Inhibition of Proteolytic Enzymes in an In Vivo Model of Metastasis

Lawrence E. Ostrowski

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INHIBITION OF PROTEOLYTIC ENZYMES
IN AN IN VIVO MODEL OF METASTASIS

by

Lawrence E. Ostrowski

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy
June
1986
Acknowledgments

I would like to thank my advisor, Dr. Richard Schultz, for his continued support and patience during this work. I would also like to sincerely thank the members of my committee for their careful reading of this dissertation and for their helpful discussions. In addition, I would like to acknowledge Dr. Bruce Persky for his excellent photography and all the members of Dr. Schultz's laboratory who helped to make this project possible. Finally, I would like to thank my Mother for helping me to survive the last six years, my friends for helping me to enjoy them, and my wife, Carole Ann, for giving me the motivation and encouragement necessary to end them.
But in looking back, at the lessons I've learned,
and the mountains I've wanted to climb,
I just look at myself to find,
I've learned the hard way every time.

Jim Croce
VITA

Lawrence Edward Ostrowski is the son of the late Feliks Joseph Ostrowski and Grace Mary Ostrowski. He was born on March 5, 1958 in Chicago, Illinois.

He obtained his elementary education at Saint Senis Grade School in Chicago, Illinois. His secondary education was completed in 1976 at Quigley South Preparatory Seminary, also in Chicago. In 1976, he enrolled at Northwestern University, Evanston, Illinois, on an Evans Scholarship. In June, 1980, he was awarded a Bachelor of Arts degree in Biochemistry and Molecular Biology.

He was accepted into the Ph.D program at Loyola University in the summer of 1980.

On April 5, 1986, he was married to Carole Ann Meyer, M.D.. After completion of his degree, he will join his wife at the University of Arizona, Tucson, where she is an intern in the Department of Pediatrics. He will begin work for G. Tim Bowden, Ph. D., in the Department of Radiation Oncology, investigating the molecular biology of metastasis.
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Electron microscopy of amelanotic lung nodules
LIST OF ABBREVIATIONS

CAM  chorioallantoic membrane
DEAE  diethylaminoethyl
DFP  diisopropyl fluorophosphate
DMEM  Dulbecco's Modified Eagle Medium
DMSO  dimethyl sulfoxide
DNS  dansyl
EDTA  ethylenediamine tetraacetic acid
E64  L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane
Ep475  L-trans-epoxysuccinyl-leucylamido(3-methyl)butane
Ep453  ethyl ester of Ep475
FCS  fetal calf serum
i.p.  intraperitoneal
IU  international units
i.v.  intravenous
KIU  kallikrein inhibitor units
PA  plasminogen activator
LIST OF ABBREVIATIONS (continued)

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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>pCMB</td>
<td>para-chloromercuribenzoate</td>
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<td>PLG</td>
<td>plasminogen</td>
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<tr>
<td>R-</td>
<td>t-boc and/or H group when preceding D-Phe-Pro-Argininal, t-boc and/or tfa group when preceding Glu-Gly-Argininal</td>
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<tr>
<td>rtPA</td>
<td>recombinant tissue type plasminogen activator</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SFCM</td>
<td>serum-free conditioned medium</td>
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<tr>
<td>SPM</td>
<td>serum-free medium</td>
</tr>
<tr>
<td>t-boc</td>
<td>tertiary-butyloxycarbonyl</td>
</tr>
<tr>
<td>tfa</td>
<td>trifluoroacetate</td>
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<tr>
<td>tPA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>UK</td>
<td>urokinase</td>
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INTRODUCTION

Metastasis is the complex process in which tumor cells growing at a primary site detach from the primary tumor, migrate throughout the body, invade into normal tissue at a distant site, and grow into secondary tumors. The process of blood-borne metastasis is one of the major obstacles to the treatment of cancer, and is illustrated in a general and very simplified fashion in Figure 1. The tumor must first invade through normal tissue until it reaches a circulatory system, or, as illustrated, can induce the formation of its own supply of blood vessels. Tumor cells must then migrate through the basement membrane surrounding the vessel in order to be released free in the circulation (intravasation). The cells must then survive transport in the circulation throughout the body, and attach at some distant site. Here the tumor cell or cells must again penetrate the endothelium and basement membrane (extravasate) and migrate into the normal tissue, where the process of growth, invasion, and metastasis begins again. It has been estimated that only a very small percentage of tumor cells released into the circulation actually survive this process and develop into secondary growths. Many investigators are studying the mechanisms which allow certain
Figure 1. The pathogenesis of metastasis (from Fidler, 1978).
tumor cells to successfully complete the difficult metastatic pathway in the hope of finding ways to interfere in this process.

Recently researchers have tried to identify phenotypic properties necessary for metastasis by comparing normal cellular properties to those of transformed cells from the same tissue or by comparing the properties of tumor cells from the same origin with different metastatic potential. In this way, it has been demonstrated that there is a good correlation between an increase in proteolytic activity and an increase of the metastatic capability of certain tumor lines. As more information about the types of proteolytic enzymes secreted by tumor cells became available, roles for their possible involvement in the metastatic process were postulated. Due to the complex nature of the metastatic process, especially with regards to the number of interactions between a metastasizing tumor cell and the host, (i.e. immune system, hormones and growth factors, plasma proteases and inhibitors, etc.) there have been many suggested points of protease action, some of which have been in direct contradiction. The process of metastasis can be conveniently divided into several distinct steps as illustrated in Figure 1, although it should be understood that tumors are heterogeneous and not all tumor cells need to complete all of the steps in order for metastasis to occur. Proteases have been proposed to be involved in many of
these steps and there is evidence to support these ideas. Two of the major functions which proteases may perform are the degradation of normal host tissues which would act as barriers to a migrating tumor cell, and the reattachment of the tumor cell after it is in circulation. For example, plasminogen activators can activate the serum enzyme plasminogen to plasmin, a general serine type protease which can degrade many proteins on the surface of normal cells and attack many extracellular structures such as fibrin and fibronectin. Plasmin has also been shown to activate latent collagenase, a metalloproteinase which is essential for complete degradation of the basement membrane. It has been demonstrated that some tumors secrete a type IV collagenase and this proteinase may also be a part of the successful metastatic cell's phenotype. Cathepsins, especially B, have been shown to correlate in some cases with metastatic potential, and they too can degrade a variety of substrates, including proteoglycans and type IV collagen by activation of latent collagenase. In addition, other proteases, such as elastase, cathepsin D, and neutral trypsin-like proteases, have been correlated with metastatic potential. These may again be involved in degradation of normal host tissue. Thrombin has been postulated to play an important role in the reattachment of tumor cells by converting fibrinogen to fibrin and thus creating a
fibrin clot. Many tumors have been shown to secrete factors which activate thrombin, and factors which stimulate platelet aggregation have also been isolated. This can lead to the aggregation of a tumor cell and platelets or other blood components. An embolus formed by clumping of tumor cells, platelets and fibrin would help to anchor the tumor cell at a secondary site where it can invade to form a new tumor.

Other suggested roles for proteases include, 1) defensive mechanisms against the host immune system, 2) facilitating the release of tumor cells from the primary tumor by decreasing cell-cell adhesion, or 3) modification of the cell surface of adjacent normal cells. Modification of cell surfaces can stimulate the secretion of growth factors or proteinases by the normal cells which are necessary for the tumor to metastasize. These factors may also be important to the process of angiogenesis, which is another important feature of the progression of some tumors.

Although much evidence has accumulated over the years in favor of a crucial role for proteinases in the metastatic process, the issue is still not clearly resolved. Many of the results consist mainly of correlations with little direct evidence. In other cases the literature is contradictory, or at best unclear, perhaps because of the many different model systems and types of tumors being studied.
If proteases are essential to the process of metastasis, it may be possible to inhibit the spread of cancer by inhibiting the proteases involved. Alternatively, since much of the current literature disagrees on the importance of proteases in the process of metastasis, it may be possible to determine if specific proteases are necessary for metastasis by inhibiting their action. However, the inhibition of individual proteases in an in vivo model for tumor metastasis presents several problems. Some of the most potent enzyme inhibitors (i.e., chloromethylketones) are very toxic in vivo. Other inhibitors lack the specificity required to inhibit a particular enzyme or type of enzyme, thus producing results which are not conclusive. In addition, concentrations of the inhibitors must be able to be maintained at high enough levels to significantly inhibit the protease in order for an effect to be observed. Several in vitro models have been developed to selectively examine steps or stages in the metastatic process, and these have yielded valuable information. However, as noted above, the overall process of metastasis is very complex, and observations made in vitro may not be directly relevant to the in vivo situation.

The goal of this dissertation was to further investigate the role of proteolytic enzymes in metastasis utilizing specific enzyme inhibitors. Some of these enzyme inhibitors have been newly
synthesized in this and other laboratories, and should allow for the specific inhibition of implicated proteases. By performing these studies in a relevant in vivo model system, it is hoped that these results will help to clarify the importance of proteinases in metastasis and hopefully lead to a better understanding and treatment of neoplastic disease in the future.

The specific aim of this work therefore, was to examine the role of the proteolytic enzymes cathepsin B, plasminogen activator, and thrombin in the process of metastasis by inhibiting their activity in an in vivo model system for metastasis. It was proposed to accomplish this goal by treating the experimental animals with specific proteolytic inhibitors. Because of the evidence suggesting that plasminogen activator is important to the process of metastasis, and because there exist different types of plasminogen activators with different properties, a second aim of this project was to identify and characterize the plasminogen activator(s) produced by the tumor used in the in vivo model.
1. GENERAL DISCUSSION OF PROTEASES IN METASTASIS

Although the role of proteases in metastasis has become a very active area of research over the past decade, there are still very few clear conclusions to be found in the literature, which is now quite extensive in this area (Strauli et al., 1980; Liotta and Hart, 1982; Quigley, 1979a; Nicolson, 1982; Schirrmacher, 1985). This is due in a large part to the complexity inherent in the metastatic process and therefore in any attempts to study it. It is also due in part to the large variations between investigators in the type of tumors studied and the model systems and methods used to investigate them.

Much of the evidence which supports the theory that increased production of proteases is an important component of a tumor cell's metastatic potential comes from simple quantitative experiments. Many of the earliest experiments compared the amount of certain proteases produced by a tumor with the amount produced by normal tissue. For example, Recklies et al. (1980) demonstrated that explants of malignant human breast tissue maintained in tissue culture secreted 10-fold greater amounts of cathepsin B.
than did similarly maintained explants of normal tissue or fibroadenoma. It is interesting to note that in this study, the authors detected no difference in the amounts of plasminogen activators (PA), collagenase, or cathepsin D. However, Evers et al. (1982) report a 4-fold increase in PA activity in human breast tumors compared to normal tissues. Similarly, Keiditsch and Strauch (1970) showed that mammary carcinomas display a higher level of collagenase activity in the periphery of the tumor than in the central regions, both of which were higher than normal surrounding tissue. Among the many explanations for this contradiction in the reported levels of proteinases in breast tissue, several deserve comment. First, one study (Recklies et al., 1980) maintained the tumor in tissue culture for at least two days before assaying for proteinase. The question of whether tumor cells in vitro are suitable models for in vivo tumors depends on the question asked, but is probably most suitably answered in the negative. In addition, differences in the preparation of the samples, the assays used for plasminogen activator, the size and stage of the tumor growth and the presence of normal host cells and/or proteinase inhibitors make interpretation of any study using tumor tissue difficult. Two of these authors also compared the amounts of protease secreted by primary tumors and metastases; Recklies et al. (1980) finding one third the amount of cathepsin B
in metastasis and Evers et al. (1982) finding no significant difference in PA levels. These early observations are significant in light of experiments performed by Colombi et al. (1984) which demonstrate a very high level of PA activity in adenocarcinoma tissue, but very low levels of plasma PA activity in patients bearing metastasis. The authors suggest that the tumor secretes high levels of PA in the peritumor space to degrade normal tissue, but once in the circulation, decreased levels of PA increase the chances of the tumor attaching by means of a fibrin clot, and hence increases the chance of metastasis. This illustrates that certain tumor cells may vary the secretion of proteases depending on their environment, or that more than one of the heterogeneous cell populations found in a tumor may act in a concerted manner to produce metastasis. There are many other reports in the literature which quantitate the amount and type of protease in tumor samples and compare it to that found in normal tissue, or compare the proteases secreted by malignant tumors with the proteases secreted by nonmalignant tumors of the same type (i.e., Wilson and Dowdle, 1978; Wilson et al., 1980; Kirchheimer et al., 1985; Sloane and Honn, 1984). In the majority of these, a positive correlation between the invasive state and protease production is observed. In some cases this correlation is not observed, but because of the problems with obtaining homogeneous samples of
tumor tissue, free from contaminating normal cells, proteases, or inhibitors, these results can all be questioned.

An interesting method to overcome some of these problems is to use labeled probes (radioactive or fluorescent antibodies or inhibitors) directed against the protease under study, and examine fixed sections of tumors, where the normal cells can be clearly identified. Recently, urokinase (UK) has been localized in Lewis Lung tumors to areas of invasive growth and degradation of normal tissue using antibodies directed against the enzyme (Skriver et al., 1984). This method has also been used to look at the amounts and location of proteases on tumor cells in culture (Kozlowski et al., 1984). Results from these types of studies clearly show in some instances large increases in the amount of proteases on the surface of tumor cells, a result which will be discussed further.

Because of the difficulties involved in studying samples of in vivo tumors, many researchers have studied the production, secretion, and properties of tumor produced proteases utilizing tumor lines maintained in culture. Again, most researchers find an increased amount of proteolytic activity associated with the tumor cells compared to their normal controls (Sloane and Honn, 1984; Dano et al., 1985). And again there are studies which fail to find a correlation, and reasons why this correlation might not be seen. Some enzymes are secreted as zymogens and if not activated before
the assay, their activity would not be correctly measured. Also, some cells produce both a certain protease and an inhibitor for that enzyme (Roblin et al., 1978), and again, the question of the amount of protease produced under in vitro conditions as opposed to the amount produced in vivo when the cell is actually in the process of invasion may make measurements of this type less than conclusive. This is especially important when it is realized that tumor cells change their phenotype rapidly and adapt to culture conditions, which can result in a change in metastatic ability (Honn et al., 1986; Miner et al., 1982).

Using the same primary tumor, it has been possible to select for sublines which vary in their metastatic ability and/or target organ of metastasis. This has provided another model for studying properties associated with the metastatic phenotype. The best known group of these sublines are those developed from the B16 melanoma originally isolated by Fidler (Fidler, 1973). By various selection procedures, which usually include injection into animals, collection of metastases, growth in culture, and reinjection into animals, lines selected for high lung colonization, increased invasion, brain metastasis, ovary metastasis, and others have all been developed (Fidler, 1973; Hart, 1979; Nicolson, 1982). Using these lines, different authors have demonstrated a correlation between metastatic potential or
invasion and many different properties, including increased PA, cathepsin B, type IV collagenase, Factor X activating enzymes and heparanase activity (Wang et al., 1980; Sloane et al., 1981; Liotta et al., 1980a; Gilbert and Gordon, 1983; Nakijima et al., 1983). In addition, at least one study (Nicolson et al., 1976) showed no correlation between plasminogen activator and metastasis, although all lines produced higher levels than normal cells. Since PA's activate a cascade type mechanism, it may be that only small amounts of PA are needed in order to enable a cell to metastasize, and other factors may determine the efficiency of the process.
2. PLASMINOGEN ACTIVATORS AND METASTASIS

In addition to the general experiments discussed above, there exists a large body of literature dealing with the roles of specific proteinases in metastasis. PAs are one of the most studied of these enzymes, and several recent reviews discuss these results in detail (Dano et al., 1985). There exist two distinct types of PAs in mammalian systems, the urokinase type and the tissue type (tPA). tPA is normally produced by endothelial cells lining the blood vessels and is believed to be involved in maintaining hemostasis. Urokinase type PA was originally purified from urine, but seems to have a more general role in tissue degradation and is produced by a number of normal cells during tissue remodeling processes. Both enzymes are serine proteases which act by cleaving the proenzyme plasminogen at a specific Arg-Val bond to generate the serine protease plasmin, which has a broad trypsin-like specificity. Plasminogen, which is produced by the liver, is known to occur throughout the body in significant quantities and can thus act as a large reserve of proteolytic activity. Plasminogen is the only known protein substrate of PAs. Both UK and tPA have been purified from a number of sources. Antibodies do not cross react between the two types of enzyme, but
antibodies against one type will cross react with the same type enzyme from another species. The human enzymes have been cloned and sequenced and their position on the chromosome is known (Rajput et al., 1985). The gene for murine UK has also been cloned (Belin et al., 1985). Both types of enzymes have also been shown to exist in various forms. Human UK is secreted as a one chain protein of Mr 55,000, which although analogous to the proenzyme form of most serine zymogens, has recently been shown to be active against its native substrate, plasminogen (PLG) (Lijnen et al., 1986). The one chain form is not active against small substrates, and shows low reactivity with inhibitors. In plasma, the activation of plasminogen by one chain UK is inhibited, and this inhibition can be neutralized by the formation of a fibrin clot. When cleaved by plasmin, the UK exists as a fully active 2 chain molecule of the same Mr and also as an active, partially degraded form of Mr 30,000. The tPA, (which has been shown by DNA sequencing to be very different from UK), is likewise produced in a one chain form which is reported to be partially active, although some experiments indicate it may not be (Andreasen et al., 1984). This enzyme has a Mr of approximately 70,000 and can be cleaved to a more active 2 chain form by the action of plasmin. Again, the activity of tPA towards plasminogen is increased in the presence of fibrin, and this has important implications to the
natural regulation of PA activity. A partially degraded form of tPA which retains activity has also been reported (Dano et al., 1985). In the human, both enzymes are glycoproteins. In the mouse, the UK type is not glycosylated, which results in a slightly lower Mr of approximately 48,000 (Belin et al., 1985). The larger tPA molecule contains additional peptide sequences, recently located on the heavy chain (Rijken and Groeneveld, 1986), which are responsible for this enzyme's high affinity for fibrin clots.

Although some tumor cells secrete increased amounts of tPA, urokinase is the type of enzyme most usually found associated with tumors, although in earlier studies antibodies were not always available to distinguish between the two types. One of the arguments that support the role of UK in the invasion and spread of cancer is the fact that UK is often produced by cells undergoing normal processes of invasion and tissue destruction. Thus in the involution of mammary glands after the cessation of lactation, Ossowski et al. (1979) showed a good correlation between an increase in UK PA in the mammary gland tissue and the process of involution. This was demonstrated temporally by varying the time at which the litters were removed and also by using hormone combinations which affected the beginning of involution and UK PA production in the same manner. It has been suggested that the process of involution is very similar to the invasion of
neoplastic cells, as in both processes a substantial amount of tissue destruction takes place, including the degradation of basement membrane structures. Evidence of the same nature also shows that PAs are probably involved in several stages of the reproductive cycle, including the production of PAs by granulosa cells and the oocytes in the process of follicle disruption, the implantation of fertilized eggs into the uterus (trophoblast invasion), and possibly the penetration of sperm into the egg (Huyfar et al., 1985). Another example of normal cells utilizing UK to invade comes from studies which show that stimulated macrophages and polymorphonuclear leukocytes produce UK, whereas the unstimulated cells do not (Werb et al., 1980). Thus there exist normal situations analogous to the invasion of a tumor cell into normal tissue in which it appears that PAs, particularly UK, are intimately involved. This is an important consideration in view of the fact that this situation has not yet been shown to exist for some of the other proteinases implicated in the process of metastasis, with the possible exception of collagenase.

It is also significant to note that by activating plasminogen, UK may be triggering a cascade of proteolysis, since plasmin has been shown to activate latent collagenase (Paranjpe et al., 1980) and possibly other proenzymes as well. Thus PAs are well suited to their proposed role of destroying normal tissues,
including basement membrane type IV collagen. In addition, plasmin has been shown to be capable of modifying a number of normal cellular properties by altering cell surface components. For example, plasmin treatment can stimulate cells to divide, to secrete various factors, or even to secrete additional proteases (Werb and Aggeler, 1978). Plasmin has also received some interest as an enzyme which may cleave host produced antibodies against the tumor cell (Latner et al., 1974).

The study which perhaps most strongly indicates that PAs are necessary for the process of metastasis was performed by Ossowski and Reich (1983). These experiments studied the invasion and metastasis of human carcinoma cells grown on the chorioallantoic membrane (CAM) of chicken embryos. In this model system, the tumor cells invade through the CAM, penetrate into the circulation, lodge in the lungs and develop into metastasis. The authors showed that the carcinoma produced significant amounts of UK type PA. Injecting the embryo with a specific inhibitory antibody against the carcinoma UK reduced the number of metastasis drastically (in some experiments, by 95%). Although this experiment seems to conclusively indicate an important role for UK, it must be taken into consideration the non-homology of the system used. A human carcinoma growing on a chicken egg is in a significantly different environment than a human carcinoma growing in a human. In a recent
abstract (Ossowski and Wilson, 1986), the authors have reported similar results with the same carcinoma line in nude mice, but again, the immune system plays an important role in metastasis and can not be ignored. However, the use of a specific inhibitory antibody is a promising approach which has produced strong evidence for the importance of UK to the process of metastasis.

In another system, these authors have shown that hydrocortisone, which suppresses the synthesis of PA by mouse mammary tumors in vitro, also inhibits the growth and incidence of these tumors in vivo (Mira-y-Lopez et al., 1985). One common criticism that this study shares with others of this nature is that the question of whether hydrocortisone is inhibiting the synthesis of UK and consequently inhibiting tumor growth, or if hydrocortisone is somehow affecting the growth of the tumor and consequently inhibiting the release of PA, is not clear. It was mentioned above that the production of UK by normal mammary gland tissues is also regulated by hormones, which further complicates this issue. In either case, it is apparent that an increase in the production of plasminogen activator is closely correlated to the growth of this particular tumor.

Further studies on the importance of PA to metastasis attempt to show a coordinate modification of PA production and tumorigenicity. In one of these, a clone of the B16 melanoma was
shown to have a decreased ability to form tumors when grown in culture medium containing 5-bromodeoxyuridine (Christman et al., 1975). It was subsequently shown that these cells also showed a coordinate decrease in PA expression when grown in 5-bromodeoxyuridine. These changes were both reversible. In addition, a clone maintained in 5-bromodeoxyuridine which permanently lost its tumorigenicity also produced no detectable PA. It should be noted here that as in most studies of this type another group reported the opposing view. Using a Syrian hamster melanoma which only displayed transformed characteristics in the PRESENCE of 5-bromodeoxyuridine, Rosenthal et al. (1975) report that PA was again suppressed by 5-bromodeoxyuridine, and therefore not associated with transformation. However, this paper did not give any details about whether this mutant line was actually tumorigenic. This study also illustrated that at different stages of growth in culture, different levels of PA were produced. This phenomena has been observed by other authors as well and may again be a reason for discrepancies between research groups. Further studies with the mouse melanoma line suggests that cocultivation of nontumorigenic and tumorigenic lines results in a suppression of PA production by the tumorigenic line (Newcomb et al., 1978). Furthermore when this mixture of cells was inoculated into immunocompetent mice, tumor production was suppressed; however the
same mixture produced tumors in immunodeficient mice. This may indicate a role of plasminogen activator–plasminogen (PA–PLG) proteolysis in the protection of tumor cells from the immune system. In another model system, using clones of a rat mammary carcinoma, a strong correlation between PA activity and metastatic potential was also observed (Carlsen et al., 1984). In this study there seemed to be a threshold level of PA activity, and clones with PA levels higher than this threshold produced high numbers of metastases.

Stronger evidence for the involvement of PAs in metastasis, which may or may not hold up to further scrutiny by the scientific community, comes from viral transformation studies. Some of the earliest evidence for a role of PAs in metastasis came from work done on viral transformation by Rifkin and Reich. They showed that transformation of chick embryo fibroblasts by Rous sarcoma virus (RSV) results in the production of UK (Unkeless et al., 1973), which is not produced by the normal fibroblasts. This production of UK is dependant on the development of transformation since in cells infected with non-transforming RNA or DNA viruses, no increase in UK is seen. Likewise when cells were infected with a temperature sensitive mutant strain of RSV which only produced transformation at the permissive temperature, PA production was also increased only at the permissive temperature. PA production
and transformation were both reversible with changes in
temperature, and the increase in PA was the earliest biochemical
event detected after transformation. Ossowski et al. (1974) showed
that the morphological changes observed upon transformation
depended on PA-PLG activity. In addition, the tumorigenicity of
virally transformed cells in nude mice has been shown to correlate
with PA activity. Again, some authors have claimed to the contrary
that temperature sensitive variants produce tumors even at the
non-permissive temperature (Poste and Flood, 1979), and that the
production of plasminogen activator is not related to
tumorigenicity using these same strains (Wolf and Goldberg, 1978).
However closer examination shows in the case of the temperature
sensitive mutants (Poste and Flood, 1979) the production of PA was
not actually measured under the conditions of the tumor formation,
and in the second case (Wolf and Goldberg, 1978) all of the lines
which produced tumors in the absence of high PA production grew as
noninvasive tumors encapsulated in a tough fibrous coat. Thus,
although the literature shows a close correlation with PA
production and transformation, PA production may not be necessary
for tumorigenicity, depending on the system used. However, even in
cases of low PA production there seems to be a strong indication
that its action may be necessary for invasive growth of the tumor.
Similar results have been reported using other viruses and cell
lines, and increased PA activity has also been observed in cells transformed by chemical carcinogenesis (Dano et al., 1985).

In other studies, the expression of PAs has been shown to be modulated by hormones in hormone sensitive tumors (Ossowski et al., 1979), to be induced by damage to cellular DNA (Miskin and Reich, 1980), and also to be stimulated by tumor promoters such as phorbol myristate acetate. Goldfarb and Quigley (1978) have shown that phorbol myristate acetate treatment increases the synthesis of PA in normal cells, resulting in morphological changes, and also increases the already high production of PA by transformed cells. Again, this evidence only supports a correlation between PA production and transformation or tumor growth. Likewise it has often been demonstrated that there is usually a good correlation between production of plasminogen activators and other of the transformation phenotypes studied in culture, such as anchorage independent growth, reduced cellular adhesiveness, and increased cell migration (Quigley, 1979b).

Another model system which has been used to study the role of proteinases in invasion and metastasis developed by Liotta uses a human amnion membrane to quantitate tumor cell invasion through a basement membrane (Liotta et al., 1980b). Although in most studies using this system, collagenase is the only proteinase shown to correlate with invasion, recent evidence indicates UK may also be
important. Rifkin (1986) reports that anti-UK (but not anti-tPA) antibodies prevented invasion of the amnion by B16 cells. An important consideration in interpreting these results is of course the lack of many in vivo factors, and the fact that most investigators perform these studies under slightly different conditions.
3. CATHEPSIN B AND METASTASIS

Although the evidence for an important role of cathepsin B in metastasis is not as extensive as that for PAs, research in this area indicates that cathepsin B is important in at least some of the tumor systems studied (Sloane et al., 1986). Cathepsin B is a cysteine protease with a Mr of approximately 25,000 and a broad endopeptidase activity. Normally found in the lysosomes, cathepsin B has a major function in the degradation of proteins. Cathepsin B has an acidic pH optimum of around 6, and is subject to rapid, irreversible denaturation above pH 7. Lysosomal proteinases in general may be increased in metastasis, and the possible existence of "extracellular lysosomes" may be significant in this respect. However, very little evidence exists on this subject. Original work in this field was dedicated to confirming that the existence of cathepsin B activity within tumors was a property of viable tumor cells, and not just an artifact from tumor cell necrosis or invasion of normal phagocytotic cells. Also, there were difficulties with explaining how a protease, usually intracellular and inactive at neutral pH could be involved in extracellular destruction. Thus it is significant that a cathepsin B-like protease with slightly different properties than the normal
protease has been described (Sloane et al., 1984, Mort et al., 1980). This enzyme has increased stability at higher (above neutral) pH and appears to be localized in the plasma membranes, as well as the lysosomes, and perhaps exists in a shed or secreted form. The enzyme is often found in a latent form which can be activated by pepsin treatment and/or a higher M_r form (approximately 40,000). The enzyme is immunologically cross reactive with cathepsin B under appropriate conditions, and so is described as a pro-cathepsin B or cathepsin B-like enzyme. Cathepsin B is capable of degrading the non-helical portions of collagen, fibronectin, proteoglycans and can also activate latent collagenases and therefore, like PA, is capable of initiating a proteolytic cascade. Increased secretion of a cathepsin B-like protease would therefore enable a tumor cell to degrade most extracellular structures that a migrating tumor cell may encounter. There is also evidence that cathepsin B localized in membranes may be resistant to plasma protease inhibitors (Sloane and Honn, 1984). Because of these properties, it is reasonable to propose that extracellular cathepsin B may play a role in the invasion of tumors, even in an environment (high pH, proteinase inhibitors) where lysosomal cathepsin B would not be active.

Similarly to PA, there is accumulating evidence that cathepsin B may be involved in non-neoplastic tissue destruction.
Early evidence correlated higher levels of cathepsin B activity in tissue remodeling and inflammation (Nakagawa et al., 1977; Bayliss and Ali, 1978; Davies et al., 1978). However, these studies did not distinguish between intra- or extra-cellularly located cathepsin B and therefore may simply represent an increase in lysosomal activity in these conditions. Recently, Recklies and Mort (1985) have demonstrated in culture medium from lactating mammary gland explants the existence of stable, active, high molecular weight forms of both cathepsin B and L. These authors propose that the secreted immunologically related precursor forms may represent an abnormal form which is incompletely processed. It is interesting to note that these authors found hydrocortisone inhibited the release of the cathepsin B-like enzyme from normal mammary gland explants in culture (Recklies et al., 1985) in much the same way that Ossowski et al. (1979) showed for PA. This illustrates that the practice of studying only one of the various proteases involved in the complicated process of metastasis may lead to incorrect conclusions. Further support for a normal physiological extracellular role of cathepsin B or a cathepsin B-like protease comes from a paper by Gordon et al. (1985) which demonstrates, in cultures of a human hepatoma line, that a Mr 54,000 protease immunologically and enzymatically related to cathepsin B may be responsible for the the removal of a prosegment
from proapo-lipoprotein-A-II. This indicates that a "procathepsin B-like" proteinase can accurately process natural protein substrates, and may function extracellularly in normal cells.

As mentioned above, several studies have correlated an increase in the amount of cathepsin B with tumor homogenates as compared to homogenates of normal tissues. Histochemical studies by Sylven et al. (1974) have shown the presence of cathepsin B-like material on the surface of a variety of tumor cells using an immunological technique. Poole et al. (1978) have demonstrated that the invading edges of breast tumors have a higher cathepsin B activity than the center of the malignancy. This localization would be expected if cathepsin B were playing an essential role in tumor invasion, but it does not provide causal evidence.

Increased cathepsin B has also been correlated with increased spontaneous metastasis and lung colonization in selected sublines of the B16 melanoma and murine Lewis Lung carcinoma (Sloane and Honn, 1984). There also exist reports in which this correlation failed to be demonstrated (Sloane and Honn, 1984), possibly because of similar problems in measuring the amount of cathepsin B in ex vivo homogenates, or in variations induced by growing the cells in culture in order to assay cathepsin B levels.

It has also been reported that agents which modify the metastatic potential of cell lines concomitantly affect cathepsin
B activity. Takenaga (1984) demonstrated that dimethyl sulfoxide (DMSO) increases the ability of Lewis Lung carcinoma to form experimental metastasis and also increases the amount of cathepsin B activity. However, treatment of this clone with DMSO also resulted in increased activities of other proteases, including plasminogen activator. Likewise a group of murine methylcholanthrene-induced fibrosarcomas had elevated cathepsin B activity compared to normal muscle (McLaughlin et al., 1983). The levels of cathepsin B activity did not correlate with metastatic potential. However, the authors point out that increased proteolytic activity may be important to some aspect(s) of metastasis, and still not correlate with the overall process.

In experiments using the amnion membrane model in collaboration with Dr. Persky (Persky et al., 1986), it was shown that a strong inhibitor of cathepsin B had no effect on invasion and migration through the basement membrane. This result is in agreement with that of other researchers using this model (Rifkin, 1986). This lack of an inhibition of invasion suggests that cathepsin B may not be involved. Alternatively, an effect may not be detected because of other reasons. For example, the location of cathepsin B in this system may be unavailable to small inhibitors of the type used (leupeptin), the cathepsin B activity may only be required for tumor invasion in vivo, or the "pro" form of the
enzyme secreted may be resistant to the inhibitor used.

In summary, although the number of experiments which support an important extracellular role of cathepsin B in tissue destruction by tumor cells is not as large as that for PA, the experiments follow the same type of methodology and generally support the involvement of cathepsin B in metastasis. Further research to clarify the role of this enzyme is therefore of significant interest.
Thrombin is another protease which is believed to be involved in the process of metastasis and host responses to tumors. The role of thrombin in hemostasis is becoming increasingly more complex, and the role which this enzyme may play in metastasis is at present unclear. Thrombin is a 34,000 dalton serine protease responsible for cleaving fibrinogen to fibrin at a single arg-gly bond, which results in the initiation of clot formation (Stryer, 1981). Thrombin is produced from inactive 66,000 dalton prothrombin by the action of another serine protease of the coagulation cascade, Factor Xa. Factor X can be activated by either the intrinsic or extrinsic pathway and so this is the first common step of the two pathways leading to coagulation. Unlike PA and cathepsin B, thrombin is not a tumor produced protease, but many cancer patients have coagulation disorders and many tumors have been shown to directly cause the activation of the coagulation cascade (Rickles and Edwards, 1983). Other properties of thrombin which may or may not be important to the metastatic process include evidence that (1) thrombin can stimulate the synthesis and secretion of a collagenase inhibitor from platelets and a PA inhibitor from platelets and endothelial cells (Cooper et
(2) thrombin initiates cell proliferation (Carney et al., 1985), (3) thrombin bound to thrombomodulin can activate protein C, which will result in increased fibrinolytic activity by degrading an inhibitor of PAs (Maruyama and Majerus, 1985), (4), thrombin can release proteoglycans from endothelial cells, and (5), thrombin can stimulate endothelial cells to secrete prostacyclins and tPA to again stimulate fibrinolysis (Shimada and Ozawa, 1985).

In metastasis, thrombin is proposed to increase the percentage of tumor cells released which survive transport in the circulation and attach to grow into secondary tumors (Markus, 1984). By forming a fibrin clot around the tumor cells, thrombin can lead to the aggregation of platelets and other blood components. This fibrin/platelet covering can protect the circulating tumor cell from the host immune system by masking the cell surface antigens. The larger embolus is also believed to be more easily anchored and the tumor cell can than extravasate from this stationary phase and continue to grow into a secondary tumor. It should be noted that the postulated role for thrombin and fibrin formation is in direct opposition to the postulated role of PAs. However, the possibility that tumor cells may display different phenotypes at different stages in the metastatic process must again be considered. Thus in the primary tumor, which is
being surrounded by a fibrin clot and in general is under increasing pressure to invade and expand, a high fibrinolytic/degradative activity may be favored. Once released into the circulation, where tumor cells do not survive very long, the formation of a protective fibrin clot/platelet embolus may be favored. After attachment, the degradative processes may again be the pathway leading to successful metastasis. The action of thrombin can, by this hypothesis, promote or retard the formation of metastasis, depending on the local stage of the process. These opposing pathways may also be responsible for some of the contradictory experimental results.

Evidence for the role of fibrin function in the primary tumor is limited at this time. Histological studies however have generally revealed a fibrin structure surrounding primary tumors, and when looking at a malignant tumor, places of local dissolution or absence of this structure, as well as the basement membrane, are apparent. In addition, studies such as the one by Colucci et al. (1981) give support to the theory that under these conditions, the fibrin observed is an impediment to tumor migration and metastasis. In their studies, different sublines of a murine sarcoma of varying spontaneous metastatic potential were shown to display an inverse correlation between ability to metastasize and ability to activate coagulation of plasma. This activation was
proposed to be due to a tissue factor, and the authors concluded that the fibrin acts as a barrier to the primary tumor. Other tumors have been shown to produce a tissue factor; however several of these studies used tumor extracts which may have contained host derived tissue factor. For example, macrophages have been shown to express increased tissue factor activity when stimulated by the presence of cancer, and the fibrin formed may affect macrophage migration. Studies which attempt to correlate the amount of tissue factor with metastatic potential may therefore not actually be measuring tumor tissue factor.

In an interesting series of experiments, Tanaka et al. (1982) tested the effects of tranexamic acid and UK injections on the spontaneous and experimental metastasis of several tumor lines. When rabbits received i.v. injections of V2 carcinoma cells, treatment with UK reduced the number of pulmonary metastases. When rabbits with primary V2 footpad tumors received i.v. UK, the number of lymph node metastases was increased. In further studies using the Lewis Lung carcinoma, these authors again reported an increase in lung metastases from a primary footpad tumor when the animal was treated with UK, but also reported a decrease in lung metastases when the animals received tranexamic acid, an inhibitor of the plasminogen to plasmin conversion. Thus there is strong evidence for the proposal of conflicting roles for fibrin, and
therefore thrombin, in metastasis; a suggested inhibitory role of fibrin in the early stages and an enhancing role of fibrin in the later stages. It is interesting that in experiments using a spontaneous model, injection of UK did not prevent tumor cells from lodging in the lungs in larger numbers, but inhibition of fibrinolysis did prevent them from escaping the primary tumor. This would suggest that the local degradation of surrounding fibrin may be more important to the process of metastasis then that of fibrin formation and attachment. It may be that attachment is controlled by other factors, such as damage to the endothelium and exposure of laminin (Terranova et al., 1982). However, other studies (see below), have not always supported this hypothesis.

There are also a number of studies which utilized inhibitors of coagulation such as heparin or warfarin, or perhaps more drastic measures such as infusion of plasmin or defibrinogenation of the animal, to attempt to determine the role of thrombin and fibrin formation in metastasis. Because of the many possible side effects of these treatments, results from these experiments will only be summarized here. In general, treatment with an inhibitor of thrombin (or in some cases, inhibitors of platelet aggregation (Honn et al., 1986) reduce the number of metastasis. These studies are usually most convincing when performed on i.v. injected cells.
In spontaneous models, the results are less clear, and often questionable because the drug used has other effects on the primary tumor besides the inhibition of thrombin (Markus, 1984). For example, heparin has been shown to be antimitotic, to facilitate immune responses (Gorelik et al., 1984), and to have profound influences on angiogenesis (Folkman, 1985; Folkman et al., 1983). Warfarin inhibits the synthesis of many proteins and by decreasing the formation of γ-carboxyglutamate may have pleiotropic effects (Hilgard, 1977).

Recently a number of tumors and tumor lines have been shown to produce a cysteine protease which is capable of directly activating Factor Xa (Gordon and Cross, 1981; Falanga and Gordon, 1985). This activity has been purified from extracts of the V2 carcinoma, and similar factors have been reported to occur in tissue extracts of human breast, colon, kidney, vagina and lung cancer, transformed hamster fibroblasts, Lewis Lung carcinoma, B16 murine melanoma, and Ehrlich ascites JW sarcoma cells. This cancer "procoagulant" has not been found in extracts of normal tissue. Using murine B16 melanoma and Lewis Lung tumor cells, Tohgo et al. (1985) have shown the importance of this procoagulant in the probable mechanism for platelet aggregation. And in several examples, including selected sublines of the B16 melanoma (Gilbert and Gordon, 1983), platelet aggregation has been shown to
correlate with metastasis. Further research into the occurrence and possible function of this interesting proteinase will certainly be forthcoming.

Evidence such as that briefly presented here demonstrates the profound influence of the hemostatic state of the host on cancer invasion and metastasis. It is clear that tumors or transformed cells are capable of causing the activation of both the coagulation and fibrinolytic pathways, perhaps at different stages. In a study by Kohga (1978) of ten rat ascites lines, the highest incidence of metastases after i.v. injection was obtained from the cells with both the highest fibrinolytic and thromboplastic activities. Before it is possible to clinically interfere in these processes, it is also clear that much more research on the actual mechanism of cancer metastasis needs to be done in order to identify the crucial steps or times for intervention.
5. PREVIOUS INHIBITOR STUDIES

One of the most exciting prospects of research on the mechanism of tumor cell metastasis is the hope that an increased understanding will lead to treatment modalities which may prevent this process. Based on the evidence currently available, researchers have attempted to reduce metastasis through the use of protease inhibitors. Demonstration of a clear reduction of metastasis by inhibition of a particular protease would also provide conclusive proof that the enzyme was participating and further our understanding of the metastatic process. Alternatively, lack of an effect would indicate that the protease was not essential to the overall process. Experiments performed to determine if protease inhibitors do have antimetastatic effects have yielded mixed results. Because of the complex nature of metastasis and the possible interplay of various host systems and the many proteolytic cascades, many of these studies cannot be clearly interpreted. Some of these have been mentioned already, additional examples are discussed below.

Although \textit{in vitro} experiments may have limited relevance to an \textit{in vivo} process, they do provide valuable preliminary evidence. Various inhibitors of proteases have been tried in all of the \textit{in
vitro model systems discussed, again with various degrees of effectiveness. In collaboration with Dr. Persky (Persky et al., 1976), it was shown that high concentrations of urokinase and cathepsin B inhibitors had no significant effect on the invasion of B16 melanoma cells through an amnion basement membrane. It was, however, possible to completely inhibit invasion by the use of ethylenediamine tetraacetic acid (EDTA), an inhibitor of metalloenzymes such as collagenase. Rifkin (1986) has also reported no effect of leupeptin on the invasion of B16 cells in this system, and a complete inhibition by EDTA. However, he was also able to inhibit invasion using an anti-UK antibody, but not with an anti-tPA antibody. Since leupeptin at high concentrations inhibits UK, the use of an antibody to inhibit UK must differ in some way. Perhaps UK is not completely inhibited by the reversible inhibitor leupeptin, especially when the cells are bound tightly to the amnion, or the antibody may have other effects which result in the differences observed between the two studies. Collagenase has also been shown to be important by the work of Thorgeirsson et al. (1982). Quigley has also studied the effects of proteinase inhibitors on the degradation of extracellular matrix by tumor cells utilizing metabolically labeled subendothelial matrix synthesized by normal chicken embryo fibroblasts (Quigley et al., 1986). In this system, aprotinin has no effect unless plasminogen
is present, in which case aprotinin normalizes the rate to that observed without added plasminogen. Diisopropylfluorophosphate (DFP), L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), and 1,10-phananthrolone, all show some inhibition of the degradation, however, even with all three inhibitors present, some degradation still occurs. This indicates that either the cells are producing protease in a localized way in an area where they are inaccessible to the inhibitors, or the cells are producing a protease which is not sensitive to the inhibitors used. Pearlstein et al. (1981) showed that a specific thrombin inhibitor (dansylarginine N-(3-ethyl-1,5-pentanediyl)amide could inhibit the action of thrombin activated by tumor cells and thereby prevent their aggregation with platelets. DiStefano et al. (1982) demonstrated leupeptin inhibition of a membrane protease involved in lysing of normal red blood cells by tumor cells, as did Zucker et al. (1985) using EDTA and minocycline (inhibitors of metalloproteinases). These experiments, as well as many others not mentioned here, show that specific steps in metastatic processes can be blocked by protease inhibitors, and it is reasonable to assume that a similar situation exists in vivo.

Several important factors must be taken into account when attempting to inhibit proteases in vivo. First, unlike in a test tube, the inhibitors must be free of toxic effects, both to the
host and to the tumor cells. Secondly, the inhibitors must show specificity towards the protease of interest. This is a major drawback of most of the studies reported so far. Many of the components of the complement, coagulation and fibrinolysis pathways have identical or similar primary specificity, principally for the basic amino acids arginine and lysine. Thus an inhibitor like leupeptin is effective against more than one of the enzymes postulated to be involved in metastasis. Leupeptin is a potent inhibitor of cathepsin, but at higher concentrations it can also inhibit many other enzymes (for further discussion see results).

An interesting report by Gasic et al. (1983) used an extract of leech salivary gland to inhibit lung colonization by Sarcoma T241 cells. The extract was shown to contain anticoagulation and antiproteinase activities, however other anticoagulants (heparin or atroxin) did not result in a significant inhibition of colonization. Thus these authors conclude the antiproteinase activity of the extract is responsible for the inhibition of experimental metastasis, however, the proteases involved cannot be identified.

Leukocyte neutral protease inhibitor (LNPI), shown to inhibit collagenase, elastase and chymotrypsin-like neutral protease, was reported to significantly inhibit the spontaneous metastasis of
Lewis lung carcinoma (Giraldi et al., 1977a). Further studies are needed to identify the enzyme(s) inhibited in this system. Giraldi et al. (1977b) also reported an inhibition of metastases formation from Lewis lung carcinoma transplants by treating animals with derivatives of N-diazoacetyl-glycine. This compound is an irreversible inhibitor of some proteolytic enzymes, but no experiments to identify the enzymes inhibited were reported.

Turner and Weiss (1981) in a very detailed study of Lewis Lung carcinoma, showed aprotinin increased both spontaneous and experimental metastasis. Aprotinin is a broad spectrum protease inhibitor capable of inhibiting trypsin, plasmin, and also kallikreins. They also showed treatment with aprotinin after i.v. injection of tumor cells increased the retention of the tumor cells in the lungs and liver. This was suggested to be a result of inhibiting plasmin, which would allow a larger number of tumor cells to remain attached to the endothelium by a fibrin clot. A larger number of stably arrested cells would then give rise to a greater number of metastases. In contrast, Latner et al. (1974) reported aprotinin inhibited tumor growth and metastasis of a malignant fibrosarcoma. Also, Giraldi et al. (1977c) reported a significant inhibition of spontaneous metastasis of the Lewis Lung carcinoma following i.p. injection of aprotinin. Administration of leupeptin and pepstatin by the same protocol showed no effect,
which suggested the lack of an essential role of cathepsins B and D. Leupeptin, pepstatin, and antipain, also had no inhibitory effect on the spontaneous metastasis of hamster cells transformed by Human Herpes Simplex virus (Harvey et al., 1977). Leupeptin was effective, however, in reducing by fifty percent the number of experimental metastasis of a hepatoma cell line in rats in experiments by Saito et al. (1980). In the same system, chymostatin and elastatinal had no inhibitory effect. The authors suggest that the reduction by leupeptin is due to an inhibition of thrombokinase (Factor Xa), and histological examination of the lungs of animals treated with leupeptin prior to injection of tumor cells showed significantly fewer tumor cell thrombi. However, leupeptin inhibits cathepsin B 1000-fold more effectively than Factor Xa, and at the levels used in these experiments, leupeptin may be inhibiting other enzymes as well (see results and discussion). Therefore the mechanism of action of leupeptin in this system is unclear. It is again clear that protease inhibitors have produced different results, sometimes in seemingly identical systems. This may be due to differences in methods (i.e., Giraldi et al. (1977c) used 200,000 KIU of aprotinin, compared to 5000 KIU used by Turner and Weiss (1981)), animal or tumor variations, and may depend on the stage of metastasis being examined. It may also be a consequence of the fact that the concentrations of the
inhibitors used have not been carefully monitored. It has been shown that proteinase inhibitors can be removed from circulation rapidly (Tanaka, 1983), and also that administration of some inhibitors may cause a compensatory overproduction of certain enzymes (Sutherland and Greenbaum, 1983; Aoyagi et al., 1983; Tanaka et al., 1984). Thus the time of the inhibitor injection may be extremely important in terms of the proteolytic status of the experimental animal.

A different approach to studying protease production and metastasis has been used by Giraldi et al. (1985a,b). In these experiments, animals bearing different tumors of varying metastatic potential were treated with proven cytotoxic or antimetastatic drugs. The levels of proteases (surface neutral protease, PA, cathepsin B) were assayed in the tumor before and after treatment. These experiments demonstrated no correlation between protease levels and metastases formation, nor did they reveal any correlation between inhibition of metastasis and inhibition of protease activity. These experiments do not directly test the role of proteases in metastasis.

Recently nafazatrom, an antithrombotic compound (Bayer 6575) which was shown previously to be antimetastatic (Honn et al., 1982), was shown to interfere with the degradation of endothelial matrix by B16 amelanotic melanoma (B16a) cells (Maniglia et al.,
These experiments did not identify the enzymes responsible for degradation of the matrix. In addition, Maniglia et al. report that nafazatrom had no inhibitory effect on the ability of i.v. injected B16a cells to form lung colonies, indicating that the effect of nafazatrom on spontaneous metastasis was not due to its' antithrombotic properties. These results illustrate the difficulty involved in studying the complex process of metastasis.
6. INHIBITORS USED IN THIS WORK

Leupeptin, acetyl-Leu-Leu-Arginine-aldehyde, is often used as a general protease inhibitor in studies on metastasis. It has been shown to be nontoxic and has been shown to have potent \textit{in vivo} effects which correlate with its antiproteinase activity. For example, leupeptin has been used experimentally to treat pancreatitis (Jones \textit{et al.}, 1982), and 12 mg/kg three times a week was effective at preventing the onset of muscular dystrophy in a mouse model (Sher \textit{et al.}, 1981). Leupeptin is a tripeptide aldehyde in which the normally C-terminal carboxylic acid is converted to an aldehydic function (for structure, see Appendix A). This compound is a potent inhibitor of both serine and cysteine proteases with a primary specificity for arginine or lysine. The mechanism of inhibition by tri-peptide aldehydes has been shown to occur by a transition state analog process in which the active site nucleophile (S\textsuperscript{-} or O\textsuperscript{-}) forms a covalent bond with the aldehydic carbon (Kennedy and Schultz, 1979; Frankfater and Kuppy, 1981). This intermediate is partially stabilized by the forces which normally stabilize the proposed transition state, but cannot be cleaved to yield products because of the aldehyde.
substitution. It can, however, dissociate to starting materials, so these tripeptide aldehydes are not irreversible inhibitors. It has also been shown that, even among proteases of identical primary specificity, altering the secondary or tertiary binding residues can have profound effects on the affinity of a protease for a particular tripeptide. For example, Bajuz et al. (1978) have shown that compounds of the structure Phe-Pro-Arg have very high binding constants to thrombin, and as mentioned above, leupeptin has an extremely high affinity for cathepsin B. Studies on chloromethylketones demonstrated that sequences of the formula Glu-Gly-Arg were very effective at inhibiting UK (Coleman et al., 1979). However, chloromethylketones are toxic in vivo. Patel et al. (1983) have demonstrated by synthesizing various tripeptide aldehyde affinity resins that these peptides are capable of selectively binding a specific enzyme based on secondary specificity. Likewise, Kabi manufactures a series of tripeptide p-nitroanilide substrates for many blood enzymes with a primary specificity for arginine which exhibit selectivity based on the other two residues chosen. Because of these features, i.e. low toxicity, high specificity, and potency as inhibitors, it may be possible to use these tripeptide aldehyde inhibitors in a model for metastasis to distinguish the roles of proteases implicated in metastasis.
Another inhibitor which was used in these studies, Ep453, was developed by Hanada et al. (1983). A derivative of E-64, Ep453 is a peptide epoxide (for structure, see Appendix A) which is an irreversible inhibitor of cysteine proteases such as cathepsins B and L, and may also be used in vivo to evaluate the roles of cysteine proteases in metastasis.
MATERIALS AND METHODS

MATERIALS

Common chemicals and reagents were purchased from Sigma (St. Louis, MO) or Scientific Products (McGaw Park, IL). Leupeptin (acetyl-Leu-Leu-Argininal) was a gift of Dr. W. Troll (New York University Medical Center) or was purchased from Sigma (synthetic). D-Phe-Pro-Argininal, t-boc-D-Phe-Pro-Argininal, t-boc-Glu-Gly-Argininal, and Glu-Gly-Argininal (trifluoroacetate salt) were all synthesized by members of Dr. R.M. Schultz's research group. Derivatives of E-64 (L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane) were a gift of Dr. W. Tanaka (Institute of Microbial Chemistry, Tokyo, Japan). Kabi substrates S-2251 (H-D-Val-Leu-Lysine-p-nitroanilide), S-2444 (pyro-Glu-Gly-Arginine-p-nitroanilide), and S-2238 (H-D-Phe-Pipecolyl-Arginine-p-nitroanilide), were purchased from Helena laboratories (Beaumont, TX). Human urokinase (Abbokinase, UK) was purchased from Abbott laboratories, (North Chicago, IL) and recombinant human tissue-type plasminogen activator (rtPA) was a gift of Genetech (San Francisco, CA). Reagents for polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). Triton X-100 was from Kodak (Rochester, NY). All cell culture reagents were from Gibco (Grand...
Island, NY) unless otherwise noted. Tissue culture flasks were from Corning Glass Works (Corning, NY) or Falcon (Oxnard, CA). Filter units were from Nalgene (Rochester, NY) and Millipore (Bedford, MA). Female C57BL/6 mice 6-8 weeks of age were purchased from Harlan Industries (Indianapolis, IN), Cumberland View Farms (Clinton, TN) or Charles River (Wilmington, MA) depending on availability. Animals were maintained by the Loyola University Medical Center Animal Research Facility under fully accredited (American Association for Accreditation of Laboratory Animal Care) conditions with unlimited food and water.

CELL CULTURE

B16-F10 murine melanoma cells, obtained from the Frederick Cancer Center (Frederick, MD), were used for all experiments. This subline of the spontaneously arising B16 melanoma was derived by ten successive in vivo cycles of i.v. injection into a lateral tail vein, isolation of tumor nodules growing in the lungs, growth in culture, and reinjection into animals (Fidler, 1973). Cells were cultured as adherant monolayers in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal calf serum (FCS) and 100 U penicillin-100 mcg streptomycin per ml (complete media). Cultures were grown in humidified 5% CO₂- 95% air at 37° C in a Forma Scientific (Marietta, OH) water-jacketed incubator. All cell culture solutions were sterilized by filtration. Under these
conditions, the observed doubling time was about 16 hr, which agrees with published values (Fidler, 1975). Cells were routinely passaged when confluent using 0.25% trypsin (crude, porcine pancreas, Sigma) and 1mM ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS) (2 ml for one minute). Cultures usually contained about 10 million cells at this time and were split 1:30. Cells were cultured in 75 cm$^2$ flasks in 10 ml of complete media.

CELL COUNTING AND TRYPAN BLUE DYE EXCLUSION TEST FOR CELL VIABILITY

Cells were counted using an improved Neubauer hemacytometer (C. A. Hausser and Son, Philadelphia, PA). Cells were suspended in PBS and diluted to an appropriate concentration (approximately $1 \times 10^5$). One drop of the cell suspension was placed in each chamber of the hemacytometer and the individual cells were counted with an Olympus (Tokyo) light microscope under a magnification of 100X. The number of cells in the original solution was calculated by multiplying the average of at least two determinations by the appropriate dilution factors.

When making a determination of cell viability by the trypan blue dye exclusion test (Phillips, 1973), 1 ml of the cell suspension was mixed with 0.1 ml of a 0.4% trypan blue solution
and dispersed with a Pasteur pipette 10 times. A drop of this mixture was placed in the hemacytometer chamber. After 4 min the number of stained and unstained (viable) cells were counted, and the percentage viability determined. A minimum of one hundred cells were counted to determine viability.

**CRYOPRESERVATION OF CELLS**

Semiconfluent cultures of B16-F10 cells were used to prepare frozen samples for storage. Flasks containing 2-6 x 10⁶ cells were harvested using a 0.25% trypsin and 1mM EDTA in PBS wash for 1 min, followed by addition of 5 ml complete media to inactivate the trypsin. Cells were collected by centrifugation (IEC clinical tabletop) at 2500 x g for 3 min in sterile 15 ml polystyrene tubes (Corning Glass Works, Corning, NY). The cell pellet was then resuspended by gentle pipetting in 1 ml freezing media (DMEM containing 20% FCS and 10% dimethylsulfoxide at 0°C) and pipetted into a sterile 2 ml cryotube (Nunc, Vangard Int., Inc., Neptune, NJ). The cryotube was sealed, wrapped in paper towels, placed inside a styrofoam container and placed into a -20°C freezer for 12-16 hr. The container was then placed into a -80°C freezer for an additional 12-16 hr. Following this slow freezing procedure, the cryotubes were placed into a liquid nitrogen storage tank. Several flasks of cells were frozen at the same time, and after at least three days, one or more of the frozen
samples were thawed and tested for viability. This was done by thawing the frozen cryotube rapidly by immersion in a 37°C waterbath for several min. The cell suspension was then mixed with 5 ml of complete media warmed to 37°C in a 15 ml tube and centrifuged as above. This procedure removes excess DMSO. The cell pellet is then resuspended in several ml of complete media and added to a flask containing 10 ml complete media. This procedure always resulted in a confluent culture of cells in two days or less, indicating that the frozen cells were highly viable. Because there are reports that B16 melanoma cells are phenotypically unstable, after periods of 1-5 months, all growing cultures were discarded and a sample of frozen cells was removed from storage and used to initiate new cultures.

**EXPERIMENTAL METASTASES**

The effect of the protease inhibitors on metastases was assessed utilizing the B16 melanoma model for experimental metastasis as described by Fidler (1978) and outlined below.

Briefly, 2-3 x10^5 B16-F10 cells were seeded in 10 ml of complete media. After three days, the semi-confluent cells were collected by a mild trypsinization, washed in complete media, resuspended in PBS, counted and diluted to 500,000 cells/ml. Originally the cells were kept at room temperature; in later
experiments the cells were placed in a sterile septum tube and kept on ice to improve viability. Cells treated in this way were always greater than 85% viable by the trypan blue exclusion method, and were often used to initiate cultures AFTER all the mice had been injected with no apparent decrease in viability. Female C57BL6 mice 6-8 weeks old were obtained from Harlan, Cumberland View Farms, or Charles River. For tumor cell inoculation, the mice were placed (in their cages with access to water) under a 150 watt light bulb until their tail veins were clearly dilated (approximately 15 minutes). Individual animals were removed alternately from the control and treated group and gently placed in a small dish-washing basket with their tails drawn through an opening in the side of the cage. This insured that each group of animals received injections of cells which had been in suspension for equal time. The tail was swabbed with 70% ethanol in water, straightened across a cork the same height as the tail, and 0.2 ml of the cell solution (containing 100,000 or 500,000 cells per ml) was injected slowly into the lateral tail vein. A 0.25 ml glass syringe was used for the injections with a clean 27 gauge 0.5 inch needle used for each animal. An injection was considered successful when there was no resistance to the injection. Any animals which showed blebbing of the skin or struggled excessively were eliminated from the study. The number
of animals successfully injected varied between experiments, but averaged about 70%. The cell suspension was inverted repeatedly during the experiment, and microscopic examination of the cell suspension after the conclusion of the injections revealed no cell clumping. Animals were sacrificed after 15 or 21 days, the lungs were removed, rinsed in PBS, dissected into lobes, and the tumor colonies were counted under a 30X binocular dissecting scope. Only surface colonies were counted on both sides of the lung lobes. Because of the presence of melanin, the black melanoma colonies were easily distinguishable. When amelanotic colonies were present, lungs were dissected as above and stained overnight with Bouins solution (250 ml formalin, 750 ml saturated picric acid, 5 ml glacial acetic acid) before counting. This staining procedure colored the normal lung tissue yellow, while leaving the tumor nodules white so that they could be counted. Electron microscopy was performed (Dr. Persky, Loyola University Medical Center) on amelanotic nodules and the presence of stage III pre-melanosomes was taken as conclusive proof that the cells forming the nodules were melanomas (see Appendix C). Results were analysed by the Students t-test and Mann-Whitney U test (Colton, 1974). Autopsies were routinely performed on animals from the different experiments. Internal organs, subcutaneous sites, and popliteal lymph nodes were grossly examined.
TREATMENT OF MICE WITH PROTEASE INHIBITORS

Protease inhibitors were introduced into mice by the following procedures in order to determine their effect on the process of experimental metastasis. Treatment with the protease inhibitors was always begun before injection of the tumor cells. All protease inhibitor solutions were sterilized by filtration. When protease inhibitors were introduced by repeated i.p. injection, the animals received injections 12 hr apart for a total of 13 injections. The tumor cells were injected 1 hr following the second injection of inhibitor. Using this protocol, animals received the following protease inhibitors and dosages in separate experiments: leupeptin at 5, 25, 37.5, or 50 mg/kg in 0.2 ml PBS; t-boc-D-Phe-Pro-Argininal at 12.5, 25, or 37.5 mg/kg in 0.2 ml PBS; D-Phe-Pro-Argininal at 37.5 mg/kg in 0.2 ml 5% DMSO, 10% ethanol, 85% PBS; Ep453 at 50 mg/kg in 0.05 ml DMSO. Animals treated with the UK inhibitor t-boc-Glu-Gly-Argininal received 8 injections of 30 mg in 0.2 ml PBS over a 36 hr period (approximately every 4 hr) beginning 1 hr before the tumor cell injection. Animals treated with the UK inhibitor (tfa)Glu-Gly-Argininal received 8 injections of 30 mg in 0.2 ml PBS over a 25 hr period (approximately every 3 hr) beginning 1 hr before the tumor cell injection. In all experiments, control animals received equal volumes of the drug vehicle by the identical schedule.
When osmotic pumps were used to deliver the protease inhibitor solutions, the pumps were implanted (see below) at least 12 hr before the tumor cell injection. The solutions loaded in the pumps in separate experiments were as follows: leupeptin, 0.1 M in PBS; Ep453, 0.08 M in propylene glycol; D-Phe-Pro-Argininal, 0.25 M in 40% DMSO, 60% PBS. All control animals were implanted with pumps containing the drug vehicle. The pumps contained a minimum of 200 ul of protease inhibitor solution and administered the solution to the animals at a flow rate of 1 ul/hr.

**IMPLANTATION OF OSMOTIC PUMPS**

When osmotic mini-diffusion pumps (Alzet, Model 2001) were used to deliver protease inhibitor solutions, they were implanted according to the manufacturers instructions as described below. The mice were anesthetized with an i.p. injection of 0.2 ml pentobarbital solution (6.5 mgs./ml PBS) and their backs were shaved. The area was swabbed with Betadine (povidone-iodine 10%, Purdue Frederick Co.) solution and a small incision was made through the skin. A subcutaneous tunnel was formed using blunt forceps, and the pump was inserted towards the animal's head. The opening was then closed with two 9 mm wound clips. Occasionally, when the skin was pulled tight around the pump, the wound would reopen to the sides of the wound clips. In these cases, the animal
was anesthetized again and the wound repaired. Pumps were removed by reversal of these procedures after delivery of the drug was complete. The pumps were handled with sterile gloves and none of the mice showed any sign of infection or discomfort.

**ASSAY OF PROTEASE INHIBITOR CONCENTRATIONS IN VIVO**

In order to determine the in vivo concentrations of the protease inhibitors, the serum levels of the inhibitors were measured by the following procedures.

Mice were injected i.p. with the protease inhibitor solution to be tested and sacrificed at selected time points by CO₂ suffocation. Alternatively, mice were surgically implanted with osmotic pumps and were sacrificed on days 1-5 after the implantation. Blood samples (approximately 1 ml) were collected immediately by cardiac puncture after surgically opening the rib cage to expose the heart. Blood was collected using a 22 gauge needle, allowed to clot, and centrifuged in an Eppendorf microfuge for 15 minutes at 4° C. The serum was collected, diluted (usually 1:2 depending on the sample size) with the buffer used in the enzyme assay, and filtered through an Amicon YM-5 membrane (5,000 Mwt. cut-off) using the Amicon Micropartition system to remove serum protein inhibitors. The filter units were centrifuged for at least 30 minutes in a clinical tabletop IEC centrifuge at 2500 x g at 4°. A sample of the filtrate (usually 200 ul) was assayed for
the presence of the injected protease inhibitor by incubating for 3 minutes with enzyme-buffer solution and then adding the appropriate p-nitroanilide substrate. The rate of hydrolysis of substrate was monitored at 405 nm in a Perkin-Elmer spectrophotometer at 25°C and compared to the control rate obtained using serum from mice treated with only the drug vehicle. Leupeptin and Ep453 were assayed by their ability to inhibit papain. Papain, 100 ul of 2.8 x 10^{-6} M which was activated by a 30 minutes incubation in an equal volume of 30 mM dithioerythritol-15mM EDTA at pH 5.2, was mixed with the serum sample to be assayed and enough 0.3 M Na citrate/phosphate buffer pH 6.2 to bring the total volume to 1.1 ml. After addition of 30 ul of a 1 mg/ml solution of N-benzoyl-arginine-p-nitroanilide (BAPNA), the rate of hydrolysis was measured. For the assay of tFA- and t-boc Glu-Gly-Argininal compounds, the hydrolysis of 10 ul of 5 x 10^{-4} M S-2444 by urokinase (1.5 I.U. UK) was observed in a total volume of 315 ul 0.1 M Tris-0.1 M NaCl pH 7.8 containing 0.1% Triton-X 100 and the sample to be tested. The thrombin inhibitors t-boc- and H- D-Phe-Pro-Argininal were assayed against thrombin (20 mU bovine thrombin (Pentax)) in a total volume of 1.160 ml 0.2 M PO₄ buffer pH 8.0 containing 50 ul 1 x 10^{-3} M S-2238 and the serum sample to be tested. Concentrations of inhibitors were estimated by comparison to rates obtained with known amounts of inhibitor.
added.

**COLLECTION OF SERUM-FREE CONDITIONED MEDIUM**

For studies of the secreted B16 melanoma plasminogen activators, cells were seeded in 75cm\(^2\) or 150cm\(^2\) flasks containing 10 or 25 ml complete DMEM. When semi-confluent, the media was removed and the cell surface was washed twice with 4-5 ml PBS, after which the flasks were reincubated with 10 or 25 ml of the same media without serum (serum-free medium, SFM). After 24 hours, the serum-free conditioned medium (SFCM) was collected and the cells were reincubated for a second 24 hour period. The SFCM was again collected, after which these flasks were discarded. Sometimes media was collected for a third day if the cell layer was still attached and morphologically healthy by microscopic examination. If the cells were heavily confluent, incubation in SFM often resulted in sheets of cells releasing from the dish, and this media was always discarded. Variations to try to improve the yield and efficiency of this procedure, such as selection procedures to develop a cell line which would be maintained in SFM or recycling of cell cultures after a passage in SFM, and the use of serum supplements (Ultroser G., LKB) were attempted. However, none of these attempts proved fruitful (see Appendix B). After collection of the SFCM, it was centrifuged at 6000 rpm and the
supernatant was frozen at -20°C until further study. In recent experiments, the SFM (Sigma) was supplemented with 4.5 g glucose/L to increase the nutritional properties of the SFM and/or 30 KIU/ml of aprotinin (Sigma) to inhibit proteolytic degradation of the plasminogen activator.

**PLASMINOGEN ACTIVATOR ASSAYS**

Three separate assays were used for determining PA activity. The first measures the ability of PA to produce zones of lysis on a fibrin-agar substrate by activating plasminogen, and was performed essentially as described by Lassen (1953). Agar (Kodak) 2.5% with 0.02% NaN₃ added to inhibit microbial growth was prepared by autoclaving and then aliquoted into sterile 15 ml plastic tubes (Corning), capped and stored at room temperature. Human plasminogen (Worthington) was dissolved in PBS at an initial concentration of 10 mg/ml and then treated with DFP (Aldrich) to inactivate plasmin. Solutions of plasminogen were incubated for at least one hour with DFP at a final concentration of 25 mM and then assayed using S-2251. This treatment was repeated until the plasmin activity could no longer be detected, and the solution was then dialysed against PBS, aliquoted into plastic tubes (5 ml polystyrene, Sarstedt) and frozen at -20°C until use. Fibrinogen (Bovine type IV, Sigma) was originally further purified (Laki, 1951; Mosesson, 1962), but this was found to be unnecessary. A 10
mg/ml fibrinogen solution in PBS containing 0.02% NaN$_3$ was used. Bovine thrombin (Pentex, Miles laboratories) was dissolved in 0.9% NaCl and frozen at -20° C in 1 ml fractions each containing 100 IU. For preparation of fibrin-agar plates, a tube of 4 ml agar was heated in a boiling water bath until melted. This was placed in a heated water bath at 45° C. To this tube was added 0.8 ml of PBS with 0.02% NaN$_3$ or 0.8 ml of plasminogen solution also at 45° C and 37 ul thrombin solution. The tube was inverted and then 2.5 ml fibrinogen solution was rapidly added, the solution inverted, and poured smoothly unto a petri dish (diSPo, sterile polystyrene, 10 x 1.5 cm, Scientific Products) which was warmed to 45° C by floating in the water bath. The dish was placed on a level surface and allowed to harden. For controls, it was found to be most convienant to pour the fibrin agar plate without adding plasminogen and then heating the plate to 80° C for 40-50 minutes (Lassen, 1953). This procedure denatured the contaminating plasminogen without affecting significantly the ability of plasmin or trypsin to degrade the fibrin. When plasminogen activators were present in the sample, they produced active plasmin which degraded the fibrin layer and resulted in clear zones of lysis. This test was not used quantitatively, but verified the presence of plasminogen activator activity. Plates varied in their stability but could usually be observed for 3-4 days before non-specific
lysis from contamination appeared.

The second assay used involved measuring plasminogen activation by measuring the rate of hydrolysis of S-2251 by the active plasmin formed. In this assay 100 ul of plasminogen solution was added to 1 ml of 0.05 M Tris pH 8.8 and 25 ul of S-2251 (2.5 mg/ml H2O). Absorbance was followed at 405 nm in a Perkin Elmer specrophotometer for several minutes to observe any background rate. The reaction was then repeated using a sample of the solution to be tested for PA activity and enough buffer to make the volumes equal, and the acceleration in rate was observed. Alternatively, the PA sample was added directly to the mixture and the acceleration was observed.

Finally, the urokinase-specific substrate S-2444 was used to assay the presence of UK-like enzymes and to determine inhibition constants. Generally, these assays involved mixing the sample (100 ul) to be tested in enough 0.05 M Tris buffer pH 8.8 to bring the volume to 1.1 ml. Substrate was then added (25 ul of S-2444, 5 x 10^-3 M) and the rate of hydrolysis was measured at 405 nm in a Perkin-Elmer spectrophotometer (Hitachi, Ltd., Tokyo, Japan). When using samples prepared from B16 melanoma cell cultures, this assay was usually done in the presence of 30 KIU per ml of aprotinin to inhibit other trypsin-like proteases which may have been present. Aprotinin does not affect PAs at this concentration (Dano et al.,
PURIFICATION OF B16 PLASMINOGEN ACTIVATOR

The purification procedure described here was the final procedure used to prepare samples for the experiments described, although various modifications were attempted. This procedure was based on that of Dano et al. (1980). SFCM was thawed and adjusted to pH 5.5 with acetic acid, made 0.1% in Triton X-100, and 0.01 M in ZnCl₂. This was applied at a flow rate of 1 ml/min to a DEAE Sephadex-A25 (Pharmacia) column (5-7 x 2.5 cm., Biorad) coupled to a p-aminobenzamidine affinity resin (Pierce, -aminocaproic acid spacer on agarose, 5-10 x 1 cm.) in the cold. The columns were equilibrated with 0.01 M acetate pH 5.5, containing 0.1% Triton X-100, 0.2 M NaCl, 0.01 M ZnCl₂. After the sample had been applied (usually 500-1000 ml), the columns were washed with the equilibration buffer until absorbance reached baseline or until the red dye of the media was eluted. The columns were then disconnected and the benzamidine column was washed with the same buffer made 0.5 M in NaCl (until baseline reached zero or at least 10 column volumes). The bound plasminogen activators were then eluted using 0.5 M arginine in 0.01 M acetate, 0.1% Triton X-100, 0.01 M ZnCl₂, pH 5.5 (usually 50-100 ml). This solution was then concentrated under N₂ pressure using an Amicon PM10 filter in an
Amicon stirred ultrafiltration device kept in an ice bath. If necessary, buffer exchanges were performed by repeated cycles of dilution and dialysis using the same system. Earlier variations of this procedure were performed at room temperature or were performed without addition of ZnCl₂, and eluates were concentrated by lyophilization.

**IRREVERSIBLE INHIBITION OF B16 PLASMINOGEN ACTIVATOR**

For the DFP and p-chloromercuribenzoate (pCMB) inhibition studies, 200 ul of 0.05 M Tris-.025 M EDTA pH 8.8 was mixed with 150 ul of a partially purified PA preparation. After addition of either 30 ul DFP (135 mM in isopropanol), 30 ul isopropanol, or 30 ul pCMB (277mM in H₂O) these solutions were incubated for 30 min at room temperature. 300 ul of each solution were then assayed against S-2444, and 50 ul were assayed in the plasminogen-S-2251 coupled assay. Residual DFP was inactivated by incubation of the enzyme sample with buffer before the plasminogen activation assay was performed, and this was verified by showing that the plasminogen could be activated by urokinase.

**DETERMINATION OF INHIBITION CONSTANTS**

For inhibition studies of rtPA, a 0.1 mg/ml solution in 0.01 M acetate, 0.1% Triton X-100, 0.2 M NaCl, pH 5.5, was used. To 100 ul of this solution was added the inhibitor solution and enough 0.05 M Tris-0.2 M NaCl pH 8.8 to bring the total volume to
1.075 ml. After a 3 minute incubation, 25 ul of S-2444 (final concentration $1.1 \times 10^{-4}$ M) was added, the cuvettes were inverted three times, and the rate of absorbance change was followed at 405 nm in a Gilford Response or Perkin-Elmer recording spectrophotometer for the initial 30 minute period. All inhibitors were tested at at least two different concentrations, and each was performed in triplicate. Stock solutions of inhibitors were made up as follows: tfa-Glu-Gly-Argininal, t-boc-Glu-Gly-Argininal, and leupeptin were dissolved at 1 mg/ml in 0.05 M Tris-0.2M NaCl, pH 8.8; D-Phe-Pro-Argininal, 3.7 mgs. in 1.875 ml Tris containing 25 ul DMSO; t-boc-D-Phe-Pro-Argininal, 2.4 mgs in 1.6 ml Tris containing 0.6 ml DMSO; DNS-Glu-Gly-Argininal, 4.4 mgs in 1.025 ml Tris containing 25 ul DMSO. When necessary, inhibitor solutions were diluted in Tris buffer (0.5 M, pH 8.8).

$K_i$'s were calculated by the equation below where $(I) =$ the inhibitor concentration, $v_u =$ the rate in the absence of inhibitor and $v_i =$ the rate in the presence of the inhibitor. These experiments were performed under first order conditions with substrate $< K_m$.

\[
K_i = \frac{v_u}{v_i} - 1
\]
Inhibition constants against partially purified B16 melanoma PA and concentrated SFCM were estimated in essentially the same way, except for the partially purified preparation, the Tris buffer contained 0.25 M EDTA to remove any remaining Zn$$^{++}$$, and the reaction times were 60 and 100 minutes respectively, due to the lower enzyme concentration.

**POLYACRYLAMIDE GEL ELECTROPHORESIS**

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the purity and molecular weights of plasminogen activators in samples prepared from the B16 melanoma by comparison to standards of known molecular weight. The procedure was that of Laemmli (1970) as detailed by Crowe et al. (1978). All electrophoresis equipment and reagents were from Bio-Rad (Richmond, CA). Gels were 16 x 18 cm x 0.75 cm thick and were composed of a stacking gel (4-5 cm) of 3% acrylamide and a resolving gel of 11% acrylamide. Electrophoresis was performed at 25 mA for 6 hr or at 10 mA for 14-16 hr in a running buffer of 0.025 M Tris, 0.192 M glycine pH 8.3 containing 1% SDS. Acrylamide stock solution (30% acrylamide-0.8% bisacrylamide in distilled water) was filtered through Whatman (Clifton, NJ) fluted paper and stored in the dark at 4° C. The lower gel buffer was 1.5 M Tris pH 8.8 containing 0.4% SDS and the
stacking gel buffer was 0.5 M Tris pH 6.3 containing 0.4% SDS. Polymerization was initiated by the addition of 75-150 ul of 10% ammonium persulfate and 10 ul of N,N,N',N'-tetramethylethlenediamine. Samples for electrophoresis were diluted 1:1 in sample buffer (sample buffer contained: 10 ml glycerol, 23 ml SDS (10%), 2 ml bromphenol blue (0.1%), 8.3 ml stacking gel buffer, 6.7 ml H2O). Protein molecular weight standards (Bio-Rad, Richmond, CA) were as follows: lysozyme, 14,400; soybean trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; ovalbumin, 45,000; bovine serum albumin, 66,200; phosphorylase B; 92,500. Gels were stained in 46% methanol, 8% glacial acetic acid, 0.1% Coomassie Blue (500 ml). Gels were destained in several washes (250 ml) of 20% methanol-8% glacial acetic acid.

VISUALIZATION OF PLASMINOGEN ACTIVATORS BY ZYMOGRAPHY

When gels were overlayed on fibrin-agar plates to visualize the position of PAs, the procedures of Granelli-Piperno and Reich (1979) were followed as detailed by Crowe et al. (1978). This procedure consisted of running the samples on SDS-PAGE under the usual conditions (see above), but instead of fixing and staining, the gel was soaked in a 2.5% v/v solution (250 ml) of Triton-X 100 for at least 1 hour, with gentle agitation. This replaces the SDS with Triton X-100 and allows the PAs to regain enzymatic activity.
This gel is then rinsed in dH₂O and carefully overlayed on a fibrin-agar substrate prepared in a petri dish as described above, only in a 10cm square dish (Lab Tek) using double the amount of solutions. The plate was then incubated at 37° C in the humidified cell culture incubator. When plasminogen activators were present in the electrophoresis gel, they diffused into the fibrin-agar substrate and activated plasminogen to plasmin. The plasmin then produced clear bands of lysis which allowed the positions of plasminogen activators in the original electrophoresis gel to be determined.

**ANTIBODY STUDIES**

Goat anti-human tPA and goat anti-human UK IgG antibodies were purchased from American Diagnostica (batch #s 8312/0105 and 8412/12). 5 mgs of each were reconstituted in 1 ml of sterile filtered 0.05 M Tris pH 8.8 and mixed well. Each antibody solution (15 ul) and 15 ul of Tris were added to 15 ul of the purified PA preparation in individual microcentrifuge tubes and vortexed. Tubes were incubated for 3.5 hours at room temperature, and then 10 ul of each solution was spotted in wells punched on a fibrin agar plate, in duplicate. The fibrin agar plate was then incubated at 37° C and examined regularly over the next 5 days for signs of lysis.
PROTEIN DETERMINATION

Protein was determined by the method of Lowry (1951) or by using the Pierce BCA reagent and comparing to standard curves. When Triton X-100 was present in the Lowry assay, SDS was added at a concentration which would prevent precipitation (Bonsall and Hunt, 1971).
RESULTS

SELECTIVITY OF TRIPEPTIDE ALDEHYDE INHIBITORS

Peptide aldehydes have been synthesized with sequences designed to inhibit proteolytic enzymes implicated in the metastatic process. The enzymes chosen for investigation in this study were cathepsin B, thrombin, and urokinase. The peptide aldehydes, t-boc-D-Phe-Pro-Argininal, D-Phe-Pro-Argininal, (R-D-Phe-Pro-Argininal), (tfa)Glu-Gly-Argininal, t-boc-Glu-Gly-Argininal, (R-Glu-Gly-Argininal), and leupeptin, were tested for their ability to inhibit the selected proteases believed to play a role in metastasis. From the determined $K_i$ values in Table 1, it is clear that leupeptin is an extremely potent inhibitor of cathepsin B. The $K_i$ of leupeptin for cathepsin B of approximately $4 \times 10^{-9}$ M indicates that leupeptin is an extremely potent inhibitor of cathepsin B. Likewise the $K_i$'s of the R-D-Phe-Pro-Argininal compounds against thrombin of $10^{-8}$ M show that these compounds should be able to effectively inhibit the activity of thrombin in vivo. The $K_i$ values of the R-Gly-Gly-Argininal compounds for UK are higher than those of the thrombin and cathepsin B inhibitors, but they are still selective for UK (see below). By using higher concentrations they should still be
Table 1. Inhibition constants of tripeptide aldehydes against proteinases implicated in the metastatic process (from Ostrowski et al., 1986).
### Inhibition Constants of Peptidyl Aldehydes Against Selected Protease Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ac-Leu-Leu-Argininal</th>
<th>D-Phe-Pro-Argininal</th>
<th>Boc-D-Phe-Pro-Argininal</th>
<th>Glu-Gly-Argininal</th>
<th>Boc-Glu-Gly-Argininal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>4.7x10^{-6}</td>
<td>1.1x10^{-8}</td>
<td>1.5x10^{-8}</td>
<td>&gt;2x10^{-4}</td>
<td>&gt;2x10^{-4}</td>
</tr>
<tr>
<td>Factor Ia</td>
<td>5.7x10^{-6}</td>
<td>5.2x10^{-5}</td>
<td>3.9x10^{-6}</td>
<td>&gt;2x10^{-4}</td>
<td>3.1x10^{-4}</td>
</tr>
<tr>
<td>Urokinase</td>
<td>7.7x10^{-5}</td>
<td>2.0x10^{-4}</td>
<td>2.2x10^{-6}</td>
<td>2.1x10^{-5}</td>
<td>5.4x10^{-5}</td>
</tr>
<tr>
<td>Plasmin</td>
<td>1.8x10^{-6}</td>
<td>2.6x10^{-5}</td>
<td>2.6x10^{-6}</td>
<td>&gt;2x10^{-4}</td>
<td>1.6x10^{-4}</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>3.7x10^{-9}</td>
<td>&gt;2x10^{-5}</td>
<td>&gt;2x10^{-5}</td>
<td>&gt;2x10^{-5}</td>
<td>&gt;2x10^{-5}</td>
</tr>
</tbody>
</table>
effective at inhibiting UK in an **in vivo** model for metastasis.

As mentioned previously, leupeptin can also inhibit other enzymes believed to be involved in metastasis. Thus the $K_i$ values of leupeptin against thrombin, UK, factor Xa, and plasmin were also determined. These are given in Table 1. The $K_i$ value which is closest to that of cathepsin B was obtained for plasmin $(2 \times 10^{-6} \text{ M})$. The ratio $K_i$ plasmin / $K_i$ cathepsin B is indicative of the specificity of leupeptin and shows that leupeptin is approximately 500-fold more effective at inhibiting cathepsin B than plasmin. This selectivity of leupeptin is even greater for the other enzymes proposed to be involved in metastasis. Likewise, D-Phe-Pro-Argininal has a $K_i$ plasmin / $K_i$ thrombin of approximately 250. The R-Glu-Gly-Argininal compounds were not as effective at inhibiting UK and therefore their specificity ratio was about an order of magnitude over the other proteases. However, these compounds still were selective for UK.

**EFFECT OF CATHEPSIN B INHIBITORS ON METASTASIS**

An attempt to inhibit experimental metastasis with leupeptin was made in a manner similar to that used by Saito et al. (1980). In these experiments, leupeptin at a concentration of 50 mg/kg in 0.2 ml PBS was injected i.p. into one group of mice while the control group received an equal volume of PBS. Mice were injected
with the drug solutions thirteen times, once every 12 hours. Tumor cells, (100,000) were injected into the lateral tail vein of each animal approximately 1-3 hrs. after the second drug injection. As shown in Table 2, experiment 1, the leupeptin treated group showed a significant reduction in the number of metastatic lung colonies (by Student's t-test). This agrees with the results of Saito et al. (1980) who also observed a 50% reduction in lung colonies using the same protocol with a hepatoma cell line in rats. However, further experiments with leupeptin failed to yield significant inhibition (as analysed by Student's t-test for differences about a mean and the Mann-Whitney U test). This can be seen in Table 2 experiments 2-7. It will be noticed that in each case, except experiment 7, leupeptin produced a decrease in the average number of lung colonies. (In experiment 7 the colonies had grown so large that an accurate count was difficult. At this time, the criteria used in counting resulted in a minimal number, so that two or more colonies which had grown together were counted as one, possibly resulting in the decrease compared to the leupeptin treated group. In future experiments, the time before sacrifice was shortened to avoid this problem and the maximal number of metastases were determined). However, in only the first experiment was this difference significant. It is interesting to note that in experiments 2, 3, and 5 (Table 2)
### Table 2

Effect of i.p. Injected Leupeptin on Experimental Metastasis

<table>
<thead>
<tr>
<th>Experimental Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Lung Metastases per Animal</th>
<th>Mean (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>13,17,21,21,37,47,51</td>
<td>30±6</td>
</tr>
<tr>
<td>Leupeptin (50)</td>
<td>0,7,10,15,17,18,22,31</td>
<td>15±3</td>
</tr>
<tr>
<td>2. Control</td>
<td>66,68,69,88,108,180</td>
<td>97±18</td>
</tr>
<tr>
<td>Leupeptin (5)</td>
<td>32,38,66,72,83,125,146</td>
<td>80±16</td>
</tr>
<tr>
<td>Leupeptin (50)</td>
<td>26,32,36,46,69,111,168</td>
<td>70±20</td>
</tr>
<tr>
<td>3. Control</td>
<td>3,8,18,22,25,41,54,60,70,73,84,92,120,133,299</td>
<td>74±19</td>
</tr>
<tr>
<td>Leupeptin (5)</td>
<td>4,12,14,18,28,33,47,66,70,76,77,79,81,86,101,106,120</td>
<td>60±9</td>
</tr>
<tr>
<td>4. Control</td>
<td>1,23,33,41,54,59,121</td>
<td>47±14</td>
</tr>
<tr>
<td>Leupeptin (50)</td>
<td>5,6,23,44,103</td>
<td>36±18</td>
</tr>
<tr>
<td>5. (20,000 cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0,0,0,1,1,1,9,10,10</td>
<td>3.6±1.5</td>
</tr>
<tr>
<td>Leupeptin (25)</td>
<td>0,0,0,1,1,1,1,3</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>6. Control</td>
<td>1,3,8</td>
<td>4±1.8</td>
</tr>
<tr>
<td>Leupeptin (5)</td>
<td>0,1,2,2,5</td>
<td>2±0.8</td>
</tr>
<tr>
<td>Leupeptin (50)</td>
<td>0,1,1,5</td>
<td>1.8±1.1</td>
</tr>
<tr>
<td>7. Control</td>
<td>5,6,6,14,15,20,21,36,36,41,60</td>
<td>24±5</td>
</tr>
<tr>
<td>Leupeptin (37.5)</td>
<td>0,10,18,22,28,39,40,43,44,49,54</td>
<td>32±5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers in ( ) indicate mg/kg injected i.p. every 12 hours for 5.5 days. Control animals received injections of drug vehicle.

<sup>b</sup>Value is significantly different from control by Student's t-test at P < 0.05.
where different amounts of tumor cells were injected (20,000) or lower amounts of leupeptin (5 or 25 mg/kg) were used, this trend continued.

To verify this lack of a statistical reduction in experimental metastasis using a strong cathepsin B inhibitor, the peptide epoxide Ep453 was used to treat mice in the same fashion as leupeptin. In plasma, the Ep453 is rapidly converted to the more soluble compound Ep475 by esterases (Hanada et al., 1983). This conversion occurs in less than 30 minutes (Ostrowski et al., 1986), and the Ep475 produced is a potent, irreversible inhibitor of cathepsins and other cysteine proteases. Again, the inhibition of cathepsin B by this procedure produced no reduction of metastasis as shown in Table 3.

Although this protocol has been shown to reduce metastasis in other systems, the possibility that the dose of leupeptin used was not sufficient to inhibit cathepsin B in this model must be considered. Previous data has shown that leupeptin has a short halftime in vivo (a halftime of 12 minutes has been reported, Tanaka, 1983), although lower doses than that used here have had lasting effects on dystrophy (see literature review). In an attempt to determine if the dosage used was sufficient to inhibit cathepsin B, the blood levels of leupeptin were determined by the procedure detailed in the methods section. As shown in Table 4,
### Table 3

Effect of i.p. Injected Ep453 on Experimental Metastasis

<table>
<thead>
<tr>
<th>Experimental Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Lung Metastases per Animal</th>
<th>Mean (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15, 31, 44, 46, 48, 54, 97, 113, 131, 139, 142</td>
<td>78±14</td>
</tr>
<tr>
<td>Ep453 (50)</td>
<td>8, 36, 37, 42, 42, 51, 60, 60, 80, 127, 129, 153, 180</td>
<td>77±15</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number in ( ) indicate mg/kg injected i.p. every 12 hours for 5.5 days. Control animals received injections of the drug vehicle.
Table 4

Turnover of Leupeptin in Mouse Plasma following i.p. Injection

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Leupeptin conc. (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>$4.9 \times 10^{-5}$</td>
</tr>
<tr>
<td>1.0</td>
<td>$3.67 \times 10^{-5}$</td>
</tr>
<tr>
<td>2.0</td>
<td>$1.54 \times 10^{-5}$</td>
</tr>
<tr>
<td>4.0</td>
<td>$2.52 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Mice were sacrificed at the indicated times after an i.p. injection of 50 mg/kg of leupeptin and serum levels of the drug were determined as described in methods.
leupeptin is originally present at very high levels (5 x 10^{-5}M) following the injection. At this level, leupeptin will inhibit all of the proteases possibly involved in metastasis which were targeted for study (Table 1). After 6 hr, the level of leupeptin was below the limit of detection in this assay, but may have still been present in amounts capable of inhibiting cathepsin B. Although the evidence still argues strongly against a role of cathepsin B, an alternative method of drug delivery was used to further test this hypothesis.

In the following experiments (Table 5), leupeptin was delivered by continuous infusion using Alzet mini-osmotic pumps. The pumps were loaded with an amount of leupeptin equivalent to that delivered by injections, and the determination of the blood level of leupeptin showed a steady level of 3 x 10^{-6}M for at least 5 days. In the first experiment (Table 5) only a small number of animals were tested, and although the variation is quite large, there was no significant reduction of metastases in the leupeptin group. This experiment was repeated twice using larger groups of animals (Table 5, experiments 2 and 3). Again no significant difference in the number of metastases was observed between the control and the leupeptin treated animals.

The peptide epoxide (Ep453) was also infused continuously by osmotic pump to see the effect of a second potent inhibitor of
Table 5

Effect of Continuously Infused Leupeptin On Experimental Metastasis

<table>
<thead>
<tr>
<th>Solution Infused&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Lung Metastases per Animal</th>
<th>Mean (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>43, 47, &gt;300, &gt;300</td>
<td>172±74</td>
</tr>
<tr>
<td>Leupeptin (0.1 M in PBS)</td>
<td>7, 15, 29, 222</td>
<td>68±51</td>
</tr>
</tbody>
</table>

2. Control (PBS) 34, 46, 64, 71, 80, 96, 111, 132, 133, 153, 157, 236
                 Leupeptin (0.1 M in PBS) 74, 91, 102, 109, 109, 121, 127, 137, 155, 250
                 Mean 109±16

3. Control (PBS) 1, 2, 2, 3, 4, 4, 5, 6, 7, 7, 7, 8, 8, 10
                 Leupeptin (0.1 M in PBS) 0, 0, 1, 2, 2, 3, 4, 6, 20
                 Mean 5.4±0.7

<sup>a</sup>Solution in () infused into animal from Alzet mini-osmotic pumps surgically implanted into mice. Rates of infusion were 1 uL/hr over 5 to 7 days. Steady-state concentration of leupeptin in mouse plasma during infusion of inhibitor was 3 x 10<sup>-6</sup> M.
### Table 6

**Effect of Continuously Infused Ep453 On Experimental Metastasis**

<table>
<thead>
<tr>
<th>Solution Infused&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Lung Metastases per Animal</th>
<th>Mean (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PG)</td>
<td>13, 22, 27, 33, 44, 49, &gt;300, &gt;300</td>
<td>98±44</td>
</tr>
<tr>
<td>Ep453 (0.08 M in propylene glycol (PG))</td>
<td>17, 25, 25, 43, 66, 79, 90, 105</td>
<td>56±12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Solution in () infused into animal from Alzet mini-osmotic pumps surgically implanted into mice. Rates of infusion were 1 uL/hr over 5 to 7 days. Steady-state concentration of Ep453 in mouse plasma during infusion of inhibitor was 3 x 10⁻⁷ M.
cathepsin B on metastasis. In this experiment the plasma level of Ep475 was $3 \times 10^{-7}$ M. As shown in Table 6, this concentration of an irreversible inhibitor of cathepsin B also had no significant effect on the formation of metastatic lung colonies.

**EFFECT OF THROMBIN INHIBITORS ON METASTASIS**

In experimental models for metastasis, prior evidence has indicated that the attachment of circulating tumor cells is an important step leading to metastasis. Treatment with anticoagulation drugs or drugs which inhibit platelet aggregation have been shown in some instances to reduce metastasis. However, many of these compounds have other effects in addition to their anticoagulation properties. Since thrombin is believed to play an important role in the attachment of circulating tumor cells and tumor cell/platelet emboli, the specific thrombin inhibitors t-boc-D-Phe-Pro-Argininal and D-Phe-Pro-Argininal were injected into mice by the same protocols that were used for inhibitors of cathepsin B. Injection of 50 mg/kg of t-boc-D-Phe-Pro-Argininal was fatal to 5 of the mice in experiment 1, Table 7. This experiment was continued using injections of 12.5 mg/kg. Because of this toxicity, slightly lower doses of these compounds (37.5 or 25 mg/kg) were used. These doses were tolerated well by the
Table 7

Effect of i.p. Injected R-D-Phe-Pro-Argininals on Experimental Metastasis

<table>
<thead>
<tr>
<th>Experimental Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Lung Metastases per Animal</th>
<th>Mean (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13, 17, 21, 21, 37, 47, 51</td>
<td>30±6</td>
</tr>
<tr>
<td>Boc-D-Phe-Pro-Argal (1x50, then 12.5)</td>
<td>9, 29, 47</td>
<td>28</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1, 23, 33, 41, 54, 59, 121</td>
<td>47±14</td>
</tr>
<tr>
<td>Boc-D-Phe-Pro-Argal (37.5)</td>
<td>12, 25, 26, 31, 36, 48, 62, 79, 118, 180</td>
<td>62±16</td>
</tr>
<tr>
<td>3. (20,000 cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0, 0, 0, 1, 1, 1, 9, 10, 10</td>
<td>3.6±1.5</td>
</tr>
<tr>
<td>Boc-D-Phe-Pro-Argal (25)</td>
<td>0, 1, 1, 1, 1, 2, 2, 2, 2, 3, 6, 14, 17, 36</td>
<td>6.3±2.7</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5, 6, 6, 14, 15, 20, 21, 36, 36, 41, 60,</td>
<td>24±5.3</td>
</tr>
<tr>
<td>D-Phe-Pro-Argal (37.5)</td>
<td>7, 18, 23, 24, 29, 38, 46, 62, 62, 64,</td>
<td>37±6.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers in () indicate mg/kg injected i.p. every 12 hours for 5.5 days. Control animals received injections of the drug vehicle. Argal is an abbreviation for argininal.
animals. In experiment 3 (Table 7), several of the mice (3) died before the end of the experiment, and several were lethargic with difficulty in hind leg motion. Several of these animals were autopsied and appeared normal, except for the tumors. In 4 animals on which complete autopsies were performed, metastases were found outside the pleural cavity in three animals; 2 of these were in the kidneys and one each in the lymph node and at a subcutaneous site. It is believed that the effects seen in these animals were due to tumor burden, possibly due to the length of this experiment (21 days). Similar rear body effects on motion were observed in animals of the control groups and treated groups. It was also noticed that in experiment 2 (Table 7) in which the higher dose of the t-boc-D-Phe-Pro-Argininal compound (37.5 mg/kg) was used, the animals in the treated group appeared to have bruises on their abdominal region. This differed from the control and leupeptin animals where the abdominal region toughened with scar tissue from the repeated injections. Some of the animals receiving this dose (37.5 mg/kg) of the t-boc-D-Phe-Pro-Argininal also showed susceptibility to bleeding when handled by the tail.

In Table 7 it can be clearly seen that the specific inhibitors of thrombin had no significant effect on metastasis. This was the result when t-boc-D-Phe-Pro-Argininal or D-Phe-Pro-Argininal was used, and did not vary when the number of tumor
cells injected was decreased (experiment 3, Table 7). In fact, in 3 of the experiments (experiments 2, 3, and 4, Table 7), these compounds showed a slight promotion effect, causing the number of lung colonies to increase by an average of 40%.

Again, in order to make sure that the concentrations of drug in vivo were sufficient to inhibit thrombin, the blood levels were assayed following i.p. injection. A 37.5 mg/kg injection of t-boc-D-Phe-Pro-Argininal resulted in plasma levels of $1 \times 10^{-6}$M after 30 minutes and $4 \times 10^{-7}$M after 1 hr. After 1 hr, no inhibitor could be detected. Therefore, these experiments were repeated using the osmotic pump procedure described previously. Loading the pumps with a 0.25 M solution of D-Phe-Pro-Argininal resulted in a steady state level of inhibitor at a concentration of $4 \times 10^{-7}$M. This is higher than the $K_i$ of this compound for thrombin, but lower than the $K_i$ values for the other implicated proteases (Table 1), so that in these experiments thrombin should be effectively and specifically inhibited. This treatment increased the number of lung colonies dramatically as shown in Table 8. In two separate experiments the treated group averaged over 300 individual lung colonies, while the control groups averaged close to ten.
Table 8

Effect of Continuously Infused D-Phe-Pro-Argininal On Experimental Metastasis

<table>
<thead>
<tr>
<th>Solution Infuseda</th>
<th>Number of Lung Metastases per Animal</th>
<th>Mean (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (40% DMSO)</td>
<td>4,4,4,6,8,9,12,13</td>
<td>7.5±1.3</td>
</tr>
<tr>
<td>D-Phe-Pro-Arginal (0.25 M in 40% DMSO-PBS)</td>
<td>229,&gt;300,&gt;300,&gt;300,&gt;300,&gt;300,&gt;300,&gt;300</td>
<td>&gt;300b</td>
</tr>
<tr>
<td>2. Control (40% DMSO)</td>
<td>7,8,11,12,13,18</td>
<td>12±1.4</td>
</tr>
<tr>
<td>D-Phe-Pro-Arginal (0.25 M in 40% DMSO-PBS)</td>
<td>104,&gt;300,&gt;300,&gt;300,&gt;300,&gt;300,&gt;300,&gt;300</td>
<td>&gt;300b</td>
</tr>
</tbody>
</table>

aSolution in () infused into animal from Alzet mini-osmotic pumps surgically implanted into mice. Rates of infusion were 1 uL/hr over 5 to 7 days. Steady-state concentration of D-Phe-Pro-Argininal in mouse plasma during infusion of inhibitor was 4 x 10⁻⁷ M. bValue is significantly different from control at P < 0.001. Argal is an abbreviation for argininal.
EFFECT OF PLASMINOGEN ACTIVATOR INHIBITORS ON METASTASIS

Because of the higher $K_i$ values of the R-Glu-Gly-Argininal inhibitors against their targeted protease, urokinase (Table 1), higher in vivo concentrations of these compounds were needed to examine their effect on metastasis. The blood level of these compounds was measured following i.p. injections as described in the methods section. It was found that after a 30 mg injection, the compound was only detected (as measured by inhibition of urokinase, lower limit $1 \times 10^{-5}$ M) for 1-2 hr. Due to these features of the R-Glu-Gly-Argininal compounds, (high $K_i$ values, rapid turnover in vivo), it was impossible to use osmotic pumps to deliver these compounds. Accordingly, maximum doses (30mg) tolerated by the animals were injected i.p. every 2-4 hours for the first 1-1.5 days. The results are shown in Table 9. The free (tfa) Glu-Gly-Argininal compound appeared to be more toxic than the t-boc-Glu-Gly-Argininal. At these doses, both compounds appeared to make the mice lethargic and somewhat stiff in motion (in the (tfa) Glu-Gly-Argininal experiment, 2 of the smallest mice died; these mice received a higher dose per kg and were also clearly not as vigorous as the others before the experiments). However all the mice recovered rapidly (within 24 hours) after the
Table 9

Effect of i.p. Injected R-Glu-Gly-Argininals on Experimental Metastasis

<table>
<thead>
<tr>
<th>Experimental Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Lung Metastases per Animal</th>
<th>Mean (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>122,165,177,180,220,230,260,&gt;300,&gt;300,&gt;300</td>
<td>226±20</td>
</tr>
<tr>
<td>Boc-Glu-Gly-Argal (30 mg i.p. per animal; 8 times over 36 hr)</td>
<td>62,68,154,160,178,&gt;300,&gt;300</td>
<td>174±37</td>
</tr>
<tr>
<td>2. Control</td>
<td>87,89,122,128,149,189</td>
<td>127±13</td>
</tr>
<tr>
<td>H-Glu-Gly-Argal (30 mg i.p. per animal; 6 times over 25 hr)</td>
<td>214,279,&gt;300,&gt;300,&gt;300</td>
<td>279±17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers in ( ) indicate mg/kg injected i.p. every 12 hours for 5.5 days. Control animals received injections of the drug vehicle. Argal is an abbreviation for argininal. <sup>b</sup>Significantly different from control at P < 0.001.
injections had ceased. These inhibitors also failed to reduce metastasis, even at the high doses used in this experiment. The promotion of lung colonization seen in experiment 2, although significant, must be considered with caution due to the possible toxic side effects of the high doses of drug used in this study.
RESULTS OF PLASMINOGEN ACTIVATOR STUDIES

Because of the strong evidence for a role of PA in metastasis, attempts were made to study the PAs secreted by the B16 melanoma in culture. The susceptibility of the secreted enzyme to inhibition by the tripeptide aldehyde inhibitors was estimated using concentrated media prepared by different procedures. Those reported (Table 10) were performed using an Amicon concentrate (500 ml concentrated to 2.5 ml) and were determined under the conditions described in methods. Inhibition constants were determined by measuring the rate of hydrolysis of S-2444 in the presence and absence of inhibitor. This substrate (S-2444) has been shown to be specific for UK with a $K_m$ of $6 \times 10^{-5}$ M (Kabi). Preliminary data (Table 11, Figure 2) indicated that although plasminogen is the only known protein substrate for both UK and tPA, they have different specificities for small substrates. The $K_m$ of S-2444 determined against human rt-PA was approximately $1.5 \times 10^{-3}$ M. This indicates that S-2444 is a 25-fold better substrate for UK than for tPA. Thus, these $K_i$'s give an estimate of the susceptibility of the UK-like enzyme present in serum-free conditioned medium (SFCM) to inhibition by the aldehydes used in this study. Several of these inhibitors gave
TABLE 10
Inhibition of urokinase-like activity in concentrated SFCM by tripeptide aldehydes

<table>
<thead>
<tr>
<th>tripeptide aldehyde</th>
<th>$K_i , ^a , M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(tfa)Glu-Gly-Argininal</td>
<td>$3.0 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$(1.59 \times 10^{-4}, 2.64 \times 10^{-4}, 4.76 \times 10^{-4})$</td>
</tr>
<tr>
<td>t-boc-Glu-Gly-Argininal</td>
<td>$4.0 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$(4.5 \times 10^{-4}, 3.4 \times 10^{-4})$</td>
</tr>
<tr>
<td>DNS-Glu-Gly-Argininal</td>
<td>$2.1 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$(9.5 \times 10^{-5}, 3.2 \times 10^{-4})$</td>
</tr>
<tr>
<td>D-Phe-Pro-Argininal</td>
<td>$9.8 \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>$(1.43 \times 10^{-4}, 5.37 \times 10^{-5})$</td>
</tr>
<tr>
<td>t-boc-D-Phe-Pro-Argininal</td>
<td>$2.5 \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>$(9.3 \times 10^{-7}, 4 \times 10^{-6})$</td>
</tr>
<tr>
<td>leupeptin</td>
<td>$1.6 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$(3 \times 10^{-4}, 2.59 \times 10^{-5})$</td>
</tr>
</tbody>
</table>

$^a$ Inhibition constants were determined in 1.1 mls. 0.05 M tris pH 8.8 containing 30 KIU of aprotinin and 100 ul concentrated SFCM. Substrate (S-2444) was at a final concentration of $1.1 \times 10^{-4}$ M. The $K_i$ is the average of the individual values in parentheses.
Table 11

Determination of $K_m$ of S-2444 for rtPA

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>INITIAL RATE (v)$^a$</th>
<th>S/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>M x 10^3</td>
<td>M/min. x 10^7</td>
<td>min x 10^{-4}</td>
</tr>
<tr>
<td>3.6</td>
<td>3.3</td>
<td>1.1</td>
</tr>
<tr>
<td>1.82</td>
<td>2.7</td>
<td>0.67</td>
</tr>
<tr>
<td>0.91</td>
<td>1.7</td>
<td>0.54</td>
</tr>
<tr>
<td>0.45</td>
<td>1.1</td>
<td>0.41</td>
</tr>
</tbody>
</table>

$^a$ Reactions were performed in a final volume of 1.225 mls. of 0.05 M tris buffer pH 8.8, using a stock substrate solution of 1 x 10^{-2} M and 25 ul of a 0.1 mg/ml solution of rtPA. See Figure 3 for plot of data.
Figure 2. Determination of $K_m$ of S-2444 for rtPA.

A plot of $S/v$ vs. $S$ gives a straight line with a correlation coefficient of 0.99 by linear regression. From the Hanes plot, $S/v = K_m/V + S/V$, the slope = $1/V$ and the intercept = $K_m/V$, giving a $K_m$ of $1.5 \times 10^{-3}$ M.
\[ \frac{S}{v} \text{ min} \times 10^{-4} \]

\[ \frac{1}{v} \]

\[ -K_m \]

\[ K_m \]

\[ \text{Substrate} \times 10^4 \text{ M} \]
similar \( K_i \)'s against this preparation, but in this crude
preparation it is probable that more than one enzyme was taking
part in the reaction. Electrophoresis gels of the concentrated
media showed many protein bands, and when overlayed on fibrin agar
indicator gels, several bands of lysis were observed. This is
clearly illustrated in Figure 3. No lysis was observed in fibrin
plates in which the plasminogen had been denatured. These bands
varied in appearance but always consisted of a combination of the
three shown in Figure 3. These bands could be interpreted in
several ways (partially degraded forms, different enzymes, enzyme
inhibitor complexes), but it is now clear that they represent
synthesis of both types of PA (tissue and urokinase) by the B16
cells.

In order to obtain a better estimate of these \( K_i \)'s, several
attempts were made at purifying the enzyme; these were hampered
principally by the low amounts of activity present in the initial
starting material. Serum free conditioned media contained
approximately 4000 to 5000 mU/L of UK-like activity as measured
by S-2444 hydrolysis. Attempts to grow cells in SFM for extended
lengths of time or in media containing serum supplements proved
unsatisfactory, and the small amounts of conditioned media
obtained by traditional cell culture were inadequate for detailed
study. After initial attempts using a peptide aldehyde column,
Figure 3. Photograph of electrophoresis gel stained in Coomassie Blue after overlaying on fibrin agar indicator gel. Sample was concentrated B16 melanoma SFCM.
the use of a p-aminobenzamidine column was found to result in reproducible binding of the enzyme activity. After several modifications were attempted, the purification procedure outlined in the methods section was used to produce a preparation which produced a single major band of lysis following gel electrophoresis and zymography on a fibrin-agar plate (Figure 4). Mouse urine (25 ul) was electrophoresed in an adjacent lane, and produced two bands of lysis corresponding to the low (30,000) and high (48,000) molecular weight forms of urokinase. Samples were always electrophoresed in triplicate and the gels were sliced into three equal sections. One of the gel sections was stained in Coomassie Blue. The two remaining sections were prepared for zymography. One was overlayed on a fibrin-agar gel to detect PA activity and the other was overlayed on a fibrin-agar plate in which the plasminogen had been denatured to control for plasminogen independant lysis. No lysis was observed on the control plates. It is clear from the position of the lysis bands that the sample (50 ul) contained a qualitatively different plasminogen activator. From the position of mouse tissue type PA (see Figure 5), it was concluded that the majority of the PA activity in the partially purified sample was of the tissue type. This single-step chromatography procedure resulted in a yield of about 30% and about a 100-fold purification, although these
Figure 4. Photograph of fibrin agar underlay showing bands of lysis produced by overlaying with electrophoresis gel containing samples of partially purified B16 PA (lanes 1 and 2) and mouse urine (lane 3).
numbers are based in S-2444 hydrolysis activity and the protein determinations were obtained in the presence of detergents and high concentrations of other materials. A second preparation purified from a different batch of SFCM, resulted in a single band of lysis when electrophoresed and overlayed on a fibrin-agar plate. This single partially purified activity is shown clearly in Figure 5, where samples of concentrated SFCM, partially purified PA from SFCM, mouse urine, and mouse lung homogenate were electrophoresed on the same gel. Mouse urine is known to contain only UK and mouse lung is known to show tPA and UK PA activity. Comparing these "standards" to the B16 samples, it is clear that both enzymes are present in the sample of concentrated medium while the partially purified preparation only shows a tPA band. The Coomassie Blue stained gel is shown for comparison in Figure 6. That this preparation consisted solely of tPA was demonstrated by showing that the plasminogen activator activity was completely inhibited by anti-tPA antibody and completely resistant to anti-UK antibody. After incubation with the specific antibody preparation, samples were assayed for their ability to activate plasminogen and produce lysis on a fibrin-agar plate. The result is shown in Figure 7, which was photographed 5 days after the samples were applied. The complete inhibition of lysis by anti-tPA antibody clearly indicates that this sample contained only mouse tPA.
Figure 5. Fibrin agar underlay showing bands of lysis produced by electrophoresing mouse urine (lane 1), concentrated B16 SFCM (lane 2), partially purified PA from B16 SFCM (lane 3), and homogenized mouse lung tissue (lane 4). This pattern of lysis did not change after five days.
Figure 6. Stained electrophoresis gel corresponding to that overlayed in figure 15. Samples are urine (lane 1), concentrated SFCM (lane 2), partially purified PA from B16 SFCM (lane 3), homogenized mouse lung tissue (lane 4), and molecular weight standards (lane 5). Molecular weights of standards are: $a = 92,000$, $b = 66,000$, $c = 45,000$, $d = 31,000$, $e = 25,000$, and $f = 17,000$. 
Figure 7. Antibody inhibition of lysis on fibrin agar indicator gel by partially purified PA from B16 SFCM. Wells 3 and 6 are controls, wells 2 and 5 contained anti-tPA antibody, and wells 1 and 4 contained anti-UK antibody.
Direct activation of plasminogen by a partially purified preparation of mouse tPA using the coupled plasminogen-S-2251 assay is shown in Figure 8. The effect on this activation by pretreatment with the irreversible inhibitors DFP and pCMB is also shown. DFP completely inhibited the activity of the mouse tPA sample, while pCMB had no effect. These inhibitors gave the same result when the hydrolysis of the synthetic substrate S-2444 (Figure 9) was measured. These results confirm the serine-type nature of the isolated PA activity. Inhibition constants for the tripeptide aldehydes determined against this partially purified preparation are listed in Table 12.

Inhibition constants for a purified sample of recombinant human tPA (rtPA) were also obtained under the same conditions. These are shown in Table 13. An example of the inhibition experiments is shown in Figure 10. It can be noted that the specificity of the inhibitors against this purified preparation of human rtPA, consisting of a mixture of one and two chain forms, follows the same pattern as that for the partially purified PA preparation from the B16 melanoma. These results indicate that tPA (Table 13) has a very different specificity towards small peptides than urokinase (Table 1), even though their natural substrate is identical (plasminogen).
Figure 8. Spectrophotometer tracing showing the direct activation of plasminogen by a partially purified preparation of B16 melanoma PA and the effect of DFP and pCMB. The control reaction is shown in A), inhibition by DFP is shown in B), and lack of an effect of pCMB is shown in C). Arrow indicates point of addition of UK to verify DFP treatment was not inhibiting plasmin.
Figure 9. Spectrophotometer tracing showing the hydrolysis of S-2444 by a partially purified preparation of B16 melanoma PA before and after incubation with the irreversible protease inhibitors DFP and pCMB. A) is the control reaction, B) is after treatment with DFP, and C) is after treatment with pCMB.
A

B

C

Abs

Abs

Abs

minutes
### TABLE 12

Inhibition of a partially purified preparation of B16 melanoma tPA by tripeptide aldehydes.

<table>
<thead>
<tr>
<th>tripeptide aldehyde</th>
<th>$K_i^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(tfa)Glu-Gly-Argininal</td>
<td>$1.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>t-boc-Glu-Gly-Argininal</td>
<td>$7.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>DNS-Glu-Gly-Argininal</td>
<td>$1.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>D-Phe-Pro-Argininal</td>
<td>$8.3 \times 10^{-5}$</td>
</tr>
<tr>
<td>t-boc-D-Phe-Pro-Argininal</td>
<td>$5.5 \times 10^{-6}$</td>
</tr>
<tr>
<td>leupeptin</td>
<td>$3.3 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

$K_i$s were estimated in a final volume of 1.1 ml of 0.05 M Tris-0.025 M EDTA pH 8.8 containing $1.1 \times 10^{-4}$ M substrate (S-2444). 100 ul of the enzyme solution was incubated with the inhibitor to be tested and 30 KIU aprotinin in buffer for 15 minutes before the reaction was started by addition of substrate.
TABLE 13

Inhibition of human rtPA by tripeptide aldehydes

<table>
<thead>
<tr>
<th>tripeptide aldehyde</th>
<th>$K_i \text{ a M}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(tfa)Glu-Gly-Argininal</td>
<td>$3.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>$3.97 \times 10^{-4}$ b (2)</td>
<td>$3.34 \pm 0.64 \times 10^{-3}$ c</td>
</tr>
<tr>
<td>$1.19 \times 10^{-3}$ b (2)</td>
<td>$3.33 \pm 0.10 \times 10^{-3}$ c</td>
</tr>
<tr>
<td>t-boc-Glu-Gly-Argininal</td>
<td>$1.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>$5.1 \times 10^{-4}$ b (2)</td>
<td>$1.85 \pm 0.11 \times 10^{-3}$ c</td>
</tr>
<tr>
<td>$2.05 \times 10^{-4}$ b (2)</td>
<td>$1.66 \pm 0.35 \times 10^{-3}$ c</td>
</tr>
<tr>
<td>DNS-Glu-Gly-Argininal</td>
<td>$2.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>$6.36 \times 10^{-6}$ b (3)</td>
<td>$3.19 \pm 0.3 \times 10^{-5}$ c</td>
</tr>
<tr>
<td>$6.36 \times 10^{-6}$ b (3)</td>
<td>$2.39 \pm 0.05 \times 10^{-5}$ c</td>
</tr>
<tr>
<td>D-Phe-Pro-Argininal</td>
<td>$1.7 \times 10^{-5}$</td>
</tr>
<tr>
<td>$8.97 \times 10^{-5}$ b (2)</td>
<td>$1.78 \pm 0.07 \times 10^{-5}$ c</td>
</tr>
<tr>
<td>$3.59 \times 10^{-5}$ b (3)</td>
<td>$1.60 \pm 0.09 \times 10^{-5}$ c</td>
</tr>
<tr>
<td>t-boc-D-Phe-Pro-Argininal</td>
<td>$2.1 \times 10^{-7}$</td>
</tr>
<tr>
<td>$1.24 \times 10^{-6}$ b (3)</td>
<td>$1.88 \pm 0.09 \times 10^{-7}$ c</td>
</tr>
<tr>
<td>$1.24 \times 10^{-7}$ b (3)</td>
<td>$2.35 \pm 0.2 \times 10^{-7}$ c</td>
</tr>
<tr>
<td>leupeptin</td>
<td>$9.2 \times 10^{-6}$</td>
</tr>
<tr>
<td>$4.6 \times 10^{-5}$ b (3)</td>
<td>$8.92 \pm 0.32 \times 10^{-6}$ c</td>
</tr>
<tr>
<td>$1.84 \times 10^{-5}$ b (3)</td>
<td>$9.44 \pm 0.21 \times 10^{-6}$ c</td>
</tr>
</tbody>
</table>

$^{a}$ is the average of two $K_i$ values obtained using the two inhibitor concentrations designated as $b$. The individual $K_i$ values designated as $c$, are shown ± standard deviation. ($N$) is the number of experimental runs.
Figure 10. Spectrophotometer tracing showing the inhibition of rtPA activity by D-Phe-Pro-Argininal. A) is the control; B), C), and D) were in the presence of 3.6 x 10^{-5} M D-Phe-Pro-Argininal.
DISCUSSION

Metastasis can be envisioned as a series of individual steps, although it is not necessary to assume that the tumor cells which metastasize participate in each of these. To metastasize, the cells of a growing tumor must invade through normal tissue. This would often include degradation of a fibrin capsule found around many primary tumors which is apparently formed outside the circulatory system. When the tumor cell reaches a blood vessel, or induces the formation of its own vascular system, it must degrade the basement membrane and intravasate into the circulation. The tumor cells in circulation must then attach to the endothelium or subendothelial matrix, again degrade the basement membrane, and extravasate into the normal tissue. Here the tumor cell(s) will divide and grow into a secondary tumor mass. It should be clear that the model used here only tests the hypothesized roles for proteases in the steps following the release of tumor cells into the circulation. Thus the roles of proteases in increasing the survival of tumor cells in circulation, the attachment of these cells, and the invasion of these cells into normal tissue from the bloodstream will be considered here.
The experiments reported here utilized the B16-F10 melanoma cell line, which was selected for its high potential to form lung colonies after i.v. injection. The B16 melanoma system, originally described by Fidler (1973), was chosen for these experiments for a number of reasons. Principle among these was the fact that the B16 melanoma developed spontaneously in the C57Bl6 mice which are used in the experimental model. By using a natural tumor line in a syngeneic host, no assumptions about the role of the immune system need to be made, and the model is perhaps more relevant to the natural disease condition. Also, this cell line has been used in many studies and some of the correlations/contradictions of increased protease and increased metastasis were performed on sublines of the original B16. This tumor also metastasizes spontaneously so the current study could easily be expanded to include the initial steps in metastasis. In addition, the use of mice instead of rats or guinea pigs allowed the total amount of the difficult to synthesize aldehydes to be kept reasonable.

However, the model must be considered in terms of the work proposed. The aim of this research was to test the hypothesis that certain proteases participate in the processes of attachment (thrombin) and invasion (cathepsin B, plasminogen activator) of circulating tumor cells. It is clear that following i.v. injection
of tumor cells they must first arrest in the lungs and then extravasate into the lung parenchyma in order to develop into metastases. Therefore, the model does include the relevant steps of the metastatic process under study, and agents can be tested for their effects on this process. Another aspect of the model which must be considered is the ability to determine whether or not a specific treatment is producing an effect. In this work, a change in the number of metastatic colonies would be considered evidence of an effect on the metastatic process. Therefore it is important that the method for determining the number of lung colonies be accurate and reproducible. As stated by Fidler (1978), and discussed in the references therein, most lung tumors in mice appear on the surface and can be counted with a dissecting microscope. In two of these studies (Wexler, 1966; Wood et al., 1953), detailed microscopic examination of lung sections were compared to observations of surface colonies and found to give similar results. Thus prior evidence indicates that the enumeration of surface lung colonies would give an accurate indication of the total number of metastases, and any significant changes in the average number of metastases would be detected by this procedure (for example, see Figure 11). However, the treatments used in these experiments may result in an altered distribution of metastases, either within the lung or to other
Figure 11. Photograph illustrating lung lobes dissected into lobes for counting of metastases. The upper lung (row a) contains >300 individual metastatic colonies, while the lower lung (row b) only contained 7. Animals were selected from those shown in table 5.
organs. The absence of extrapulmonary metastases was routinely verified by gross autopsies. However, in order to address the possibility of an altered distribution of metastases within the lung, two alternate possibilities have been considered. The first method, which has been used to study the initial arrest of tumor cells in organs (Fidler 1978), involves radiolabeling of the tumor cell inoculation with $^{125}$-iodo-deoxy-uridine. This procedure may have resulted in a more accurate determination of the number of injected tumor cells remaining in the lungs, and should be considered for future experiments. Another possibility when working with melanoma cells would be to use melanin as a measure of metastasis. This procedure has not been reported in the literature, and so would require preliminary evidence to verify its usefulness. Also, the presence of amelanotic nodules in some experiments (as observed in this work) may result in difficulties in interpretation or in comparisons between experiments. Melanin is also a difficult compound to extract from tissue, requiring repeated refluxing in 6 N HCL (Jimbow et al., 1984).

The number of metastases varied between control groups in the experiments reported here. This probably was a result of using animals from different vendors, using cells which had been maintained in culture for different lengths of time, and inadvertant differences in the exact stage of growth and treatment.
of the tumor cells when injected. Because the control for each experiment was always performed identically to the experimental group, this variation in average number of lung colonies should not affect the conclusions of this work. In addition, as stated above, the agents used are being tested for their ability to inhibit the attachment and invasion of the injected tumor cells. Since every tumor cell which develops into a metastases must complete these steps, it is the relative number of successful metastases between control and experimental animals which is important to consider, not the total number.

In this work, tripeptide aldehydes were used to inhibit the proteolytic enzymes cathepsin B, PA, and thrombin in an in vivo model for metastasis. These compounds have been shown to be potent and specific inhibitors of these enzymes in vitro. Treatment of animals with these inhibitors in a model for experimental metastasis would be expected to give clear evidence as to the importance of the inhibited enzymes in metastasis. For example, if leupeptin is the only compound to inhibit metastasis, it would be a strong indication that cathepsin B is involved. Alternatively, if D-Phe-Pro-Argininal is the only compound to inhibit metastasis, it would support the hypothesis that thrombin is important to the lodgement of i.v. injected tumor cells and thus is a crucial participant in the process of metastasis. If both D-Phe-Pro-
Argininal and leupeptin produce a significant reduction in metastasis, it would indicate that cathepsin B and thrombin are important to the process of lung colonization. Or if these inhibitors reduced metastasis when used at higher concentrations, plasmin might be suggested to play a role in metastasis. By using a group of similar enzyme inhibitors of known specificity, it will be possible to clearly evaluate the effects of inhibiting specific enzymes on the metastatic process. This contrasts some of the earlier studies which used general protease inhibitors or inhibitors of unknown specificity.

As noted above, cathepsin B has been implicated as an enzyme capable of much extracellular tissue destruction, including the degradation of type IV collagen. The production of high levels of cathepsin B has been shown to correlate with high levels of metastasis, and previously was believed to play a role in the coagulation and platelet aggregation of circulating tumor cells. If a cathepsin-B like protease is crucial to the metastatic process, then inhibition of its activities should lead to a reduction of metastasis. In the experiments reported here, this is clearly not the case. Using the strong cathepsin B inhibitor leupeptin, and following a protocol which was reported in the literature to result in significant reduction of metastasis, it was repeatedly demonstrated that in the B16 melanoma system used,
leupeptin produced no significant reduction (Table 2). Because the concentration of leupeptin following i.p. injection was found to be changing rapidly with time, these experiments were repeated using mini-osmotic pumps to maintain the concentration of leupeptin at a steady state level above the $K_i$ of cathepsin B but below the $K_i$ of other proteases for which there is a significant amount of evidence to support a role in metastasis. In these experiments therefore, the effect of leupeptin should be to selectively and efficiently inhibit cathepsin B. In previous work using leupeptin, the in vivo concentration was not determined. From the turnover data reported here, it is clear that following i.p. injection of leupeptin, many proteolytic enzymes would be inhibited. Thus these prior experiments (Saito et al., 1980) do not conclusively indicate the involvement of a particular protease. Again, in my experiments there was no reduction in lung colonies in the treated group versus the untreated (Table 5). Finally, both the injection and osmotic pump studies were repeated with a second potent inhibitor of cathepsin B, Ep453. This irreversible inhibitor, at the concentration used ($3 \times 10^{-7}$ M), would inhibit any soluble cathepsin B in the circulation. These experiments also failed to show any significant reduction of metastasis (Table 6). Taken together these results give strong evidence against an essential role for cathepsin B in the process
of lung colonization by B16 melanoma cells. These results are in agreement with previous experiments using leupeptin and pepstatin which also failed to show a reduction in metastasis, although these did not report the in vivo concentration of inhibitor (Giraldi et al., 1977c; Harvey et al., 1977).

There are at least two explanations for these results which would still be consistent with a role of cathepsin B in metastatic processes. First, the cathepsin B may not be completely inhibited by the circulating drugs. This may be possible if the circulating tumor cells attach to the substrate to be degraded first and then use a membrane bound form of cathepsin B to degrade it. For example, the cells may attach to the endothelium of small capillaries in the lungs and then utilize a membrane bound form of cathepsin B to degrade the basement membrane. Liotta and others (Terranova et al., 1984) have shown the importance of tumor cell attachment to the process of metastasis. It has also been shown (Quigley et al., 1986) that when transformed cells are grown in contact with a normal subendothelial matrix, their degradation of this matrix cannot be completely inhibited by small protease inhibitors. In addition, under certain experimental conditions, enzymes are not susceptible to inhibition by protease inhibitors (Campbell et al., 1982; Steven et al., 1982). This could also apply to an extracellular enzyme secreted locally at the point of
degradation. In these localized extracellular spaces, the cathepsin B may be extremely resistant to soluble inhibitors. This could be due to the close localization of substrate and enzyme, which has the effect of increasing the substrate concentration. Because peptide aldehydes act as competitive inhibitors, at a high enough substrate concentration, their effect can be negated. Leupeptin has been shown to be ineffective at inhibiting B16 melanoma invasion in the amnion model at concentrations 100-fold higher than the $K_i$ for UK, but antibodies against UK are effective inhibitors in this system (Rifkin, 1986). However, leupeptin has been shown to be effective at inhibiting intracellular degradation of proteins, and especially intralysosomal proteins (Salminen et al., 1984). The substrate concentrations in the lysosome would be expected to be quite high, and therefore it is unclear as to how an extracellular enzyme would not be inhibited at the levels of leupeptin ($3 \times 10^{-6}$ M) used in this experiment (Table 5). In addition, leupeptin has been shown to be effective at reducing protein degradation in models for muscular dystrophy (Sher et al., 1981) at much lower doses than those used here. The use of two inhibitors with different mechanisms of action and properties also argues against the suggestion that cathepsin B is not inhibited in these experiments. A second possibility is that other enzymes, such as
PA, can degrade the same substrates as cathepsin B. For example, even if cathepsin B is inhibited 99%, tumor cell secreted UK may still be able to activate collagenase and degrade the basement membrane so that the overall process of metastasis is not inhibited. In this case increased cathepsin B might be involved in metastasis but may not be essential, or may not be involved in the rate limiting step. Another alternative is that increased release of cathepsin B-like enzymes may just be a phenotypic aberration of tumor cells. In addition, these experiments do not rule out a role of cathepsin B in the growth, invasion, or metastasis of the primary tumor. It is interesting to note that in the experiments which utilized injections, we did in one example note a statistical decrease in lung colonization, and consistently observed a reduction in the average number of lung colonies. It can be postulated that the high initial concentrations of leupeptin are inhibiting other proteases which are essential to the metastatic process. However, because the concentration of leupeptin drops rapidly, the enzyme is not inhibited long enough to significantly reduce metastasis. This might provide a partial explanation for the disagreement between the data reported here and that of Saito et al. (1980). Saito et al. (1980) (using a different tumor line) report that leupeptin administered i.p. resulted in a decreased number of pulmonary
emboli following i.v. injection of tumor cells, and attributes this to the inhibition of thrombokinase. At the high initial concentration of leupeptin, factor Xa would be inhibited, as well as other enzymes. The effect of leupeptin on metastasis at concentrations higher than those used here should be investigated further. In other experiments, leupeptin has had mixed effects, but the concentrations in vivo were not previously reported so that any conclusions from these experiments must be considered speculative.

Treatment of experimental animals with the specific thrombin inhibitors R-D-Phe-Pro-Argininal by the i.p. protocol resulted in no significant change in the number of metastases, whereas administration of these compounds by the continuous infusion method resulted in a dramatic and significant increase in metastases. Again referring to the data of Saito et al. (1980), inhibition of thrombin activity would also be presumed to block the action of factor Xa on increased fibrin formation. The observation of an increased number of pulmonary nodules after treatment with a thrombin inhibitor contradicts a large body of evidence which supports the idea that fibrin formation and platelet aggregation are important steps in the process of successful blood borne metastasis. However, as discussed above most of the previous studies used coagulation inhibitors which had
many other effects in addition to inhibition of thrombin activity. Also, tumor cells have been shown to attach to endothelial cells, subendothelial matrices, and aggregate platelets (Tohgo et al., 1985) without the formation of fibrin. Fibrin formation therefore may be a consequence of other properties of metastasizing tumor cells, again without being essential to the process. It has been proposed that fibrin around a circulating tumor cell may be part of the host response in an attempt to keep the tumor cell from extravasating. Fibrin could then be proposed to play a similar role of preventing invasion of the circulating tumor cell and of the primary tumor. It is also possible that thrombin has other suppressive roles in metastasis so that inhibition of this enzyme leads to a promotion of experimental metastasis. Thrombin can not only catalyze the formation of fibrin and stimulate the secretion of inhibitors of fibrinolysis, but when bound to thrombomodulin (an endothelial cell surface protein found throughout the circulation (Ishii et al., 1986)) can also cause the activation of the fibrinolytic pathway. However, thrombin bound to thrombomodulin is rapidly internalized and degraded, and an inhibitor of activated protein C has also been described (Esmon, 1983). In addition, Gelehrter and Sznycer-Laszuk (1986) showed that the direct effect of thrombin on endothelial cells was an increased secretion of PA inhibitor relative to PA, and hence a
decreased fibrinolytic activity. Also, some of the evidence which supports an enhancing role of thrombin and fibrin formation in metastasis is based on the direct observation of fibrin-containing tumor emboli in the target organs. Thus although the many diverse actions of thrombin can not be ignored, its net effect in the process of blood-borne metastasis appears to be the formation of fibrin and a tumor cell embolus. Therefore, the net effect of a thrombin inhibitor should be to inhibit the formation of fibrin and tumor cell emboli. In the experiments reported here, the continuous inhibition of thrombin by infusion of D-Phe-Pro-Argininal at a steady-state concentration of $4 \times 10^{-7}$ M resulted in a significant increase of lung metastases following i.v. injection of tumor cells (Table 8). This would indicate that thrombin and presumably fibrin formation have an inhibitory effect on metastasis.

It would be informative to examine the lungs of mice treated with D-Phe-Pro-Argininal at various times after the injection of the tumor cells, and compare the number of tumor emboli and the amount of associated fibrin with that found in untreated mice. Alternatively, the D-Phe-Pro-Argininal compounds may be having other effects. For example, the inhibition of thrombin may be resulting in a damaged endothelial cell layer, which would facilitate greater tumor cell attachment and invasion. In future
investigations, monitoring of the coagulation state of the animal may be useful to obtain a balance between inhibition of tumor cell attachment and other possible effects on the animal. Further research on the in vivo effects of this compound is also necessary.

The experiments using the R-Glu-Gly-Argininals to inhibit UK are not as conclusive as the experiments in which inhibitors of thrombin and cathepsin B were used. Because of the high $K_i$ values, large amounts of these drugs had to be injected and this may have produced toxic effects. In addition because of the large amounts of these compounds needed and the limited capacity of the osmotic pumps, the osmotic diffusion pumps could not be used which resulted in a much shorter period of drug treatment in these experiments. However, if UK were required for tumor cell metastasis in this initial period (1.5-2 days after tumor cell injection), it would be expected that the concentrations of UK inhibitors used in this experimental procedure would have resulted in a reduction in metastases. In the t-boc-Glu-Gly-Argininal experiment (Table 9), the number of lung colonies was similar in the treated and control groups, and in the (tfa)Glu-Gly-Argininal experiment there was a significant promotion of metastasis. The free compound appeared to produce more toxic effects than the t-boc compound, and so this promotion of metastasis should be
considered with caution.

The failure of UK inhibitors to reduce metastasis in these experiments can be explained by the same rationale used to explain the lack of an effect of cathepsin B inhibitors on metastasis (see above). Again, UK has been shown to exist in an active membrane bound form which may be resistant to inhibition after the cell is bound to the extracellular substrate (Vassalli et al., 1985). In addition, only a small amount of active UK is necessary to quickly activate a significant amount of plasminogen, especially if the whole process occurred on the surface of a fibrin clot surrounding a tumor cell. Also, PAs may not be essential to tumor metastasis, but may play a role which can be fulfilled by another substituting protease, such as cathepsin B. These experiments also do not address the participation of PAs in the invasion and spread of the primary tumor.

A further complication of these experiments comes from the demonstration that the B16 melanoma produces both the tPA and the UK PA. Although melanoma cells seem to be unique in their increased production of tPA, and UK is generally considered to be the enzyme involved in tissue degradation and the invasion and metastasis of tumors, this is partially an assumption and must be proven. In the experiments reported here, the tPA from the melanoma cells would not have been significantly inhibited even at
the high doses of inhibitor used. Thus it may be that the B16 melanoma uses the tPA to facilitate extracellular degradation and metastasis, or it may use both, with either enzyme alone being sufficient to complete the metastatic cascade. The experiments reported here would still indicate that UK is not an essential participant in the lung colonization process.
The production of PAs by the B16 melanoma has been correlated to the metastatic potential of this tumor cell line (Wang et al., 1980). There have also appeared contradictory reports which show a lack of correlation (Nicolson et al., 1976). Because of the strong evidence for a role of PAs in processes of invasion and metastasis, it was of interest to investigate the PAs produced by the B16 melanoma in culture. It was determined, by zymography and antibody studies, and confirmed by results from other laboratories (D. Belin, personal communication), that the B16 melanoma in culture produces both types of PAs, the tissue and urokinase type. In earlier experiments, i.e. Figure 3, recovered activity appeared to be largely UK type. In more recent experiments, (Figure 5), approximately equal amounts of both types of enzyme were present. This could represent a change in the phenotype of the cells due to the time which they have been maintained in culture or this could be due to changes in the procedure which resulted in preferential recovery. Other researchers have also reported varying amounts of synthesis of the two types of enzymes by B16 cells (D. Belin, personal communication). This situation could account for some of the contradictory evidence reported for the correlations of PA and metastatic potential in this cell line. If UK is the enzyme active in metastasis, measurements of total cellular or secreted
PA activity would not give a proper result since these would include the tPA activity (Camiolo and Greco, 1986). Measurements of secreted enzyme may also not correlate with metastasis, because in these and other experiments there is some indication that it is the membrane-bound proteases which actually participate in the local processes of tumor invasion. This situation is further complicated since both enzymes exhibit different activities in the presence and absence of fibrin, and exist in a variety of active forms which also differ in activity. Instead, antibody studies need to be performed which correlate the metastatic potential of a group of cells directly with a specific PA activity, for example, cell-associated UK activity.

Because of the evidence suggesting that PAs are involved in the process of metastasis, it was of interest to study the PAs produced by the B16 melanoma. Inhibition constants were determined for a crude preparation of concentrated media using the UK specific substrate, S-2444 (Table 10). This substrate was shown by $K_m$ values to be approximately 10-fold better for UK over tPA, and in the concentrated media, other proteases were probably present which were not completely inhibited by the concentration of aprotinin used (30 KIU/ml). Therefore these $K_i$ values only give an estimate of the inhibition of UK-like activity by the peptide aldehydes used. It has been shown by gene sequencing
however, that the UK type enzyme is very similar between species (Belin et al., 1985). Therefore the inhibition constants obtained for human UK can be used as an indicator of the selectivity and effectiveness of the tripeptide aldehydes against melanoma UK in the in vivo experiments.

Attempts to purify the enzymes were hampered largely by the low amounts of PA present in conditioned media. In preliminary experiments, the use of a Gly-Gly-Argininal (Patel et al., 1983) column was attempted with limited success. Because of the different specificity of tPA and UK (see below) it is probable that part of this result is due to the low affinity of tPA for this sequence. For this reason, a p-aminobenzamidine column was used. Because of large amounts of secreted melanin in original SFCM preparations, it was found useful to pass the medium through a DEAE-Sephadex column prior to the affinity resin. In this way melanin and possibly other contaminants were removed and the benzamidine column could be continuously reused. Further purification of the PAs was attempted using aprotinin and soybean trypsin inhibitor affinity resins, G-25 Sephadex, and HPLC columns. Again, the low amounts of activity made these studies difficult, and in several experiments the enzyme activity was unstable.

The $K_i$ values for the partially purified PA prep shown in
Table 12 show that the tPA has a markedly different specificity for small substrates than UK. This preparation was shown to consist only of tPA by the migration pattern on SDS-gel electrophoresis and the antibody study shown in Figure 7. It was shown to be able to activate plasminogen directly by the fibrin plate and the coupled plasminogen-S-2251 assay. The lack of hydrolysis of S-2251 or of significant inhibition by aprotinin is an indication of purity, at least of plasmin or other trypsin like proteases. The activity of this preparation was completely abolished by DFP and was not altered by pCMB, evidence for the serine type nature of the enzyme. The $K_i$ values follow the same specificity pattern as those obtained for purified rtPA listed in Table 13. The $K_i$ value for DNS-Glu-Gly-Argininal agrees with the $K_i$ value of $> 9 \times 10^{-6}$ M for DNS-Glu-Gly-Arginine-chloromethylketone reported by Genetech (D. Higgins, personal communication to Dr. Schultz). It will be noticed that the absolute values differ by about an order of magnitude between the rtPA and the partially purified B16 melanoma tPA. This is possibly due to the impurity of the B16 preparation. It may also be due to differences in the amount of one chain, two chain or partially degraded enzyme present. From these data it can be concluded that the murine PAs are similar to the human and that the B16 melanoma is a relevant model for human melanoma in that
both lines produce tPA. It is also clear that the *in vivo* inhibitor studies did not involve inhibition of tPA and so the role of UK was tested independently. Further studies need to be done in order to develop different compounds to be used as specific inhibitors of tPA, so that the role of tPA and UK can be assessed independently and jointly.
SUMMARY AND CONCLUSIONS

Proteolytic enzymes are believed to have an essential role in the complex process of metastasis. Much prior evidence has accumulated which supports the theory that an increase in proteolytic enzymes results in an increased metastatic potential. However, these experiments often consist of indirect correlations, such as a comparison of the amount of protease secreted by tumor cells in culture and their metastatic potential \textit{in vivo}. In this work, direct inhibition of three of the proteinases believed to be involved (cathepsin B, urokinase, and thrombin) has been investigated as a means of clarifying the role of specific enzymes in this complex process, with the long-range goal of identifying sites of possible therapeutic intervention. Inhibitors of cathepsin B (leupeptin, Ep453), thrombin (R-D-Phe-Pro-Arginalns), and urokinase (R-Glu-Gly-Arginalns), were used to treat C57BL6 mice which were injected i.v. with B16-F10 melanoma cells to model the later stages of metastasis. These inhibitors have been shown \textit{in vitro} to be potent and selective at inhibiting the proteolytic enzymes under investigation. Inhibitors were introduced by i.p. injection or continuous infusion using Alzet mini-osmotic diffusion pumps. Injection of inhibitors resulted in rapidly
changing drug levels, while infusion of inhibitors resulted in a constant, steady-state plasma level of drug for at least 5 days. The plasma level was at a concentration specific for the targeted proteinase. This is a significant improvement over previous studies of this type, which utilized general proteinase inhibitors of undefined specificity and did not determine the inhibitors' \textit{in vivo} concentration. Under these conditions, inhibitors of cathepsin B, at concentrations orders of magnitude higher their $K_i$'s, showed no effect on metastasis by any of the protocols and dosages used. The conclusion from this work is that a cathepsin B-like enzyme is not essential to the process of lung colonization even though much \textit{in vitro} work has implied that cathepsin B is involved in metastasis. Injection of inhibitors of urokinase also had no clear effect, suggesting that secreted UK may not be essential to the colonization process. However, continuous infusion of the specific thrombin inhibitor at levels higher than it's equilibrium $K_i$, resulted in a large significant increase in metastasis (300 vs. 10 lung colonies). This implies that the formation of fibrin by thrombin may have a retarding effect on the later stages of the metastatic process, in contradiction to much current literature which suggests that fibrin formation by thrombin acts to increase the attachment of circulating tumor cells in the target organ. The experiments reported here did not
address the role of proteases in the initial stages of metastasis, and further studies of this type are necessary to clearly identify the roles of proteolytic enzymes in the complete metastatic process.

Studies of conditioned medium from B16-F10 cells grown in culture revealed, by zymography and antibody tests, varying amounts of tissue type and urokinase type plasminogen activators. The tissue type plasminogen activator was isolated and compared to purified human recombinant tissue type plasminogen activator for sensitivity to inhibition by a series of tripeptide aldehydes. The two enzymes were found to exhibit similar specificity, with t-boc-D-Phe-Pro-Arginal being the best inhibitor. This differs from the urokinase type enzyme, which was most sensitive to the sequence Glu-Gly-Arginal. These results indicate the need to develop specific inhibitors for UK and tPA in order to further examine their possible roles in metastasis in vivo.
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APPENDIX A
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APPENDIX B
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Several variations of the basic procedure for collecting serum-free conditioned medium (SFCM) from the B16 melanoma were attempted. The first of these consisted of growing the cells in cycles of complete medium and serum-free medium (SFM). Thus cells were grown in complete medium until semi-confluent (as observed under the light microscope), washed in 5 ml of PBS twice, incubated in SFM for one day, after which the SFCM was collected and the cells were reincubated in complete medium. The cells maintained a healthy morphology under these conditions, but continued to increase in density when incubated in complete medium. When washed in PBS and recycled in SFM for a second day, large numbers of the cells detached from the tissue culture flask and a large quantity of cellular debris was evident in the medium. Therefore this procedure was not used, as the floating cells and debris was taken as an indication of cell lysis and death, which would release many proteolytic enzymes and other contaminants which would complicate the purification scheme. Flasks were also cycled in SFM for two days before being reincubated in complete medium, followed by another cycle of SFM with similar results.
A second possibility was to select for a population of cells which would be able to be maintained in SFM for extended lengths of time. This was attempted by culturing B16-F10 cells in complete medium until semi-confluent and then refeeding them only in SFM. These cultures approached confluency and then large numbers of the cells would release from the surface of the flask. These were removed with media changes and the cells remaining attached were re-fed in SFM. After several passages, the remaining cells (which were very scarce and no longer seemed to be replicating), were reincubated with medium containing calf serum (5% or 10%). The cells proliferated in the complete medium until a semi-confluent state was again reached, and then the selection procedure of growth in SFM only was repeated. The results were very similar to the first selection, with very few cells actually remaining attached (<10%). This procedure was abandoned for the following reasons: i) a truly stable population of cells capable of long-term culture in SFM was not identified, ii) the cells which did survive in SFM for several cycles did not appear morphologically "healthy" (small, more spindly) when compared to B16-F10 grown in complete medium, iii) when the SFCM from these cells was examined by polyacrylamide gel electrophoresis and zymography, although the amount of protein was very low, the amount of PA activity detected was also low.
The use of serum supplements was explored as a third possibility for obtaining larger amounts of SFCM efficiently. Ultroser G (LKB, Bromma, Sweden) was used for these studies because many cell types have been successfully cultured using this as a serum supplement. Ultroser G contains necessary growth and adhesion factors, hormones, binding proteins, vitamins, and mineral trace elements. B16-F10 cells grown to semi-confluency were adapted to growth in Ultroser G by first replacing one-half of their medium with SFM containing 1% Ultroser G. This was completely replaced with 1% Ultroser G in SFM after 24 hr. Under these conditions, cells maintained a healthy morphology, however the cultures did not increase in number, even with daily refeedings. Increasing the Ultroser G content to 2% resulted in increased growth of the cultures. However, this procedure was also found to be unsuccessful because of the following considerations:

i) When cultured in Ultroser G, the B16-F10 cells failed to produce notable amounts of melanin. This was a significant change from the cellular phenotype observed when cultured in complete medium. It was desirable to study the PA produced by the melanoma cells under conditions as close as possible to those used for preparing the cells for the in vivo studies. Ultroser G was thus not suitable.

ii) Electrophoresis and zymography of Ultroser G conditioned
medium revealed a significant amount of PA activity, but the activity was smeared by a large protein band and migrated slightly below the position of mouse UK. This difference was not explored further, but again indicated that the change in culture conditions was having an effect on the desired product, PA.

iii) It was not possible to maintain cells in Ultroser G for truly extended lengths of time. Thus the procedure was not much more efficient than growing the B16-F10 cells in complete media. In addition, Ultroser G became unavailable for import shortly after these studies began.
APPENDIX C

Nodules in mouse lungs which contained melanin were easily distinguishable from the normal tissue, and because of the presence of melanin, were identified as melanomas arising from the injected B16-F10 cells. However, the appearance of amelanotic nodules in some experiments required the use of another technique to verify that the nodules were in fact melanomas. As discussed by Mackay and Osborne (pg.379, 1978), the presence of premelanosomes as visualized by electron microscopy is definitive evidence which allows the tissue to be positively identified as arising from a melanoma. With the kind and generous help of Dr. Bruce Persky, samples of both amelanotic and melanotic nodules were examined by transmission electron microscopy, by techniques previously described (Persky and Chmielewski, 1986; Persky et al., 1986) and outlined below.

Mice were sacrificed by cervical dislocation and the lungs were quickly removed, dissected free of other tissues, and rapidly rinsed in phosphate buffered saline to remove debris. Samples containing amelanotic and melanotic nodules were then carefully cut from the lungs in cubes (1 mm$^3$ or smaller) and immediately immersed in modified Karnovsky fixative (1% paraformaldehyde,
1.25% glutaraldehyde pH 7.4) for 24 hr. The samples were then postfixed in 2% OsO₄ buffered in 0.072 N cacodylate buffer at pH 7.4 for 1 hr. After dehydration in increasing concentrations of ethanol, samples were embedded in Epon. Thin sections were cut from the samples and stained with uranyl acetate and lead citrate. Grids were then examined with a Hitachi H-600 transmission electron microscope.

Stage III premelanosomes were observed in all samples, therefore verifying that the nodules observed were melanomas.
The dissertation submitted by Lawrence Ostrowski has been read and approved by the following committee:

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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