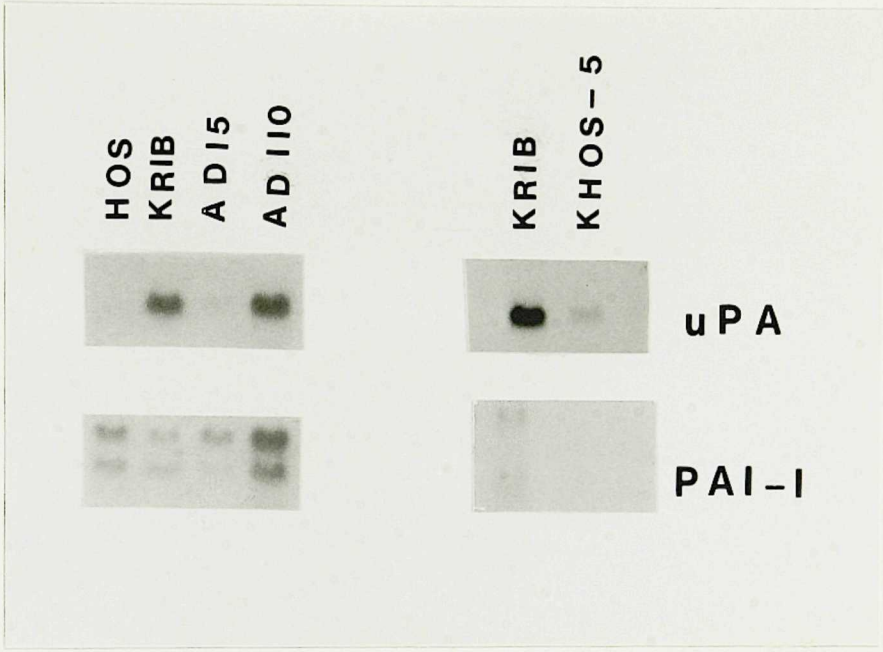
3. Possible regulatory mechanisms involved in the differential uPA expression of the HOS subclones

Northern blot analysis of steady-state mRNA levels in the HOS-derived subclones suggests two possible mechanisms for the regulation of uPA expression. One operates at the biosynthesis level whereas the other may affect the catalytic activity of the surface-bound enzyme. This can be deduced from the relationship between uPA transcript levels and those of

its inhibitor PAI-1, as shown in figure 16. Hybridization of the cytoplasmic mRNA with a uPA-specific DNA probe revealed a marked increase in the amounts of uPA transcripts in KRIB and AD110 cells compared with KHOS-5 and AD15. HOS cells, the parental line, had undetectable levels of uPA mRNA. When PAI-1 DNA probe was hybridized with the same blots, high levels of this gene transcripts were detected in AD110 compared with the other cell lines. Taken together, these results suggest that uPA biosynthesis is regulated at a pre-translational level, but the net proteolytic activity may ultimately depend on the balance between uPA and PAI-1 production.

Figure 16

Expression of uPA and PAI-1 by HOS-derived cell lines (Northern blots)




RNA samples were immobilized on Northern blots and hybridized with DNA probes for human uPA and PAI-1 genes. The left panels are from one blot, showing high transcript levels of uPA in KRIB and AD110 cells and lower levels in AD15 cells. PAI-1 expression was markedly higher in AD110 as compared with the other cell lines. The right panels are from a different blot, showing uPA and PAI-1 expression in KRIB compared with KHOS-5 cells. The transcripts levels of both genes were higher in KRIB. These samples were exposed to electrophoresis for slighly longer time than those on the gel in the left panel.

# DISCUSSION

## A. THE CELLULAR MODEL SYSTEM; ras-TRANSFORMED HUMAN OSTEOSARCOMA CELLS CAPABLE OF EXPERIMENTAL METASTASIS IN MICE

### 1. Reasons for the need to develop human cellular models for in-vivo studies of metastasis in animals

The major cause of death in most cancer patients is metastasis. Consequently, a major challenge to both clinical oncologists and cancer scientists is the development of improved methods for: a. predicting metastatic risk of tumors; b. preventing secondary spread of tumors; and, c. identifying metastatic lesions early in their development. Most of the current treatment modalities for metastasis focus on inhibition of cellular growth. They do not address the unique properties of the metastatic cells, which enable them to migrate, invade tissues and induce angiogenesis. It is possible that focusing on these processes, and on the underlying mechanisms that enable the metastatic cells to carry them out, will lead to identification of metastatic cells' properties that may serve as diagnostic and prognostic markers, or even as targets for therapeutic intervention.

While the search for unique traits of metastatic tumor cells seems well warranted, it has been hindered by several problems, inherent to the complexity of the metastatic

process. Studies of animal tumor models have not always been applicable to human malignancies because of discrepansies in drug sensitivity or developmental pattern between human and animal malignancies. Consequently, there is a growing awareness for the need to study human, rather than animal, cancer models (Poste, 1986; Eccles, 1982; Graham et al., 1978 and Kyriazis, 1978). This had been emphasized in recent years and was formally expressed in the 1985 report of the DCT Board. This report stressed that identification of properties unique to human malignancies could be more relevant and, therefore, more valuable for further applications (DCT board committee, 1985).

However, studies of human malignancies can be performed mainly by in-vitro assays, that test isolated properties, such as cellular motility, anchorage-independent growth, expression of oncogenes, or elaboration of various proteases. While all these properties were found to be associated with the metastatic phenotype, they did not always correlate with, thus could not be used as predictors for, the in-vivo behavior of the cells. Metastasis involves multifactorial interactions between the host and the tumor cells, and these make the need for in-vivo experimental conditions essential (Fidler, 1986).

In addition, it is also recognized today that factors such as the histological origin of specific tumors, their anatomical locations and developmental stages dictate

different behavior and interactions with the host enviroment (Poste, 1986 and Mareel, 1983). For this reason, it may not be possible to identify one uniform characteristic for all metastatic cancers. Instead, malignancies should be subclassified and examined according to their common characteristics, such as histological origin or metastatic target organ.

The work presented here involved the development of a new cellular model system, consisting of human osteosarcoma cells which metastasize efficiently in athymic mice. This cellular model presents an advantage over other currently employed model systems not only in being of human origin, but also in originating from a sarcoma (i.e., mesodermally-derived tumor). While sarcomas are frequently aggressive and highly metastatic in patients, none so far has been described as metastatic in experimental animals. Therefore, the HOS-derived cells could, potentially, enable identification of features unique to human sarcomas which could not be addressed to date.

## 2. Rationale for the methodologies employed in these studies for isolating human tumor cells with metastatic ability in mice

A major obstacle for conducting in-vivo studies of

metastasis with human tumor cells has been the fact that such cells rarely metastasize efficiently in experimental animals (Nicolson and Poste, 1983; Giovanella and Fogh, 1985 and Sharkey and Fogh, 1979). The results of the research presented here show that metastatic variants of human osteosarcoma (and potentially of other human cancers) can be obtained by a combination of two experimental approaches, namely, neoplastic transformation by ras oncogenes, followed by selection in-vivo of the more aggressive cell variants.

While the activated Ki-ras and Ha-ras oncogenes have been shown to confer metastatic potential to established rodent cells (Barbacid, 1987), this has not been reported in human cells. The data presented here show that human osteosarcoma cells became metastatic in nude mice following transformation by activated ras oncogenes (Table II). v-Ki-ras transformation was associated with the infection of HOS cells with the non-producer strain of MuSV (Rhim, 1975). It could be argued, therefore, that the acquired metastatic potential (as observed with KHOS and its subclones), was not a direct consequence of ras oncogene transformation but rather of other viral factors or selection processes through the prolonged in-vitro conditions. The studies presented here showed, however, that this was not the case. HOS cells acquired metastatic competence shortly after gene transfer of the activated human Ha-ras oncogene alone, as observed with AD15. These findings indicate that ras oncogenes were indeed

involved in the acquisition of metastatic behavior by HOS cells.

The ras transformants were transplanted subcutanously in athymic mice. Out of the cells which formed subcutanous tumors, some exhibited metastatic ability when re-injected intravenously. This is consistent with the hypothesis that metastatic variants exist in the heterogenous tumor cell population and they could disseminate upon the appropriate selective conditions (Fidler and Kripke, 1977 and Weiss et al., 1983). Interestingly, the metastatic variants were not necessarily those with the higher expression of ras oncogenes, a phenomenon which is discussed later.

The metastatic potential of the HOS subclones was examined by an experimental metastasis assay that addresses the late events of blood-borne tumor dissemination (i.e., adherence to the endothelial cells, extravasation and subsequent colonization). This method offers an advantage over the spontanous metastasis assay when the primary tumor growth is rapid and mortality occurs before metastases have developed. Usually, the quality of the studies is not compromised since the correlation between the two methods is high. Furthermore, the experimental metastasis assay seems particularly appropriate for the osteosarcoma cellular system in that naturally-occurring sarcomas (more so than other tumor types) disseminate almost exclusively via hematogenous routes (Jaffe et al., 1978). Tested in this assay, the HOS-derived

subclones formed primarily lung metastases, a characteristic seen also in patients with osteosarcoma (Jaffe et al., 1978 and Belli et al., 1989).

The human origin of the pulmonary tumors had to be carefully assessed because most human tumors do not metastasize in experimental animals and when they do, it is often by fusing with the host cells or by inducing horizontal neoplastic transformation of host tissues (Goldenberg and Pavia 1981 and 1982). The cells obtained from lung tumors were shown to be of human origin since they expressed the human-specific HLA transcripts and antigens but not the corresponding murine-specific MHC (Figure 12). They also expressed the ras oncogenes (Ki-ras when KRIB cells were injected and Ha-ras when AD15 cells were injected), which normally are not present at high levels in lung tissue (Figure 13). In addition, the tumor cells were shown to produce collagen, a charateristic of mesodermally-derived cells, which is consistent with the histological origin of HOS (Robey and Termine, 1985) (Figures 10 and 11).

## 3. Characteristics and specific advantages of the HOS cellular model

The HOS-derived cellular model system is particularly

suitable for comparative studies, since it provides an array of malignant phenotypes in cells which have all originated from the same parental line. In this cellular system, KRIB line had the highest metastatic potential, followed by the less aggressive AD15 and KHOS-5 lines, as judged by the percentage of tumor-bearing mice in the recipient population, the number of metastatic lesions per animal, the size of the lung metastases, and their growth rate. The other two transformants- AD and AD110- represented an earlier stage of tumor progression, as they were tumorigenic but non-metastatic. In contrast, the parental HOS cells were neither tumorigenic nor metastatic in mice (Table I and II). The availability of various malignant phenotypes in this cellular model is an advantage over most other systems used in in-vivo studies. Usually, only the extreme cellular phenotypes are available (i.e., metastatic vs. benign counterparts). Alternatively, studies are conducted using cell lines which are not of the same origin. The HOS sublines are, therefore, better suited for comparative studies of fully-metastatic as opposed to less aggressive tumor cell subpopulations. From the clinical standpoint, such comparison is more relevant, addressing an urgent problem for both diagnosis and prognosis.

For studies based on quantitative analysis of metastasis (i.e., effects of chemical reagents, hormones, biological substances or physical conditions), KRIB seemed to be the cell line of choice out of the three metastatic

sublines described here, because it satisfied several important requirements. These include high incidence of tumor-bearing mice in the recipient population, formation of numerous, macroscopic lung lesions, relatively rapid growth rate of these lesions, and stability of the metastatic phenotype during passages in culture. This is in contrast with other available human cellular models. For instance, HEp-3 cells lose their metastatic ability and even their tumorigenicity in chick embryos, when maintained in culture. In order to preserve their in-vivo behavior, these cells have to be serially passaged in-vivo. Moreover, HEp-3 cells form microscopic colonies only, which requires special experimental strategies in order to evaluate their colonization efficiently and accurately (Ossowski and Reich, 1983). Another human line, the A375 melanoma, forms visible pulmonary lesions in athymic mice but the tumor yield is relatively low. In addition, like the HEp-3, A375 melanoma cells tend to lose their aggressive phenotype in prolonged culture conditions, a phenomenon attributed to the heterogeneity of the cell population (Kozlowski et al., 1984). KRIB cell line, thus, appears advantagous with regard to these aspects.

In addition to the properties mentioned above, the HOS-derived cells offer yet another advantage in that they can be readily transfected by gene transfer methods, in contrast to most other human established lines. In the studies described here, this feature made it possible to transfect

HOS cells with EJ-ras and with the geneticin-resistant genes.

Gene transfer is potentially a powerful approach for testing directly the involvement of specific genes in a variety of processes, such as differentiation, tumor progression, hereditary diseases or metabolic pathways. Considering the histopathological origin of HOS, this cellular model could be suitable for studies related to the etiology of human sarcomas, such as the role of the retinoblastoma "suppressor" oncogene. Aberrations which inactivate this gene have been implicated in predisposition to osteosarcoma (Link and Eilber, 1987 and Friend et al., 1986). HOS-derived cells could be employed also in studies of genes involved in differentiation, since specific markers for bone cells' differentiation are well characterized (e.g., the metabolism of vitamin $D_3$, receptors of parathyroid hormone and the synthesis and deposition of collagen [Rodan et al., 1987; Howard et al., 1981; Wergedal and Baylink, 1984 and Robey and Termine, 1985]). Finally, gene transfer techniques can also be used to insert genetic markers for selection purposes or for tracing the cells' route following inoculation.

4. Relevance to human osteosarcoma

The behavior of HOS sublines in mice mimics several

aspects of the pathology of osteosarcoma as observed in clinical cases. In patients, metastatic osteosarcoma cells disseminate primarily via hematogenous routes and colonize almost exclusively in the lungs (Link and Eilber, 1987; Malawer et al., 1985 and Jaffe et al., 1983). This has been the case in the HOS subclones experimental metastasis in mice as well. It could be argued that the preferential colonization in the lungs in this model is the outcome of physical trapping, due to the methodology used (e.g., intravenous implantation) rather than the expression of a unique homing pattern. This does not seem to be the case, however, since although all the different cell lines were introduced via the same route, only KRIB, AD15 and KHOS-5 formed pulmonary colonies while AD and AD110 did not. Evidently, the former cell lines possess certain qualities which enable them to settle and grow in the lungs whereas the latter lines do not.

Histologically, in most cases of osteoblastic osteosarcoma, the tumor stroma is intercepted by osteoid production and calcification areas, but tumors composed of less differentiated cells lack obvious deposition of bone material. Worst prognosis is associated with tumors which appear cystic, with necrotic areas in the center surrounded by viable,large and poorly-differentiated cells with little evidence for osteoid formation (Dahlin and Unni, 1977 and Malawer et al.,1985). This histopathological picture is similar to that seen in the pulmonary KRIB lesions (Figure 7),

and may reflect the extremely aggressive nature of these cells. In addition, Ki-ras expression was lowest in KRIB, the cells with the most aggressive phenotype (Figure 13). A similar correlation was noted in clinical biopsies where decreased levels of Ki-ras mRNA and p21 were observed in metastases compared to their corresponding primary tumors (Spandidos and Kerr, 1984; Gallick et al., 1985 and Ohuchi et al., 1986). The reason for this phenomenon is not yet known. However, this inverse correlation suggests that while ras oncogenes may be instrumental in early stages of neoplastic transformation, their expression at high levels is not obligatory for the progression into the metastatic phenotype.

The fact that the cells' behavior in mice resembles the clinical picture of metastatic osteosarcomas in human patients indicates that the selection procedures described above did not produce major modifications in the cells' inherent pathological pattern. For this reason, other experimental findings of the HOS behavior in mice may prove relevant and applicable for osteosarcomas and related cancers. In particular, studies of the HOS system may elucidate mechanisms of osteosarcoma cells' colonization or sensitivities to specific drugs, that can be exploited in order to eradicate the metastatic cells or to block their spread more effectively. Osteosarcoma is the third most common neoplasm in adolescents, with highly aggressive manifestations and poor prognosis. Over 80% of the patients develop

metastatic lesions within the first year, for which the best known treatment today is adjuvant chemotherapy. However, even among those patients which respond to this treatment, the majority eventually relapse within 4 years (Taylor et al., 1985; Goorin et al., 1985 and Carter, 1984). Identifying alternative, more effective treatment strategies remains an urgent need.

This research project was not intended, however, to focus on aspects specific to osteosarcoma only, but rather to explore general characteristics of human cancer metastasis, using the HOS cellular system as a model. Like the HOS sublines, various types of sarcomas and carcinomas disseminate through the blood circulation; the lung is the target organ for secondary colonization of numerous malignancies. For these reasons, studies of the metastatic behavior of the HOS sublines in athymic mice may have implications for a large spectrum of human metastatic diseases.

## B. uPA INVOLVEMENT IN THE EXPERIMENTAL METASTASIS OF HOS SUBCLONES IN ATHYMIC MICE

### 1. Arguments for a putative role of uPA in metastasis and current discrepansies

What enables the metastatic cells to disseminate and colonize in distant organs? Clearly, they have to complete successfully several descrete processes including detachment from the primary tumor mass, migration through usually impermeable biological matrices, invasion into and then out of blood vessel walls, and rapid growth in the environment of the secondary site. One compelling hypothesis is that the ability of metastatic cells to perform these processes is associated with elevated production of endogenous proteolytic enzymes. Metastatic tumor cells are known to elaborate a variety of proteases, whose activities may facilitate specific stages of the metastatic spread (Quigley, 1979; Goldfarb, 1982; Reich et al., 1988; Chen and Chen, 1987, Nicolson,1982 and Dano et al., 1978). For instance, collagenase type IV can facilitate degradation of and invasion through the basement membrane (Liotta et al., 1981), high levels of elastase may determine the ability of tumor cells to invade and colonize the lungs (Zetter, 1990), whereas heparinase may be involved

in invasion of the liver parenchyma (Nakajima, 1983). Apart from their role in tissue degradation, proteases are implicated in promoting cellular motility and migration as well by generating small chemotactic peptides. For example, stromelysin, a bone metalloprotease, is thought to enhance motility and migration of neoplastic osteoblasts by producing locally chemotactic fibronectin fragments (Galloway et al., 1983 and Murphy et al., 1981). Likewise, proteolytic fragments produced by plasmin have been implicated in chemotaxis of endothelial cells and the sprouting of blood capillaries (Goldfarb et al., 1986).

Of the various tumor-associated proteases known so far, uPA appears to be the most relevant. This is because its substrate, plasminogen, is abundant in the plasma as well as in the interstitial fluid, making it possible for uPA to exert its activity in almost any organ (Dano et al., 1985 and Pollanen et al., 1988). In addition, uPA can initiate degradation of virtually all connective tissue components via the direct and indirect actions of its product, plasmin. Moreover, apart from its involvement in tissue breakdown, plasmin has also been implicated in angiogenesis and in enhancing cellular motility by generating chemotactic peptides (Goldfarb et al., 1986 and Liotta et al., 1981).

In the past 20 years, numerous studies have shown an association between uPA expression and metastasis. To mention only a few, in a study of biopsies from colon adenocarcinomas,

adenomatous polyps, and normal mucosa, increased uPA activity was found in the metastatic lesions compared with either the benign or the normal tissues (DeBruin et al., 1987). Cells obtained from metastatic melanoma lesions in human patients were shown to produce high levels of uPA (Markus et al., 1984). Induction of neoplastic features by tumor promotors such as phorbol esters was accompanied by increased production of uPA in the Swiss 3T3 murine fibroblasts (Dano et al., 1985). However, such correlation was not consistent in all studies and, in fact, in some cases the data are even conflicting. In one study, for instance, a highly metastatic subline (F10) of the murine melanoma B16, was found to secrete significantly more uPA than another subline (F1), having a low metastatic potential (Wang et al., 1980). In contrast, other groups were unable to find any differences in uPA production between the two sublines (Nicolson, 1976 and Hart, 1979). Likewise, in Lewis lung carcinoma cells, secreted uPA levels were found to correlate with their metastatic potential in two independent studies (Carisen et al., 1984 and Eisenbach et al., 1985), whereas another group reported no such correlation (Whur et al., 1980). Evidently, the putative role of uPA in metastasis is still a controversial issue, and requires thorough evaluation and more carefully-designed experimental approaches.

## 2. Methodology approaches and their interpretations in studies of metastasis and uPA expression

The conflicting reports on uPA involvement in metastasis may reflect the fact that uPA is not a universal property, common to all tumor types in all species. However, contradictions may also arise from differences in the experimental methodologies employed and their interpretations. uPA production was determined by its mRNA levels in some studies, by immunological detection assay (ELISA) in others, and yet other reports were based on analysis of uPA activity secreted by the tumor cells. Obviously, this could lead to conflicting conclusions. The net activity of uPA does not always correlate with the enzyme transcription level or the total biosynthetic product because of modulation by post-transcriptional factors, such as pro-uPA activation or the presence of PAI-1. For this reason, conclusive results regarding uPA involvement in metastasis have to be based, ultimately, on measurements of the actual enzymatic activity.

Subcellular localization of uPA is another important parameter. When bound to outer membrane receptors, uPA is more stable and may initiate focal degradation of the pericellular enviroment, whereas the secreted enzyme is less stable, and diffuses rapidly.

Likewise, the criteria used for determining metastatic

potential may sometimes be misleading. In particular, in-vitro experiments, based solely on soft-agar growth, cellular motility, or invasion through synthetic filters may not be sufficient, as these properties do not always correlate with the metastatic behavior in-vivo. Tumor cells should be defined as metastatic if they arise from a metastatic lesion, or if they are capable of disseminating and colonizing target organ(s), when inoculated in-vivo.

3. The correlation between uPA activity and metastatic potential in the HOS cellular model- factors involved and possible regulatory mechanisms

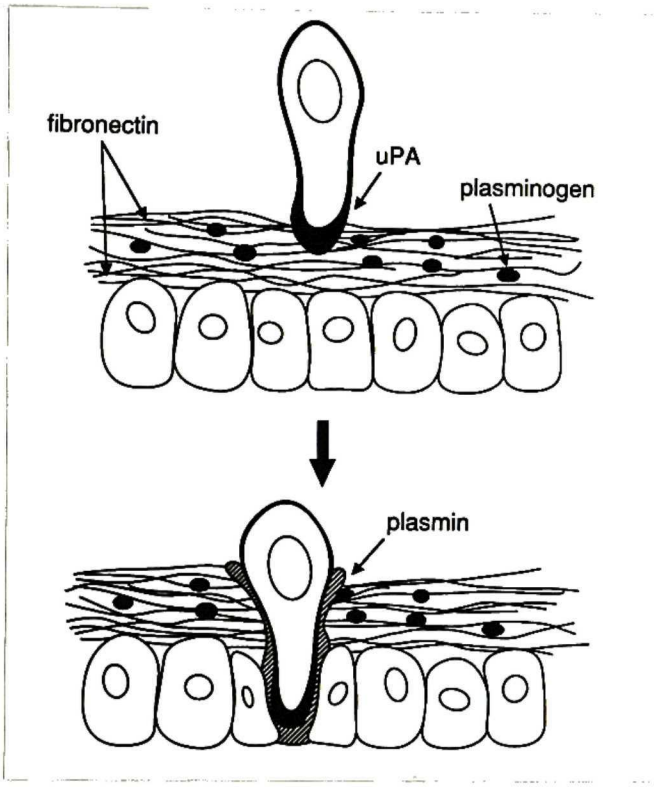
The studies presented here were carefully designed to address the considerations mentioned above. uPA expression was analysed by several parameters- transcription level, net enzymatic activity, subcellular localization, and the expression of its inhibitor, PAI-1. Evaluation of metastatic potential of the various HOS subclones was based on the in-vivo behavior in athymic mice. The results of these studies revealed a correlation between uPA activity and the in-vivo aggressiveness of the HOS-derived cells (Figure 14). Biological aggressiveness was graded by the metastatic index, a function which incorporated the period required for the

cells to form visible lung colonies, the number of these colonies per mouse, and the percentage of mice in which successful experimental metastasis was observed. This parameter (metastatic index) was strongly correlated with the surface-bound, biologically active uPA rather than the total amounts of the enzyme produced and secreted, and appeared to depend upon a threshold of uPA level (~3 Plough units/$10^6$ cells), below which the cells were not metastatic. This was most obvious in AD110 cells, which secreted uPA in levels comparable to KRIB or AD15, but had, nevertheless, markedly lower membrane-bound activity, and also exhibited relatively poor aggressiveness in-vivo (Tables I and II). Membrane-bound uPA seems to be more relevant to the metastatic capability of the cells (Pollanen et al., 1987; Hearing et al., 1988; Chen and Chen, 1987; Salonen et al., 1989 and Stephens et al., 1989). The bound form of uPA is more stable, and generation of plasmin in contact areas of the cells and the surrounding parenchyma has been suggested to enable more efficient, focal dissolution of the pericellular matrix. In addition, plasmin can generate locally high concentrations of small proteolytic products, with strong chemotactic activity (especially degradation fragments of fibronectin and laminin). As a result, cellular motility is induced and tissue invasion is facilitated. This hypothesis is depicted schematically in figure 17.

The differential expression of uPA, as observed with

Figure 17

Putative role of uPA in initiating local cellular invasion




This diagram depicts the process of cellular invasion through biological barrier. According to this hypothetical scheme, uPA is localized on the leading edge of the metastatic cell, bound to the outer membrane. It generates plasmin in areas of cell-matrix contacts, causing focalized dissolution of the connective tissue. The metastatic cells are then free to move and penetrate through the matrix and the tissue cells.

the various HOS subclones, may be regulated on several levels, such as transcription rate, mRNA stability, receptor availability, efficiency of secretion, zymogen activation, or the presence of uPA inhibitors. Analysis of Northern blots hybridized with DNA probes for uPA and PAI-1 suggests that in the HOS cellular system, uPA activity is determined by a balance between uPA transcription level and the concomitant production of its inhibitor, PAI-1. In the Ki-ras transformants, PAI-1 transcript levels were low and uPA activity was tightly correlated with its mRNA levels. However, in AD110 cells, PAI-1 synthesis was markedly elevated (Figure 16), a fact which could explain the low net catalytic activity of uPA detected in these cells. Concomitant synthesis of uPA and PAI-1 has been observed in other cases as well, and is thought to regulate and control uPA activity, especially the surface-bound form (Pollanen et al., 1987 and Blasi et al., 1987).

4. uPA plays a functional role in the experimental metastasis of KRIB cells

The correlation between net uPA activity and the metastatic potential suggested that uPA may have a functional role in metastasis. This could be tested directly either by

blocking uPA activity of the metastatic cells, or by inducing the enzyme activity in originally non-metastatic cells, and evaluating the effects of such treatments on the metastatic potential.

Several attempts in both directions have been reported in other model systems, each with some limitations. Using reagents such as DMSO or short peptide aldehydes to inhibit uPA production or activity, respectively, it has been shown that the metastatic spread was subsequently reduced (Ostrowski et al., 1986; Axelrod et al., 1989 and Ossowski, 1988). However, since these inhibitors were not uPA-specific, they may have acted through other mechanisms. In an attempt to circumvent this problem, anti-uPA antibodies were utilized in another study, in order to block the surface-bound uPA activity of metastatic murine melanoma cells (F10, a subline of B16). Subsequently, a small reduction in metastatic yield was observed (Hearing et al., 1988). Yet, these results are controversial in view of the facts that F10 metastatic potential tended to drop spontanously with time (the authors' observation, which was also confirmed in preliminary experiments for the work presented here), and that the maximal inhibition of uPA was only 30% (the antibodies were anti-human uPA rather than specific for mouse uPA). More optimal conditions were used in <u>in-vivo</u> blocking experiments of another study, where secondary lung colonization of human HEp-3 cells in chick embryos was found to be reduced following

introduction of anti-human uPA antibodies intravenously (Ossowski, 1988). These studies did not rule out, however, the possibility of non-specific effects of the antibodies on membrane structures or functions, which could have led to impaired migration. A novel approach was taken in a recent study, where cDNA of the complete human uPA gene was inserted into ras-transformed, non-metastatic murine 3T3 cells (Axelrod et al., 1989). While uPA was expressed in virtually all the transfectants, only two of them were tested in-vivo, and both were found to be metastatic. The question whether these clones were representative of the total population (in other words, whether their acquired metastatic potential was a direct outcome of uPA expression or a coincidence) was not addressed.

The hypothesis that uPA plays a causative role in metastasis needs, therefore, to be substantiated by more conclusive evidence and better controlled studies. The findings of this work have, indeed, demonstrated that uPA actually contributed to the metastatic efficiency of the osteosarcoma cells. In the in-vivo blocking experiments, surface-bound uPA activity of KRIB cells was inhibited by specific, monoclonal anti-human uPA antibodies, under conditions which produced 98% inhibition at the time of injection. This treatment resulted in a significant reduction in pulmonary colonization, as judged by the lowered incidence of tumor-bearing mice and the decrease in both number and size

of the lung colonies. Such reduction was not observed when non-neutralizing anti-human uPA antibodies were used (Table III). Since both types of antibodies were of the IgG1 subclass, neither could have initiated complement-mediated cytolysis, excluding such a mechanism as the cause for the decrease in tumor yield. Non-specific effects of the antibodies on membrane structures or functions could be ruled out as well, since the non-neutralizing antibodies were shown to bind to KRIB surface, but nevertheless did not affect the behavior of the cells. The results could not be attributed to reaction with the host uPA either, since both types of antibodies react with human but not with mouse uPA. Taken together, all these considerations indicate that the attenuation in KRIB colonization in these experiments was, indeed, a consequence of blocking the cells' endogenous uPA activity. These results imply that efficient colonization of the metastatic cells depends, in part, on their endogenous uPA activity. Furthermore, the findings suggest that as neoplastic cells produce increasingly higher levels of uPA, they may become better equipped for the metastatic process.

In the <u>in-vivo</u> blocking experiments, KRIB metastasis was suppressed, but not eliminated entirely. This is not surprising considering that uPA-dependent proteolysis may not be the sole mechanism utilized by the metastatic cells. Other proteases, such as collagenases, cathepsins and elastases have been implicated in tumor cells' dissemination as well (Reich

et al., 1988; Killion, 1989 and Zetter, 1990). Moreover, in-vivo inhibition by anti-uPA antibodies was expected to be only transient. It was probably most effective shortly after the cells had been injected, and gradually waned due to dilution and degradation of the antibodies in the plasma ($t_{1/2}$ ~ 10 hours, as reported by others [See Ossowski, 1988] and appeared even shorter in the in-vitro assay used in these studies). Therefore, the results most likely reflect the participation of uPA in early stages of the experimental metastasis, e.g., tumor cell adherance to the endothelial layer and extravasation. Other in-vivo blocking experiments, addressing the question of uPA-dependent steps, were performed in the chick embryo model, using HEp-3 cells (Ossowski, 1988). In these studies, the results have also indicated migration into the parenchyma (in that model, the chorioallantoic membrane) and invasion through blood vessel walls as the major uPA- dependent steps, although in that case, penetration into, rather than out of, the capillaries, seemed to be involved.

Taken together with the findings of uPA localization on the membrane and the production of PAI-1, it appears that uPA could contribute to the metastatic spread by inducing a stepwise process of matrix degradation and cellular invasion. According to this hypothesis, focal plasmin-generating activity of uPA forms "pathways" in the pericellular environment, through which the tumor cells are free to penetrate. However, as the migrating cell proceeds, its

leading edge encounters the secreted PAI-1, which inactivates uPA. In the absence of local proteolysis, the cell will form new connections with the matrix, a process required for further migration.

## C. SIGNIFICANCE OF THE STUDIES AND POTENTIAL APPLICATIONS

### 1. uPA as a potential diagnostic and prognostic marker

The results of this work have shown that in the HOS cellular model, surface-bound uPA activity was directly correlated with the metastatic competence of the sarcoma cells, and even reflected the degree of the cells' aggressiveness in-vivo (Figure 14) . Moreover, uPA activity proved to be a more reliable predictor for colonization potential than other parameters, such as anchorage-independent growth, morphology, motility or chemotaxis. For instance, although AD15 and AD110 cells were comparable in their proliferation in soft-agar and in their morphology, AD15 was metastatic while AD110 was not. Cellular motility and chemotaxis did not appear as reliable tests either, due to variability in the experimental results. Likewise, neither the level of ras expression nor the oncogene type seemed to be

essential determinants for metastatic potential. Transformants of either v-Ki-ras or EJ-ras were found to be metastatic, and on the other hand EJ-ras transfection yielded both metastatic (AD15) and non-metastatic (AD110) phenotypes.

The fact that uPA activity is a reliable marker for metastatic competence and aggressiveness in the HOS cellular model is significant in that it provides the first strong evidence for such a type of correlation in human sarcoma. If such a correlation is confirmed in clinical cases, then uPA activity levels could, potentially, be considered as prognostic markers to predict metastatic risk. The activity of the enzyme could be determined in cells obtained from biopsies of primary tumors. When this is not feasible (biopsy specimens are composed of a cell mixture, and it is sometimes difficult to isolate the tumor cells [Link and Eilber, 1987]), the expression of the enzyme could, alternatively, be determined by either immunohistochemistry or <u>in-situ</u> hybridization, using frozen tissue sections from biopsies. The first method measures total protein production, whereas the second method determines steady-state mRNA levels. However, when employing such methods, it is crucial to evaluate PAI-1 production as well, because the balance between uPA and PAI-1 biosynthesis appears to be a more accurate parameter for the net uPA activity.

In addition, surface-bound uPA could be used as a diagnostic marker in scanning and localizing secondary tumor

lesions. This could be done by using specific anti-uPA antibodies conjugated to radiological dyes (for the technique and clinical trials, see Baldwin et al., 1984). Moreover, if the anti-uPA antibodies are radioactively-tagged (by $^{125}I$, for example) they could be employed as cytocidal agents with preferential affinity towards metastases. In that regard, it is interesting to note that the lung, a major target organs for metastatic sarcomas, is relatively poor in endogenous uPA expression (Larsson et al., 1984). This makes scanning of pulmonary metastases with uPA as a marker all the more a valuable and promising approach.

## 2. Inhibition of tumor cells' uPA activity as a potential approach to control metastatic spread

The extent of uPA participation in enhancing metastatic ability is not yet fully characterized. However, the data presented here suggests that it may be possible to reduce the risk of metastatic spread by inhibiting uPA activity of the primary tumor cells. In some cases, metastatic cells have already detached from the primary tumor by the time diagnosis is made. However, the manipulation during surgical removal of the primary tumor has also been indicated as increasing the risk of tumor cells shedding. In such cases,

prevention could, hypothetically, be accomplished by introducing intravenously large doses of PAI-1 prior to the operation. Alternatively, if the primary tumor is anatomically accessible, either PAI-1 or anti-uPA antibodies could be introduced locally as a preventive measure.

## D. FUTURE DIRECTIONS

The results discussed above imply that uPA activity enhances the metastatic ability of tumor cells, since even a transient inhibition of this enzyme impaired their secondary colonization. This partial suppression may reflect the functional role of uPA in extravasation and migration through the lung parenchyma. However, uPA has been implicated in events beyond cellular migration and invasion. This enzyme was found to induce angiogenesis (Goldfarb et al., 1986 and Schor and Schor, 1983) and to act as a mitogen, enhancing cell proliferation (Kircheimer et al., 1989; Ossowski et al., 1979 and Sullivan and Quigley, 1986). The possible effects of uPA on these stages of the metastatic process could not be addressed by the current experimental conditions. In order to evaluate the full extent of uPA involvement in secondary colonization of tumor cells, further studies should be aimed to inhibit uPA activity in a prolonged and sustained manner.

One potential approach to achieve permanent inhibition could involve the introduction of the PAI-1 gene into metastatic cells (by gene transfer techniques), linked to a strong promoter, which will induce high production levels of PAI-1. In that way, the net uPA activity could, possibly, be reduced in spite of the intact biosynthesis of the enzyme, due to inactivation of the surface-bound uPA by PAI-1. Furthermore, such inhibition would be inherent and, thus, permanent. Another possibility for blocking uPA expression genetically is the use of short cDNA sequences of its gene in the anti-sense orientation. When driven by a strong promoter, such sequences can be transcribed at high levels, hybridize with the native mRNA, and interfere with its translation. The role of uPA in the acquisition of metastatic potential could, conversely, be tested by introducing its cDNA gene into non-metastatic cells. Obviously, a pre-requisite for such experimental approaches is that the cells can be readily transfected, a requirement that the HOS-derived sublines were shown to fulfil.

Another line of investigations should aim to evaluate the relevance of the correlative studies discussed above in clinical situations. In the experimental model described here, endogenous uPA activity of human malignant cells was found to be a reliable marker for metastatic potential. These findings are the first to provide strong evidence for such a correlation in human sarcoma cells. These studies should be

expanded, therefore, by analyzing uPA expression in biopsy specimens from human metastatic lesions and their corresponding primary tumors.

Finally, the potential approach, as outlined above, for controlling tumor metastasis by inhibiting uPA activity, requires evaluation of effective methods for in-vivo blocking of the enzyme activity. PAI-1 may appear as the agent of choice, being a naturally-occuring protein, and thus more tolerable and less immunogenic as compared with anti-uPA antibodies. However, high plasma concentrations of PAI-1 may have adverse reactions by affecting hemostasis (PAI-1 inhibits tPA as well). Further investigations should, therefore, establish the effective concentrations of PAI-1 with respect to metastatic control, its clearance rate in-vivo, and potential side effects of its use.

This work focused on one aspect of tumor secondary spread. Its main contribution is in establishing a human cellular system suitable for evaluating parameters of tumor dissemination in-vivo and in indicating uPA expression as one factor which may be functionally involved in human metastasis. These findings could be employed and expanded in the directions outlined above, in order to identify novel and improved strategies for therapy and prevention of cancer metastasis.

## SUMMARY

The data summarized here show the successful development of a human cellular model system which is adequate for in-vivo studies of metatsasis in immunodeficient mice. The experimental metastasis of the HOS-derived cells in mice resembled, to some degree, the clinical manifestations of osteosarcoma in human patients. It seems, therefore, relevant that in this model system, the metastatic potential of the cells correlated with their expression of uPA and that blocking the enzymatic activity impaired the ability of the cells to colonize the lungs. The significance of these findings could be evaluated by expanding the investigations and including correlative studies of biopsy samples from various human primary tumors and their corresponding metastatic lesions.

These studies further showed that several of the HOS sublines expressed PAI-1, a potent uPA inhibitor, concomitantly with uPA. This suggests that uPA activity may be regulated by the transcription rate of both the enzyme and PAI-1. Moreover, it implies that tumor dissemination could, potentially, be controlled by suppressing the localized uPA-mediated proteolysis through an effect on either uPA or PAI-1 production. In order to examine this hypothesis, further studies should focus on developing effective ways for blocking

uPA activity in a permanent, sustained manner both *in-vivo* and *in-vitro*.

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