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A model of non-small cell lung cancer (NSCLC) has been developed for screening and preclinical drug evaluation by implanting the A549 lung cancer cell line orthotopically into immunocompromised (SCID) mice. Aggressive metastatic sublines were then derived from metastases from the primary implant. The purpose of this project is to elucidate some of the cellular properties involved in the tumor aggressiveness of the metastatic variant cell lines. *In vitro* migration and invasion assays produced data showing no significant differences between the rates of migration or invasion of the parental and metastatic sublines. *In vivo* tumor burden experiments, however, produced data showing significant differences in the numbers and sizes of metastatic tumors formed when the three cell lines were compared in SCID mice. RT-PCR analysis has indicated that there are differences in the mRNA levels of certain matrix metalloproteinases. The A549 parental cells have matrix metalloproteinase-2 (MMP-2) but not MMP-9, while both metastatic variants show MMP-9 mRNA but no MMP-2. Western blots and gelatin zymographies also confirm these findings. RT-PCR analysis and casein zymography experiments have also shown no differences in the message or activity of urokinase plasminogen activator (uPA) among the cell lines. Multidrug resistance studies were done on the tumor cell lines in order to compare their resistance to various classes of antineoplastic drugs. These studies indicate that there is no significant difference in the resistance to doxorubicin or paclitaxel, but the parental cell line is substantially more resistant to cisplatin than either of the metastatic sublines.

PROPERTIES OF A HUMAN METASTATIC VARIANT

LUNG CANCER MODEL

Julie E. Poirot, B.S.

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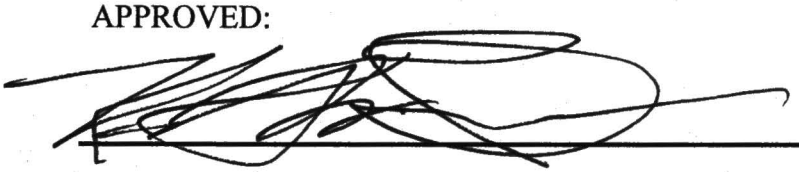

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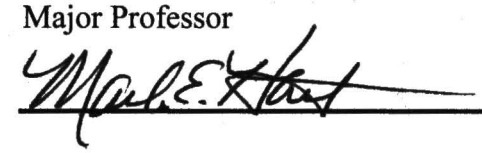

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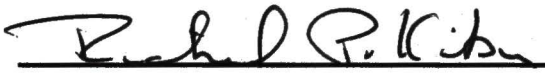

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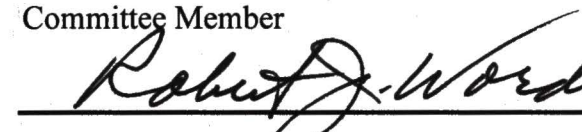

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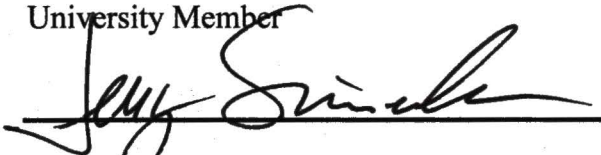

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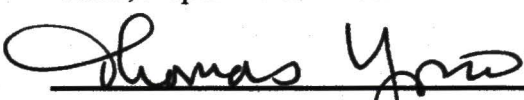

Dean, Graduate School of Biomedical Sciences

PROPERTIES OF A HUMAN METASTATIC VARIANT LUNG CANCER MODEL

THESIS

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Julie E. Poirot

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CHAPTER 1

INTRODUCTION

Metastasis

Cancer cells are defined by two heritable properties: they and their progeny (1) reproduce in defiance of the normal restraints and (2) invade and colonize territories normally reserved for other cells. It is the combination of these events that makes cancer particularly dangerous, especially the latter property of metastasis to distant organs (Albert, et al 1994). Cell migration plays a prominent role in many biological and pathological processes, including embryogenesis, the inflammatory response, tissue repair and regeneration, cancer, arthritis, atherosclerosis, and osteoporosis (Webb, Parsons et al. 2002). This process of cell migration, as well as invasion and metastasis, are biologic hallmarks of malignant tumors (Catran, et al 1990).

The process of cancer metastasis has been investigated as early as 1889 when Paget proposed the "seed and soil" hypothesis of metastatic spread. This hypothesis suggests that certain tumor cells ("seeds") grow best in certain favorable organ microenvironments ("soil"). The process of metastasis begins with the neoplastic transformation of normal cells to tumor cells. When the tumor reaches a small mass, angiogenic factors are produced to induce new blood vessel formation within the tumor to provide nutrient supply. Subpopulations of cells in the tumor then invade local host stroma, penetrate the host microvasculature, and enter the circulation. These tumor cells then extravasate out of the vasculature into the perivascular stroma, implant within the parenchyma of the target

organ, and begin to proliferate to form a secondary tumor metastasis (Goldfarb, et al 1997).

The formation of metastases is a continuous process beginning early in the growth of the primary tumor and increasing with time. Fidler and Hart (1982) have shown that the highly metastatic subpopulation of tumor cells preexists at a very early stage of development of the primary heterogeneous tumor. These highly aggressive cells may be selected because they have a higher probability of successfully producing a metastatic colony compared with the other subpopulations of the tumor cells (DeVita, et al 1993). The metastatic property of malignant tumor cells is, in part, responsible for the morbidity and mortality that accompanies advanced cancer (Goldfarb, et al 1997). At the time of diagnosis at least 50% of patients who present with primary solid tumors already have undetectable micrometastases in organ sites distant from the primary tumor (Goldfarb and Brunson 1992).

Invasion

In order for the primary tumor to metastasize to secondary sites within the body, intravasation and extravasation of the tumor must occur. This process of invasion through the extracelluar matrices (ECMs) of the host is divided into three key processes. The first step is tumor cell attachment to the ECM (Goldfarb 1986). The ECM, which is composed of collagens, proteoglycans, and glycoproteins, forms the external scaffold where both normal and tumor cells reside (McCauley and Matrisian 2000). Attachment of the cells to the ECM appears to be mediated through specific attachment glycoproteins such as laminin and fibronectin. The second step of tumor invasion is degradation of the matrix by tumor-cell derived or host-induced proteolytic enzymes. These enzymes, which can be

active in both extracellular, released forms and in cell surface associated forms, can locally degrade ECM and components including attachment glycoproteins. Finally, the last step in tumor invasion is tumor cell migration through the ECM. The direction of may be regulated to some extent by chemotactic factors from host cells, connective tissue or serum (Goldfarb 1986).

Invasion through the ECM is, therefore, caused by an internal imbalance of proteolysis (DeVita, et al 1993). There are several various proteinases capable of breaking down the ECM, but one family that appears to be particularly important for matrix degradation is the matrix metalloproteinases (MMPs) (McCauley and Matrisian 2000).

Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) were first described in 1962 when Gross and Lapiere identified an enzyme from a vertebrate with proteolytic action capable of degrading collagen (Gross 1962). It is now known that between the human MMPs and the homologues from other species virtually any component of the ECM can be degraded. They are capable of regulating the microenvironment of the tumor and their expression and activation is increased in almost all human cancers compared to normal tissues. MMPs make up a family of zinc-dependent endopeptidases with more than 20 human members and numerous homologues from other species. They are divided into eight structural classes, 6 of which are membrane bound (Egeblad and Werb 2002). The activity of the MMPs is controlled extracellularly: they are secreted in an inactive (zymogen) form and to become catalytically active the pro-domain must be removed. MMP activity is further controlled through interactions with their natural inhibitors, the

tissue specific inhibitors of matrix metalloproteinases (TIMPs) (McCauley and Matrisian 2000). Four TIMPs have been characterized in human and other species thus far. They form stable enzyme-inhibitor complexes with active MMPs. Some TIMPs can also form complexes with certain pro-MMPs, such as TIMP-1/pro-MMP-9 and TIMP-2/pro-MMP-2 (DeClerk 2000).

Two key MMPs involved in invasion of tumor cells through the ECM are MMP-2 and MMP-9. These two MMPs are subclassified as type IV collagenases/gelatinases because they are known to degrade denatured collagens (gelatins) and intact collagen type IV. They are also able to break down fibronectin, laminin, and elastin (Sawicki, Matsuzaki et al. 1998). MMP-2 (gelatinase A) is secreted in an inactive 72 kDA pro-form that is converted to a 62 kDa active form. Sato, et al. reported that MT1-MMP appears to be important in MMP-2 activation (Sato 1994). MMP-2 is also the most widely distributed MMP and is constitutively expressed by most cells (Foda and Zucker 2001). MMP-9 (gelatinase B) is secreted as a 92 kDa zymogen that is cleaved to the active 78 kDa form by MMP-3 (stromelysin-1). It is produced by inflammatory cells, including blood neutrophils and tissue macrophages, as well as by stimulated connective tissue (Foda and Zucker 2001). The importance of MMP-9 in invasion and metastasis was demonstrated by Hua and Muschel (1996) in experimental metastasis assays. These experiments showed that the number of colonies formed in the lungs of mice was reduced when MMP-9 is downregulated in cancer cells (Hua and Muschel 1996).

Urokinase Plasminogen Activator/Urokinase Plasminogen Activator Receptor

Other key proteolytic enzymes involved in tumor cell migration and invasion into the ECM are serine proteases. Two serine proteases, urokinase-type plasminogen activator and tissue-type plasminogen activator, are involved in intravasation and extravasation of tumor cells. These two types of plasminogen activators differ in both amino acid sequence and nucleotide sequence of corresponding cDNA. Almost all human plasminogen activator produced by tumor cells is urokinase-type, whereas a few tumors produce tissue-type plasminogen activator (Goldfarb and Liotta 1986).

The urokinase plaminogen activator (uPA) system is comprised of uPA, its receptor uPAR, plasminogen activator inhibitors (PAIs), and the proenzyme plasminogen (Plg) (Mazar, et al 1999). Human uPA is a highly specific serine protease that has only one well characterized natural substrate: plasminogen. uPA cleaves plasminogen to form the highly catalytic protease plasmin. Plasmin is a trypsin-like serine protease that has multiple biological substrates including fibrin, fibronectin, laminin, procollagenases, basement membrane components and casein (Goldfarb 1986).

uPA is produced by tumor cells as well as normal cells, including kidney tubule cells, phagocytic cells, fibroblasts and trophoblasts (Schmitt, et al 2000). It has several molecular weight forms including a 411 amino acid single chain zymogen also known as pro-urokinase (pro-uPA) which is approximately 56 kDa. Activation to the high molecular weight (HMW) form, which is approximately 54 kDa, requires cleavage of the Lys 158-Ile 159 bond leaving A and B chains joined by a disulfide bond. The 32 kDa low molecular weight form of uPA is made by truncating the A chain leaving an essentially pure catalytic domain. The truncation of the A chain is done by either MMP-3 or MMP-

7. Lysis by plasmin or autolysis by uPA can also truncate the HMW form forming LMW uPA (Mazar, et al 1999).

uPAR (CD 87), which is a specific cell surface receptor for uPA and pro-uPA, is pivotal to many major preoteolytic and signaling pathways associated with tumor progression (Mazar, et al 1999). It is a single polypeptide chain, highly glycosylated with a molecular weight of 55-60 kDa (Pappot, Hoyer-Hansen et al. 1997). uPAR is attached to the outer leaf of the plasma membrane via a covalent linkage of its carboxyterminus to a glycosylated phosphtidylinositol resulting in a GPI anchor. It is made up of three homologous domains. The amino-terminal domain I is the domain in which uPA binds. Besides interacting with uPA, uPAR also adheres to vitronectin and certain integrins, thereby influencing tumor cell adhesion and migration (Schmitt, et al 2000).

Upregulation of uPA, uPAR, and/or PAI-1 in cancer tissues is associated with increased malignancy. These proteins have been shown to be of high clinical value in that they are good prognostic markers suited to identify patients at risk to develop metastases. For example, in 1988-1989 Duffy et al. reported a high correlation between elevated uPA levels in the primary tumor and poor outcome of breast cancer patients (Duffy, O'Grady et al. 1988). Many independent reports have since been published confirming these results in many other solid malignant tumors.

Lung Cancer

Lung cancer is the leading cause of cancer death in developed countries (Albert, et al 1994). While the incidence of lung cancer in men is beginning to fall in the West, the incidence of lung cancer in women continues to rise and in a number of countries has overtaken breast cancer (Sethi 2002). It is now one of the most common malignancies

diagnosed in the United States. There are no signs or symptoms that are specifically diagnostic of lung cancer (Cersosimo 2002); therefore, due to the lack of diagnostic tools for early detection and of efficient treatment for advanced disease, the prognosis of lung cancer is poor with less than 15% surviving five years after diagnosis (Albert 1994). Lung cancers can be classified histologically into two main categories: small cell carcinomas and non-small cell carcinomas. The non-small cell carcinomas are further divided into squamous-cell carcinomas, adenocarcinomas, and large-cell carcinomas (Cersosimo 2002).

The A549 cell line that was used in this study is an example of a non-small cell adenocarcinoma. Adenocarcinomas are the most common form of lung cancer found in nonsmokers and in women, accounting for almost 40% of primary tumors. They are often peripherally located, involving the pleura of the lung and associated with scarring and pleural effusion. They also may grow through the pleurae to the chest wall. These tumors tend to double in size every 161 days (Cersosimo 2002). Adenocarcinomas are grayish white in color, with areas of hemorrhage and necrosis. The tumors may also have a glistening surface when sufficient mucin production is present. The cells of adenocarcinomas are usually large, with large nuclei, high nucleus-to-cytoplasmic ratio, and prominent eosinophilic nucleoli (Ginsberg 2002).

A549 Lung Cancer

The A549 non-small cell lung adenocarcinoma cell line was established in 1972 by D.J. Giard, et al. through explant culture of lung carcinomatous tissue from a 58 year old male (Giard, Aaronson et al. 1973). Studies of this cell line by Lieber, et al. in 1976 revealed that at both early and late passage levels the A549 cells had multilamellar cytoplasmic

inclusion bodies typical of type II alveolar epithelial cells of the lung. The cells were also found to synthesize lecithin with a high percentage of desaturated fatty acids using the cytidine diphosphocholine pathway. Use of this phospholipid synthesis pathway reveals that these cells probably are responsible for production of pulmonary surfactant. Together these data suggest that the A549 cell line originated from the neoplastic transformation of type II alveolar cells (Lieber, Smith et al. 1976). Other studies of this particular cell line show that the A549 cells express 3 respiratory mucin genes (MUC5AC, MUC1, MUC5B), p53, cytokeratins (polypeptides 7,8,9,18,19) and type I and II estrogen binding sites (Croce, et al 1999).

Development of Met2/Met3

Metastatic variants of the A549 cell line were then developed in order to determine some of the biological properties which are important in contribution toward enhanced malignancy of metastases. In Dr. Brunson's lab, the A549 cell line (A549 parental) was implanted orthotopically into the lung bronchi of immunocompromised (SCID) mice, and aggressive metastatic sublines were derived from metastases to regional thoracic lymph nodes in the tumor-bearing mice. Preliminary comparative data of the parental and metastatic variant (A549Met2) *in vivo* indicated marked biological differences in the capability for metastasis and decreased host survival, i.e. enhanced metastasis and lethality of A549Met2 compared to the parental A549 cells. In order to substantiate the findings in comparison studies, the A549 Met2 subline was injected into the tail vein of SCID mice and the A549 Met3 subline was then developed by harvesting the metastatic tumor cells from the lungs.

Drug Resistance

The major cause of death from cancer is metastases that are resistant to conventional therapy (Dong, et al 1994). The scattered deposits of metastases, the heterogeneity of their cellular subpopulation, and their size and age variations often limit the response to systemic anticancer drugs (Goldfarb and Liotta 1986).

Multidrug resistance (MDR) is defined as the simultaneous resistance to several structurally unrelated drugs that do not have a common mechanism of action (Gottesman, Fojo et al. 2002). It is a generic term for the variety of strategies tumor cells use to evade the cytotoxic effects of anticancer drugs. There are three main changes in cells that develop MDR: decreased intracellular accumulation of cytotoxic drugs, changes in cellular physiology affecting the structure of the plasma membrane, and changes in activity and expression of certain cellular proteins, such as the p-glycoprotein (Pgp) (Simon and schindler 1994). P-glycoprotein is a 170 kDa cell surface glycoprotein that is a product of the mdr-1 gene. It is an integral membrane protein that is mainly expressed in specialized endothelial cells with secretory or excretory functions, such as in the liver (Grude, et al 2002). In cancer cells, Pgp functions as an ATP-dependent drug efflux pump that reduces cellular accumulation of certain chemotherapeutic drugs including anthracyclines (Grude, et al 2002), colchicines, vinca alkaloids, doxorubicin, daunomycin and taxol (Bradley and Ling 1994). The quantity of Pgp expressed on the cell surface generally directly correlates with the cell's degree of drug resistance (Gerlach, et al 1987). There is also a strong correlation between detection of Pgp and a reduced probability of relapse-free survival. Studies of clinical and histological characteristics of Pgp expression have been performed which demonstrate that metastatic lesions appear to

express Pgp at levels as high or higher than those seen in primary tumors. This finding, in addition to results obtained from other Pgp studies, suggests an association between Pgp expression and tumor progression, even though the mechanism for increased expression of Pgp during cancer development is not fully understood at the present time (Bradley and Ling 1994). Further understanding of Pgp-associated MDR may, therefore, aid in the development of more successful chemotherapeutic strategies in the future (Gerlach, et al 1987).

Chemotherapeutic Drugs

One example of a chemotherapeutic drug that is extruded from the cell via the Pgp drug efflux pump is doxorubicin. Doxorubicin (adriamycin) is an anthracycline that is a very potent broad-spectrum antitumor agent. It is approved for use in treatment of a broad range of cancers including solid tumors, leukemias, and lymphomas. Doxorubicin is a DNA intercalator that inhibits DNA and RNA synthesis, produces single-stranded and double-stranded DNA breaks, alters cell membranes, and causes *in vitro* inhibition of DNA topoisomerase II (Patel, et al 1997)/(Ratain, et al 2001). It also forms free radicals causing oxidative damage to cellular proteins and stimulates apoptosis. Mechanisms of resistance to doxorubicin include increased expression of Pgp, enhanced antioxidant defense, enhanced DNA repair, overexpression of bcl-2, and mutations in p53 (Ratain, et al 2001).

Another chemotherapeutic drug administered for treatment of a wide variety of cancers including ovary, breast and lung carcinomas is paclitaxel (taxol). Paclitaxel is an antimitotic agent that binds to microtubules stabilizing them against depolymerization

(Kavallaris, et al 1997). Suppression of microtubule dynamics by paclitaxel strongly inhibits the assembly and function of the mitotic spindle, thereby preventing or slowing cell cycle progression at the metaphase/anaphase checkpoint. This disruption of the cell cycle eventually induces cell death. Despite paclitaxel's success as a valuable antitumor agent, resistance to the drug over time severely limits its effectiveness (Goncalves, et al 2001). Resistance to this drug has predominantly been associated with overexpression of Pgp drug efflux pump and the multidrug resistance associated protein (MRP) (Ratain, et al 2001).

A third anticancer drug, cisplatin, is a widely used chemotherapeutic agent. Cisplatin-based chemotherapy shows significant antitumor activity in a variety of cancers including testis, ovary, head, neck, and lung (Kartalou and Essigmann 2001). It is a well-known platinum drug that binds to many cellular components that have nucleophilic sites such as DNA, RNA, proteins and thiol-containing molecules. The process of DNA binding is thought to be the essential step in the cytotoxic reaction of the drug (Gonzalez, et al 2001). Reduced intracellular accumulation of cisplatin is often observed in cisplatin resistant cells. This reduced accumulation of the drug may arise because of decreased uptake or increased efflux. Unlike the other two drugs previously mentioned, cisplatin is not a substrate for the Pgp drug efflux pump. The exact mechanism causing the reduced accumulation of cisplatin in the cells is not fully understood at this time but it is believed that the glutathione S-conjugate export pump may efflux cisplatin conjugated with glutathione. It is also possible that an unknown 200kDa protein expressed by the cisplatin resistant cells may be involved in cisplatin efflux (Kartalou and Essigmann 2001).

Hypothesis

I hypothesize that the metastatic sublines will show increased rates of migration and/or invasion, increased levels of protease activity and expression, and differences in resistance to chemotherapeutic drugs.

Specific Aims

1. To compare the rates of migration and invasion of the A549 parental cell line and the metastatic cell lines
2. To explore some possible mechanisms of invasion and metastasis by comparing protease expression and activity in the cell lines
3. To examine chemotherapeutic drug resistance of the cell lines by comparing drug resistance *in vitro*

CHAPTER 2

MATERIALS AND METHODS

Reagents and Chemicals

WST-1, a tetrazolium salt, was used to quantify viable cell number in the migration, invasion and cytotoxicity assays, and was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Antibodies to MMP-2 (Ab-3) and MMP-9 (Ab-7) that were used to probe the Western blots were purchased from Oncogene Research Products (Cambridge, MA). The cisplatin and paclitaxel (semi-synthetic) used in the cytotoxicity assays were obtained from Calbiochem-Novabiochem (San Diego, CA). The doxorubicin hydrochloride was purchased from Sigma (St. Louis). The BB-94 MMP inhibitor was a gift from British Biotech (Oxford, UK).

Culture and Preparation of Cell Lines

The A549 parental and metastatic sublines were cultured in F12-K medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 u/ml penicilllin, 100 μg/ml streptomycin, and 2mM glutamine. The cells were cultured at 37° C with 5% CO_2. Adherent cells were detached with 1.0 mM EDTA/0.25% trypsin solution.

Migration Assays

Sub-confluent A549 cell lines were detached from the flask using EDTA/trypsin solution. Migration chambers with 8 micron filters (Becton-Dickinson Labware, Bedford, MA)

were used to determine rates of migration of the various cell lines. The bottom chambers were loaded with 0.75 ml of compete F12-K medium. A 0.50 ml aliquot of A549 cells (1.0×10^5 cells/ml) in serum-free F12-K was placed in the top well. The wells were incubated at 37°C with 5% CO_2 for 48 hrs.

After incubation, the surface of the top well was swabbed with PBS to remove any cells that did not migrate through the filter. Triplicate control wells did not have any cells removed so that the total number of cells on both the top well and bottom chamber could be calculated. WST-1 cell proliferation reagent was used to quantify the number of cells that migrated through the filter and the total number of cells in both the top and bottom chambers. Assays were performed in triplicate and results were reported as

$$\% \text{ migration} = (\# \text{ of cells migrated} / \text{total } \# \text{ of cells}) \times 100$$

Invasion Assays

This assay was performed to compare the rates of invasion of each of the cell lines. Sub-confluent A549 cells were detached from the flask using EDTA/trypsin solution. Biocoat Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA) with 8 micron pores were used. The matrigel (reconstituted basement membrane) in the top wells was hydrated with 0.5 ml of serum-free F12-K medium for 2 hours at 37°C. After hydration of the matrigel, 0.75 ml of complete F12-K medium was placed in the bottom chamber. The serum-free medium was then removed from the top wells and 5×10^4 cells in 0.5 ml of serum-free F12-K medium were plated. The plates were incubated at 37°C with 5% CO_2 for 48 hrs. After incubation, the surface of the top wells was swabbed with PBS to remove any cells that did not invade through the matrigel. The number of invading cells

was then determined using the WST-1 cell proliferation reagent. Triplicate uncoated wells for each cell line were used as controls. Conditions were the same for the uncoated wells and the matrigel coated wells. Assays were performed in triplicate and invasion results were reported as

% invasion=(# invading cells on coated wells / # migrating cells on uncoated wells) x 100

***In Vivo* Studies (SCID mice)**

Three sample groups (Par/Met2/Met3) were each made up of 7 mice in each group. Tail vein injections of 2×10^5 tumor cells per mouse were administered on day 1. Mice were sacrificed on day 43. A 1.0 mL aliquot of 40% PBS, 35% Histo prep (Fisher), 20% formalin, 5% India ink was injected into the trachea of the mice. The lungs were then harvested, bleached and preserved in formalin solution. Tumors were counted and categorized by size using an optical microscope and grid (10x10mm, $1mm^2$) (Fisher). Small tumors were defined as $\leq 1mm^2$, medium tumors as between 1 mm^2 and 4 mm^2, and large tumors as ≥ 4 mm^2.

RT-PCR

Total RNA was extracted from $1x10^7$ cells from the A549 parental and metastatic sublines using the Rneasy column from Qiagen (Chatsworth, CA). The cDNA was then synthesized using the Omniscript Reverse Transcriptase kit. For each cDNA synthesis, cells were reverse transcribed using random hexamer or oligo$(dT)_{16}$ primers in a volume of 30 μL according to the protocol provided by Omniscript. PCR amplification was done using the appropriate primers and HotStar Taq DNA polymerase. The PCR products were

run in the electrophoresis chamber on a 1% agarose gel with 20% ethidium bromide. The gel was run at 150V for 1 hour and the bands were visualized using UV light.

Concentrated Condition Medium

The A549 parental and metastatic sublines were placed in Opti-mem (Gibco) reduced serum medium for 24 hours. The supernatants were then collected, centrifuged to remove cell debris, and concentrated in Amicon Centriplus concentrators (Millipore Corp. Bedsford, MA) up to approximately 40-fold. Protein concentrations of the samples were determined using the bicinchoninic acid assay (Smith, Krohn et al. 1985). Samples were frozen at –20°C. HT-1080 control supernatants were prepared using the same protocol.

Western Blots

Concentrated condition mediums from the A549 cell lines were run on a precast 4-12% tris-glycine gel from BioWhittaker Molecular Applications (Rockland, ME) at 40 mAmps for 1 hour and 30 minutes. Proteins on the gel were then electroblotted onto a PVDF membrane and transferred for 1 hour at 100V in 4°C. The membrane was blocked overnight at room temperature on the shaker with 10% dry milk in 0.2% phosphate buffered saline-Tween 20 (PBS-T). Monoclonal antibodies (1:250 dilution) were then placed in 10% blocking for 2 hours at room temperature. The secondary antibody (1:50,000) and Extra-avidin (1:50,000) were then added to 0.2% PBS-T and the membrane was incubated at room temperature for 1 hour. The membrane was placed in equal parts Luminol/Enhancer solution and Super Signal Substrate (Pierce Chemical,

Rockford, IL) for 3 minutes. The membrane was exposed on high performance chemiluminescence film for 2-5 minutes and developed.

Gelatin Zymography

2 mg/mL of Sigma Type B gelatin was added to a 10% SDS-PAGE gel. Concentrated conditioned media (20 μg) from all of the A549 sublines was added directly onto the gel. To allow for renaturation of the active enzymes, the samples were neither boiled nor reduced prior to loading. The gels were electrophoresed at 40 mAmps for 1 hour and 15 minutes. The gels were incubated in 2.5% Triton X-100 for 1 hour at room temperature. The gels were then transferred to a 1.0% Triton X-100 buffer and incubated overnight at room temperature. Gels were stained in 0.25% Coomassie Blue in 45% methanol and 10% acetic acid overnight. Destaining was done using 30% methanol and 10% acetic acid until the bands look clear. The achromatic bands indicate the presence of active gelatinases (MMP-2 and/or MMP-9) that have cleaved the substrate gelatin. In control gels, BB-94, a general inhibitor of MMPs, was added to both wash buffers to ensure that the achromatic bands seen on the gel were due to MMP cleavage.

Casein Zymography

2% casein and 10 μg/mL of Lys plasminogen were added to a 10% SDS-PAGE gel. The concentrated conditioned medium (3.0 μg) from the three A549 cell lines was added directly onto the gel. The samples were not boiled or reduced prior to loading. The gels

were run at 40 mAmps for 1.5 hours. The gels were incubated in 2.5% Triton X-100 for 1 hour at room temperature on the shaker. They were then transferred to the second wash buffer which contained 0.1 M glycine at pH 8.4 and incubated for 4 hours at room temperature. 0.25% Coomassie Blue in 45% methanol and 10% acetic acid were used to stain the gel overnight. Destaining was done using 30% methanol and 10% acetic acid until the bands of lysis appear. The achromatic bands indicate the presence of active urokinase plasminogen activator (uPA) that has cleaved the plasminogen to plasmin, which then cleaves the casein. 10% SDS-PAGE gels without Lys plasminogen were also run at the same time to ensure that the achromatic bands were indeed from the activity of uPA.

Cytotoxicity Assays

The *in vitro* sensitivities of the three A549 sublines to cytotoxic agents were assessed using the WST-1 cell proliferation reagent. Doxorubicin (0.5–2.0 μM), cisplatin (5-50 μM), and paclitaxel (1-10 nM) were the three chemotherapeutic drugs used for comparison in this study. Concentrations used were determined following literature searches of cytotoxicity assays with the same drugs on a variety of several different cancer cell lines. Cisplatin and paclitaxel were dissolved in DMSO and doxorubicin was dissolved in H_2O. Cells (5×10^3/0.5 ml) from each of the A549 sublines were placed in each well of a 48 well plate and allowed to adhere for 24 hours. All medium was then removed and replaced with 1ml of the indicated drug solutions. Three controls (per cell line) were also used for comparison: (1) untreated cells in complete medium, (2) cells with 5 μL/ml of H_2O, and (3) cells with 5 μL/ml of DMSO. After 24, 48, and 72 hours,

the medium in each well was replaced with new medium plus WST-1 and incubated for 1 hour. Experiments were performed in triplicate and the IC_{50} of each cell line for each drug was then reported. The IC_{50} doses were calculated by determining the amount of drug needed to kill 50% of the cells.

CHAPTER 3

RESULTS

Specific Aim #1: Comparison of the rates of migration and invasion of the A549 parental cell line and the metastatic cell lines

Comparison of the Rates of Migration of the A549 Cell Lines

To compare the rates of migration of the A549 cell lines, migration assays were performed. The A549 Parental, Met2, and Met3 tumor cells were placed in serum-free medium in the top chamber of a migration well with an uncoated 8 micron pore-size filter. Complete medium was placed in the bottom well for the cells to migrate towards. The parental cells averaged 38% migration, the Met2 cells had an average migration rate of 43% and the Met3 cells averaged 45% migration. As shown in Figure 1, the rates of migration of each of the cell lines were not significantly different as determined using the one-way analysis of variants (ANOVA) test.

Invasiveness of the A549 Cell Lines

The invasiveness of each of the cell lines was determined using a matrigel invasion assay. Tumor cells from each of the cell lines were placed in serum-free medium in the top chamber of an invasion well with an 8-micron pore-size filter coated with matrigel. Complete medium was placed in the bottom well for the cells to move towards. After 48 hours of incubation, it was determined that there was no significant difference, as determined using the one-way analysis of variants (ANOVA) test, in invasiveness between the three sublines (Figure 2). Parental cells had an average invasion rate of

3.33%, the Met2 subline averaged 7.67% invasion and the Met3 cells averaged 8.0% invasion.

Assessment of Tumor Burden *In Vivo*

To assess whether the microenvironment *in vivo* may change the tumor cells' rates of migration and invasion, animal studies were performed. The tumor cells from each of the sublines were injected into the tail vein of SCID mice and the mice were sacrificed after 43 days. There was found to be no significant difference in the average number of small tumors in the cell lines, however, significant differences were found in the average number of both medium and large tumors when the A549 Met3 cell line was compared to the parental A549 cell line using the Kruskal-Wallis test (Figure 3). The average number of medium tumors in the mice injected with the Met3 cells was 54.33 tumors while the average in the mice injected with the parental cell line was 3.33. For the large tumors, the mice in the Met3 group averaged 33.83 tumors while the mice in the parental group averaged 0.50 tumors per mouse.

Specific Aim #2: Exploration of some possible mechanisms of invasion and metastasis by comparing protease expression and activity in the cell lines

RT-PCR Analysis of Proteases

Total RNA was extracted from each of the A549 cell lines. The cDNA was synthesized and PCR amplification was done using the appropriate primers for human GAPDH, MMP-2, MMP-9, TIMP-1, TIMP-2, uPA and uPAR (Table 1). Figure 4 shows that the parental cells express much less mRNA for MMP-9 than both of the metastatic sublines.

The parental cells, however, have significantly higher levels of MMP-2 than either of the metastatic sublines. Amounts of both TIMP-1 and TIMP-2 appear to be equal in all three sublines. uPA and uPAR mRNA levels, shown in Figure 5, also seem to be the same in all three cell lines. GAPDH was used as a control to ensure that equal amounts of cDNA were synthesized and loaded onto the gel for comparison between the cell lines.

Differences in Protein Expression Between MMP-2 and MMP-9

Because of the differences in mRNA levels of both MMP-2 and MMP-9 in the three cell lines, western blots were performed to look at protein expression of MMP-2 and MMP-9. Concentrated condition medium from each of the cell lines was probed using a mouse anti-human MMP-2 monoclonal antibody. A band corresponding to the active 66 kDa MMP-2 protein appeared only in the parental cell line (Figure 6A). The membrane was then stripped and reprobed with a mouse anti-human MMP-9 monoclonal antibody. The 92 kDa latent form of MMP-9 was detected in each of the metastatic sublines but not in the parental cell line active (Figure 6B). The results obtained from the western blots confirm the results seen in the RT-PCR. HT-1080 concentrated condition medium was used in these western blots as a positive control for both MMPs.

Analysis of MMP Activity

After observing differences in MMP-2 and MMP-9 mRNA and protein expression between the parental and metastatic cell lines, gelatin zymographies were done to compare the activities of the MMPs. The same concentrated condition medium used in the western blots was used for the zymography. Figure 7A show the presence of active

MMP-2 (66kDa) in the parental cell line but not in the metastatic variants. The metastatic variants, however, show MMP-9 activity with 2 bands, an active form at 82 kDa and another truncated active form at ~65 kDa. MMP-9 activity was not seen in the parental cell line (Figure 7A). BB-94, a specific MMP inhibitor, was used in Figure 7B inhibiting all activity. This inhibition of lysis confirms that the bands are definitely from MMP activity.

Determination of uPA Activity

To assess the enzymatic activity of uPA in the cell lines, casein zymographies were done. The same concentrated condition mediums used in the western blots and gelatin zymographies was used to compare uPA activity in the casein zymography. HT-1080 concentrated condition medium was used as a positive control to show the 55kDa bands of lysis indicating active uPA. All three of the A549 cell lines showed equal levels of activity of uPA (Figure 8A). In the absence of plasminogen (Figure 8B) no bands of lysis are seen indicating that the activity is specific to uPA.

Specific Aim #3: Examination of cancer chemotherapeutic drug resistance of the cell lines by comparing drug resistance *in vitro*

Comparison of Resistance to Different Conventional Chemotherapeutic Drugs

Cytotoxicity assays were performed to examine cancer chemotherapy resistance of the cell lines by comparing their resistance to different conventional chemotherapeutic drugs. The three drugs used were doxorubicin (0.5-5.0 μM), paclitaxel (1.0-10.0 nM) and cisplatin (5.0-50.0 μM). Concentrations used were determined following literature

searches of cytotoxicity assays with the same drugs on a number of cancer cell lines. By assaying the toxicity of each of the drugs after 24, 48 and 72 hours, average IC_{50} values were calculated and compared between the cell lines. Figure 9A-C illustrates the significant cytotoxic effect of doxorubicin on each of the cell lines. Average IC_{50} doses for each of the cell lines were then calculated from the results. The parental cells had an average IC_{50} dose of 0.31μM, Met2 had an average IC_{50} dose of 0.31 μM and the Met3 had an average IC_{50} dose of 0.33 μM. Therefore, no significant differences, as determined using the one-way analysis of variants (ANOVA) test, in resistance to doxorubicin were observed (Figure 9D).

Paclitaxel was the second drug tested on the cells. Killing of the cells by the drug was observed in all of the cell lines, however the drug was not as toxic as doxorubicin overall (Figure 10A-C). Average IC_{50} doses were calculated with the parental cells having an average IC_{50} dose of 10.7 nM, Met2 having an average IC_{50} dose of 10.0 nM, and the Met3 having an average IC_{50} dose of 7.9 nM. Again no significant differences were observed in the resistance to paclitaxel between the cell lines as determined using the one-way analysis of variants (ANOVA) test (Figure 10D).

The last drug tested was cisplatin. As shown in Figure 11A-C, differences were seen between the cell lines in their resistance to cisplatin. The parental cells were much more resistant to the cisplatin than either of the metastatic sublines. Average IC_{50} doses were calculated and the average IC_{50} dose of the parental cells was 28.5 μM compared to 12.0 μM for the Met2 cells and 11.6 μM for the Met3 cell line (Figure 11D). Therefore, the parental cell line is significantly more resistant to cisplatin than either of the metastatic sublines as determined using the one-way analysis of variants (ANOVA) test.

TABLE 1

Primer Sequences Used in RT-PCR Analysis

Enzyme	Forward Primers (5'-3')	Reverse Primers (5'-3')
GAPDH	TAGACGGGAAGCTCACTGGC	AGGTCCACCCTGTTGCT
MMP-2	GAGGACTACGACCGCGACAA	CCAAATGAACCGGTCCTTGA
MMP-9	AGTTTGGTGTCGCGGAGCAC	CATGAGCGCTTCCGGCACTG
TIMP-1	CTGGCATCCTGTTGTTGCTG	GGAAGCCCTTTTCAGAGCCT
TIMP-2	AGATGTAGTGATCAGGGCCA	TCACTTCTCTTGATGCAGGC
uPA	GTGGCCAAAAGACTCTGAGG	ATTTTCAGCTGCTCCGGATA
uPAR	TGTAAGACCAACGGGGATTG	CCTTTGGACGCCCTTCTTCA

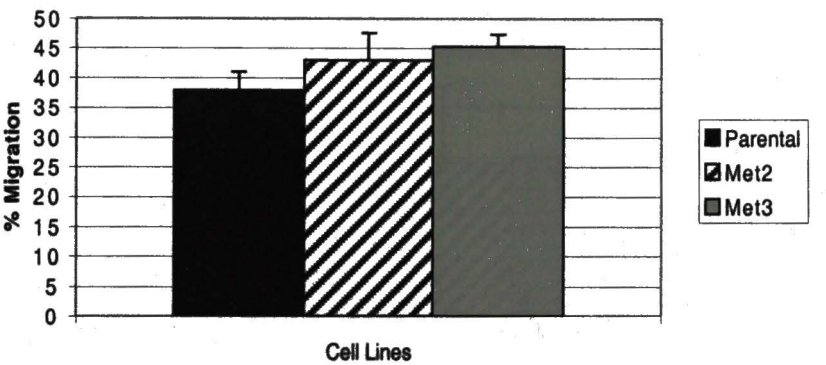

FIGURE 1. Rates of Migration of the A549 Cell Lines.

An aliquot containing 50,000 cells in 500 μL of serum-free F12-K medium was placed in the upper well of a migration chamber. Cells were allowed to move into complete medium for 48 hours. Results are expressed as % migration. Each bar represents the average of triplicate experiments (+/- SEM).

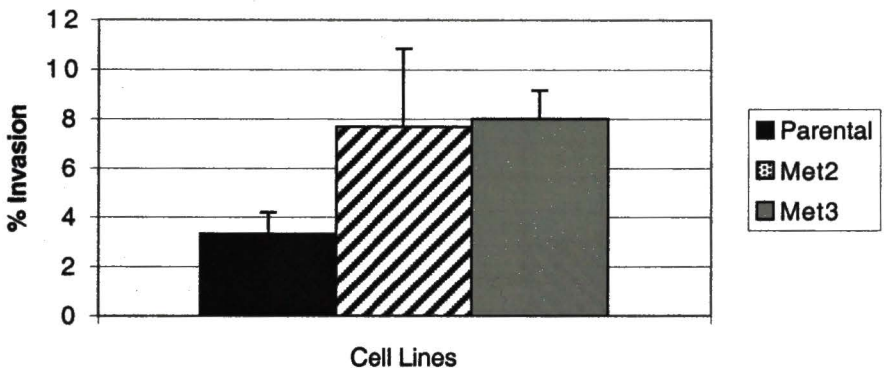

FIGURE 2. Invasiveness of the A549 Cell Lines.
An aliquot containing 50,000 cells in 500 μL of serum-free F12-K medium was placed in the upper well of a matrigel invasion chamber. Cells were allowed to invade through the matrigel into complete medium for 48 hours. Results are expressed as % invasion. Each bar represents the average of triplicate experiments (+/- SEM).

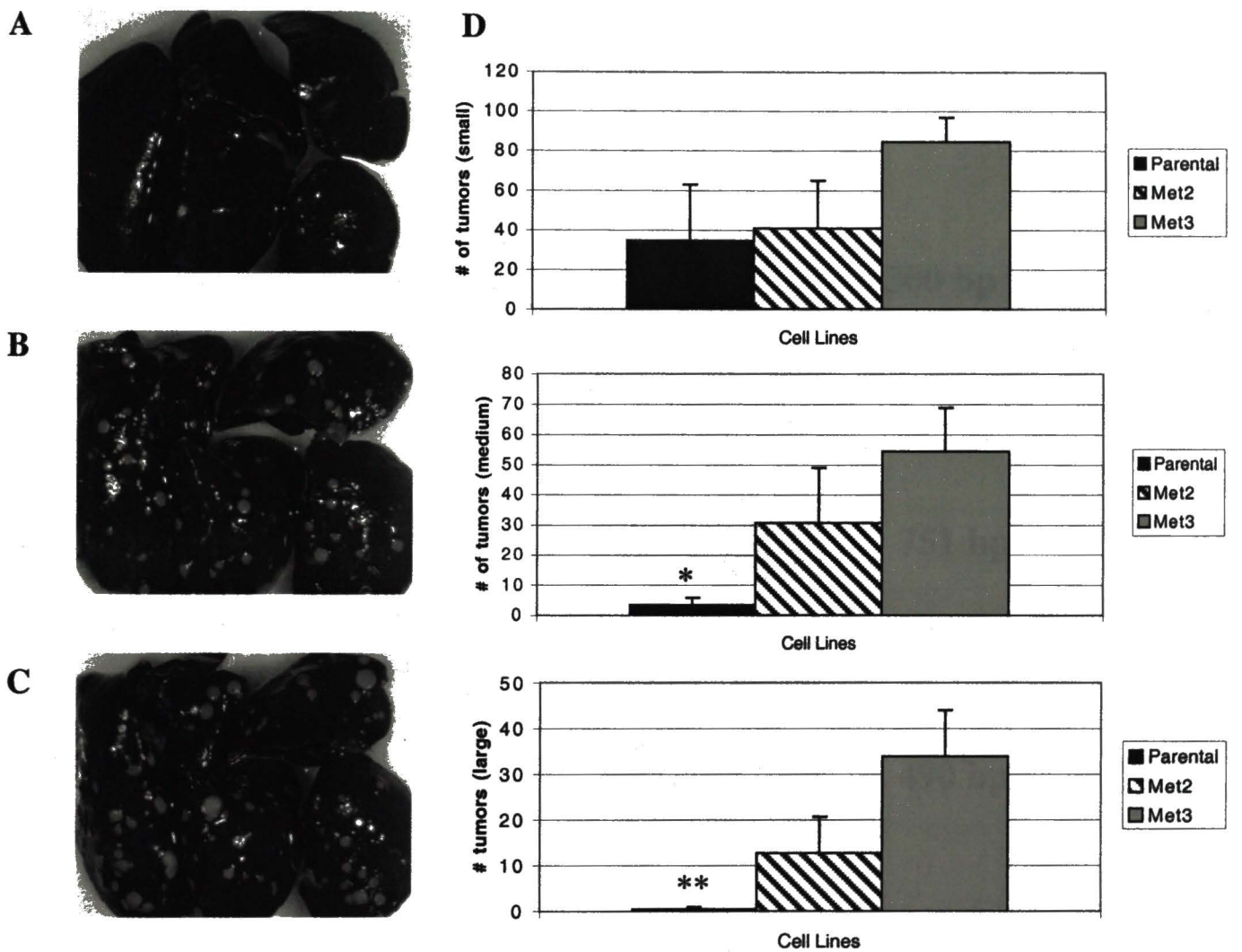

FIGURE 3. Tumor Burden *In Vivo*.

An aliquot of $2.0x10^5$ tumor cells was injected into the tail vein of SCID mice. Mice were sacrificed on day 43, lungs were harvested, and tumors were counted and categorized by size. Figure 3A-C, pictoral comparison of the tumor burden in the lungs of the mice (A:parental, B:Met2, C:Met3). Figure 3D, analyses of the tumor burden categorized by tumor size (+/- SEM).

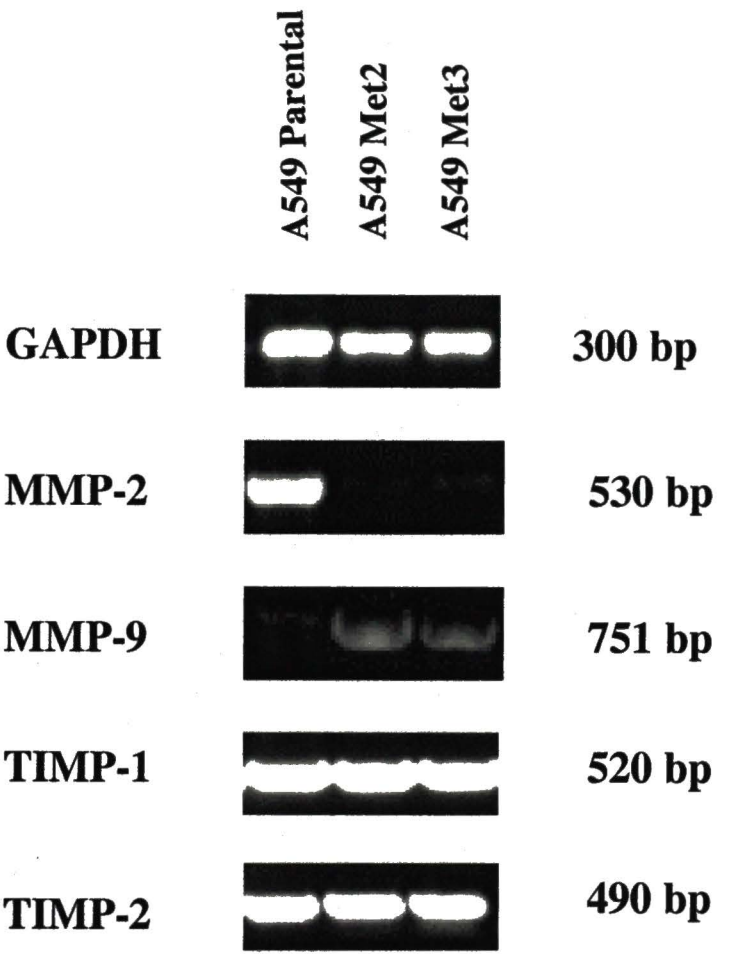

FIGURE 4. RT-PCR Analysis of MMPs/TIMPs.
Total RNA was extracted and cDNA was synthesized as described in Materials and Methods. RT-PCR analysis was done using specific primers listed in Table 1. Figure 4, analysis of MMP-2, MMP-9, TIMP-1 and TIMP-2.

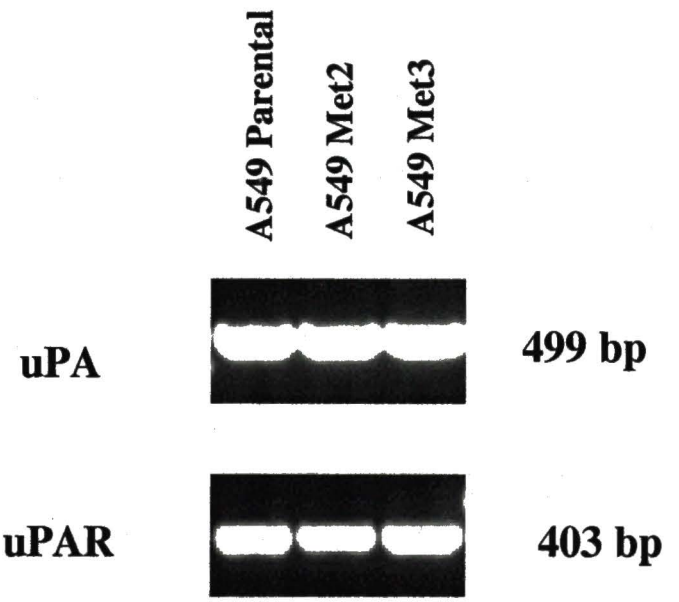

FIGURE 5. RT-PCR Analysis of uPA/uPAR.
Total RNA was extracted and cDNA was synthesized as described in Materials and Methods. RT-PCR analysis was done using specific primers listed in Table 1. Figure 5, analysis of uPA and uPAR.

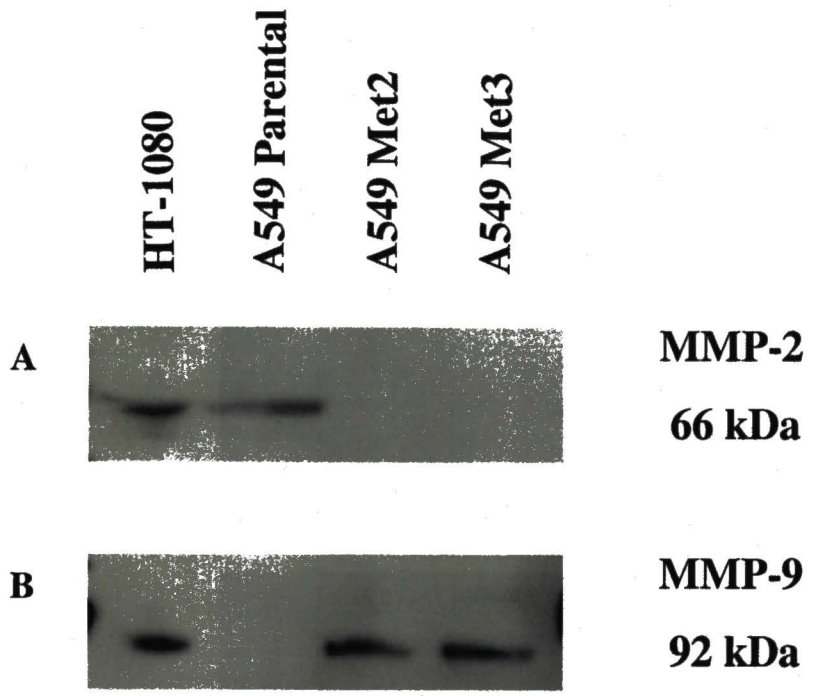

FIGURE 6. Protein Expression of MMP-2 and MMP-9. Concentrated conditioned medium from the A549 cells and the HT-1080 control cells were prepared as described in Materials and Methods. Samples were run on a 4-12% Tris-glycine gradient gel and Western blot analyses were done. Figure 6A show the MMP-2 protein at 66 kDa. Figure 6B, the membrane was stripped and re-probed for MMP-9. The MMP-9 protein was found at 92 kDa.

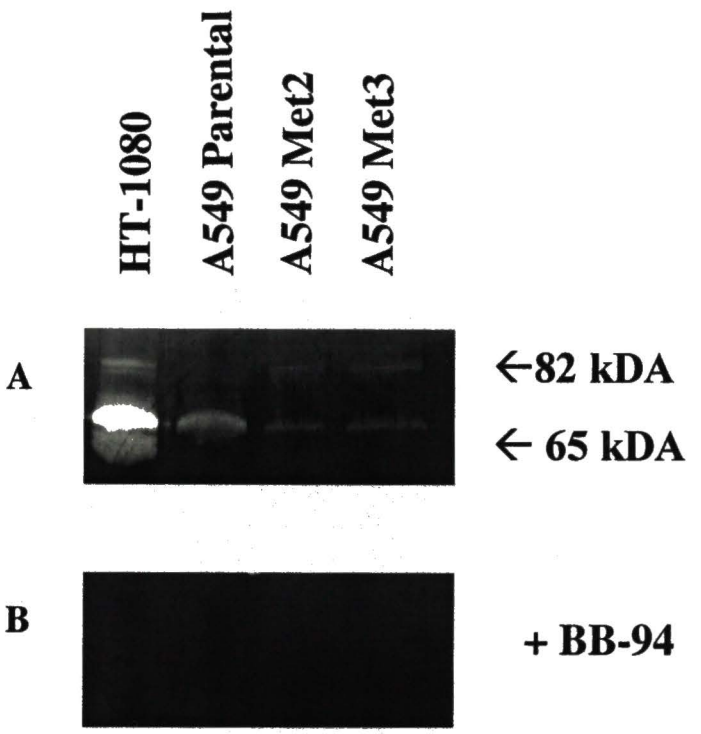

FIGURE 7. Analysis of MMP Activity.
Concentrated conditioned medium from the A549 cells and the HT-1080 control cells were prepared as described in Materials and Methods. Samples were run on a 10% SDS-PAGE gel with 2 mg/mL of Sigma Type B gelatin. Figure 7A, bands of lysis indicate MMP enzymatic activity. Figure 7B, addition of 10 μM BB-94, a specific MMP inhibitor, inhibited all activity.

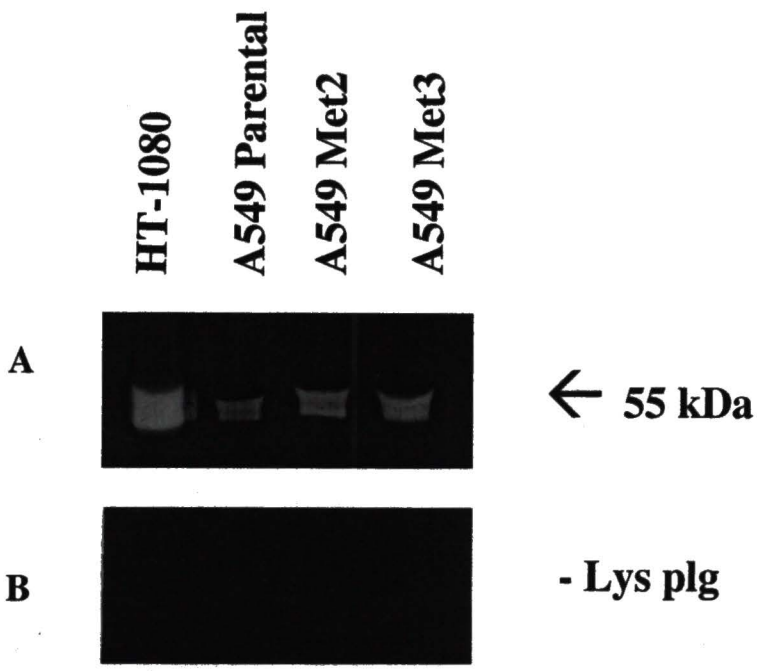

FIGURE 8. Determination of uPA Activity.
Concentrated conditioned medium from the A549 cells and the HT-1080 control cells were prepared as described in Materials and Methods. Casein was added to a 10% SDS-PAGE gel +/- plasminogen and samples were loaded directly onto the gel. Figure 8A, uPA activity (~55kDa) was seen in all cell lines. Figure 8B, gel made without plasminogen shows no bands of lysis.

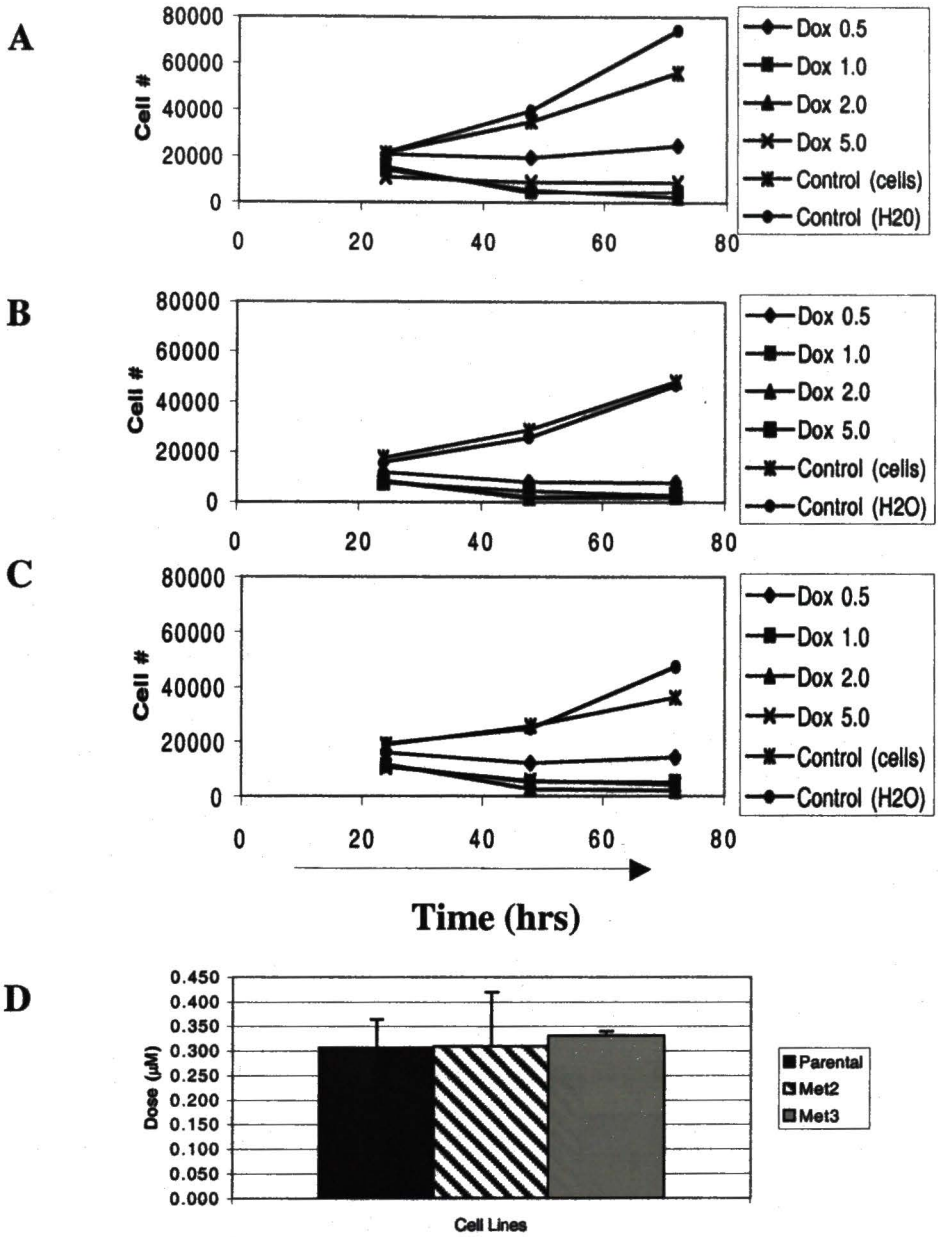

FIGURE 9. Cytotoxicity of Doxorubicin.
Cytotoxicity assays were performed to compare the resistance of the cell lines to various concentrations of doxorubicin. Figure 9A-C, toxicity of doxorubicin on the cells was compared after 24, 48, and 72 hours of incubation with the drug (A:Parental, B:Met2, C:Met3). Figure 9D, average IC_{50} doses from each of the cell lines were calculated using the data obtained.

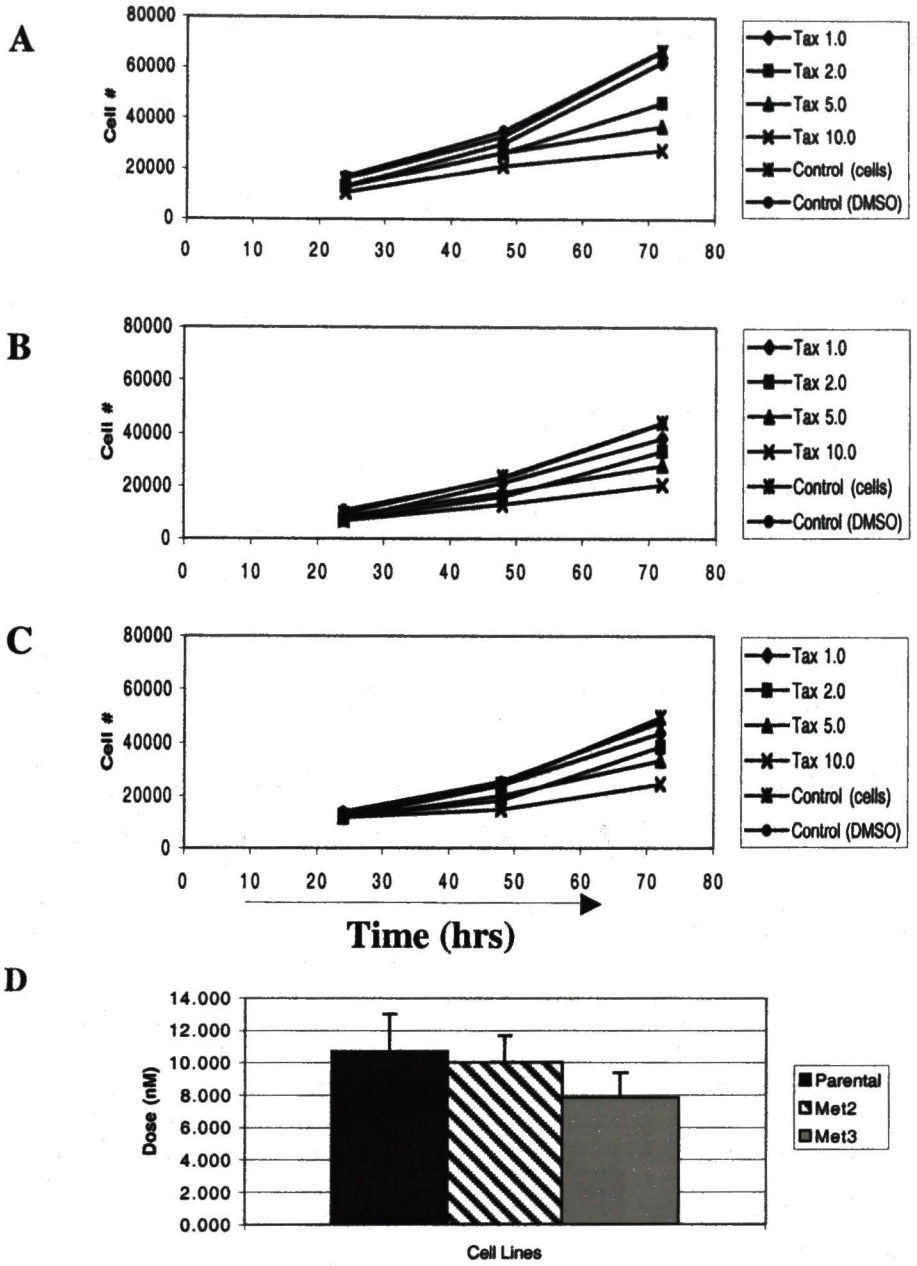

FIGURE 10. Cytotoxicity of Paclitaxel.
Resistance of the tumor cells to paclitaxel was examined using different concentrations of the drug. Figure 10A-C, killing of the cells was observed after incubation with the drug for 24, 48, and 72 hours (A:Parental, B:Met2, C:Met3). Figure 10D, data obtained from the cytotoxicity assays was used to calculate the average IC_{50} dose for each of the cell lines.

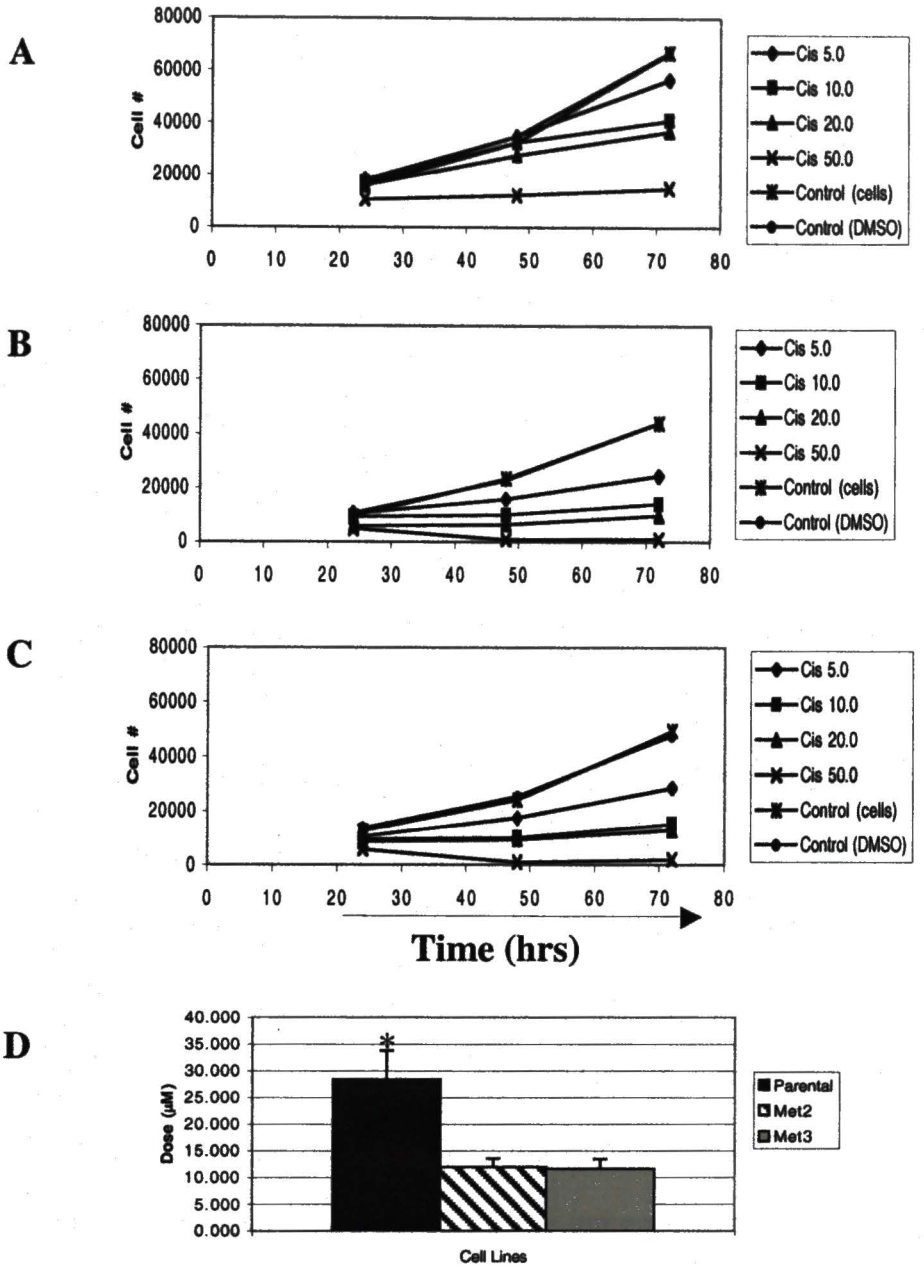

FIGURE 11. Cytotoxicity of Cisplatin.
Various concentrations of cisplatin were added to each of the cell lines to determine their resistance to the drug. Figure 11A-C, cell viability was calculated after addition of the drug for 24, 48, and 72 hours (A:Parental, B:Met2, C:Met3). Figure 11D, IC_{50} doses of cisplatin for each of the cell lines were calculated from the cell viability experiments.

CHAPTER 4

DISCUSSION

The main focus of this study was to examine key cellular components that might contribute to the malignancy of the A549 non-small cell lung cancer cell line. In order to aid in elucidating the aggressive properties of the lung tumors, two metastatic sublines (Met2/Met3) were developed to compare with the parental A549 cell line. Metastasis and invasion are biologic hallmarks of malignant tumors (Catran, et al 1990). These processes would therefore be crucial in the enhanced aggressiveness of the tumor cells. To compare the tumors' aggressiveness, the rates of migration and invasion between the three cell lines (Par/Met2/Met3) were determined using *in vitro* assays. Unexpectedly, it was found that there was no significant differences between the tumor cells' *in vitro* rates of migration or invasion. To determine if the *in vivo* microenvironment would have an effect on the tumor cells' rates of invasion and metastasis, the tumor cells were injected into the tail vein of SCID mice. After approximately six weeks, the mice were sacrificed and the tumors in the lungs were counted and categorized by size. The trends found in the tumor burden *in vivo* were as expected with the parental cells producing fewer tumors than the Met2 cells, and with the mice injected with Met2 cells having fewer tumors than the mice injected with Met3 cells. Significant differences were found in the number of both medium and large tumors with the Met3 cell line producing substantially more tumors than the parental cell line. The number of small tumors formed from each of the cell lines, however, was not statistically significant when the three cell lines were compared. The differences in the

number of larger tumors, but not in the number of small tumors, might be attributed to an increased *in vivo* growth rate of the metastatic sublines compared to the parental cell line. Data obtained previously from population doubling experiments and growth curves *in vitro* show the growth rates of the cell lines to be equal, however, when the tumor cells are placed in an *in vivo* microenvironment differences in growth rate among the cell lines may exist. Another important conclusion that could be drawn from these studies is that the significant differences in the numbers of medium and large tumors in the parental and Met3 injected mice could contribute to increased rates of mortality. The reason being that the larger tumors are much more lethal than the smaller tumors.

Next, in order to explore some of the mechanisms of invasion and metastasis, protease expression and activity was compared between the cell lines. Results obtained from RT-PCR analyses of the three cell lines indicate that the parental cell line expresses MMP-2 but does not express significant amounts of MMP-9, while the metastatic sublines express significant amounts of MMP-9 and virtually no MMP-2. The inhibitors of MMP-9 and MMP-2, TIMP-1 and TIMP-2, had equal amounts of the mRNA message in each of the cell lines. The differences found in MMP-2 and MMP-9 mRNA message between the cell lines was confirmed by Western blot analysis of actual protein expression and by gelatin zymography experiments illustrating the different activities of the gelatinases. These findings corroborate the data obtained from the *in vivo* tumor burden experiment. Previous research has shown that MMPs generate growth-promoting signals for the tumors. Specifically, it has been shown that tumor cell proliferation was decreased in MMP-9 deficient mice compared to wild-type mice (Egeblad and Werb, 2002). This difference in growth-promoting signals may explain the differences found in the number

of medium and large tumors in the mice injected with the metastatic cells, which have increased expression and activity of MMP-9, compared to the number found in the mice injected with the parental cells, which have little or no MMP-9 protein expression or activity. It could also be postulated that MMP-9's effect on tumor angiogenesis may play a pivotal role in the formation and growth of larger tumors. Bergers, et al has reported that in MMP-9 deficient mice tumor number and tumor size are decreased when compare to wild-type mice. They also show that MMP-2 deficient mice develop smaller tumors without an effect on tumor number. This data, in addition to data showing increased secretion of vascular endothelial growth factor (VEGF) induced by MMP-9, illustrate that both MMPs contribute to tumor growth, however, MMP-9 also affects the frequency of initial angiogenic switching or of progression to solid tumors (Bergers, Brekken et al 2000). MMP-9's induction of microvessel formation *in vivo* could therefore explain the differences seen in the *in vivo* tumor burden experiment presented previously.

Finally, because the major cause of death from cancer is metastases that are resistant to conventional therapy (Dong, et al 1994), resistance to conventional chemotherapeutic drugs was compared between the cell lines. No substantial differences were observed in the IC_{50} doses of the cell lines to either doxorubicin or paclitaxel. All of the cell lines were sensitive to the drugs when incubated for 24-72 hours. These findings are significant in that most metastatic tumor cells over-express the P-glycoprotein (Pgp) drug efflux pump, which pumps these two drugs out of the cell reducing their intracellular accumulation of the drugs. These two metastatic sublines, however, are sensitive to the drugs indicating that their intracellular accumulations of the drugs are not reduced by the Pgp drug efflux pump. A third chemotherapeutic drug, cisplatin, was also used to

examine its effects on the cell lines. A significant difference was seen in the parental cell line's resistance to the drug when compared to both of the metastatic sublines. These results indicate that the parental cell line utilizes some mechanism that the other cells do not which make it more resistant to the drug. Since cisplatin is not extruded from the cell via the Pgp drug efflux pump, other proteins/pumps that could contribute to the cell's resistance to the drug might include over-expression of the glutathione S-conjugate export pump to pump the drug out of the cell, over-expression of metallothioneins which bind to cisplatin and affect its sensitivity or reduced expression of Bax which would decrease rates of tumor cell apoptosis (Kartalou and Essigmann, 2001).

In conclusion, results obtained from these experiments illustrate the importance of *in vivo* cancer models for meaningful studies of tumor biology. The *in vitro* work on migration and invasion did not correlate with the tumor burden experiments *in vivo*. The drug studies also show that when tumor cells are selected on the basis of increased metastasis alone these cells are not always more resistant to conventional chemotherapeutic drugs. Further investigations into protease expression and activity *in vivo* and mechanisms of resistance of the parental cell line to cisplatin could also be performed. Results obtained in these investigations point to a critical need to evaluate biological properties of tumors in an *in vivo* environment, to confirm the importance of data obtained in *in vitro* experiments. Hopefully, insights gained into critical cellular targets associated with increased malignancy of the tumor cells may lead to more efficacious drugs for use in the clinic against aggressive lung cancer.

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