Stony Brook University

Elevation of seprase expression and promotion of an invasive phenotype by collagenous matrices in ovarian tumor cells

A Dissertation Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Genetics

Stony Brook University

December 2007

Stony Brook University

The Graduate School

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Abstract of the Dissertation

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2007

Tumor cells do not constitutively exhibit invasive activity, but rather, can be transiently induced to adhere and form lesions. We report here that the expression of seprase, a dominant EDTA-resistant gelatinase in malignant tumors, is dependent on tumor cell exposure to type I collagen gel (TICg), and this interaction can be simulated by mAb C27, an antibody against β1 integrin that induces cross linking of the integrin subunits. The induced seprase expression of ovarian tumor cells influences their collagen remodeling and invasion capability. Importantly, tumor cells with low seprase expression, due to manipulation by RNA interference, showed a reduction of TICg remodeling in the gel contractility assay, inhibition of tumor cell invasion through TICg as shown by a transwell migration assay, and inhibition of peritoneal membrane lesion formation in an *in vivo* mouse model. Thus, collagenous matrices in the tumor cell niche induce the expression of seprase and initiate tumor invasion cascades.

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LIST OF ABBREVIATIONS

TICg - type I collagen gel

mAb - monoclonal antibody

RNAi - RNA interference

ECM - extracellular matrix

TIC - type I collagen

a.a. - amino acid

MMP - matrix metalloprotease

DPP4 - dipeptidyl peptidase IV

DMEM - Dulbecco's modified Eagle's medium

PBS - phosphate buffered saline

CCC - cancer cell culture

FACS - fluorescence assisted cell sorting

GFP - green fluorescent protein

RT-PCR - reverse transcription polymerase chain reaction

i.p. **-** intraperitoneal

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

BSA - bovine serum albumin

TT - Trizma-Tween-20

GFRM - growth factor reduced matrigel

$\mathbf{TIC_{FRAG}}$ - type I collagen fragment

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CHAPTER 1
Literature Review

INTRODUCTION

Seprase, or fibroblast activation protein α (FAPα), is a type II transmembrane serine protease that exhibits both dipeptidyl peptidase (DP) and endopeptidase type gelatinase activities. It is an inducible protease that is produced by stimulated fibroblasts during wound healing as well as actively invading tumor cells. A wide variety of tumor cells express this protease including melanoma, carcinoma of the breast, ovary, colon and rectum. In fact, seprase has been localized to the invadopodia of active cells and associated with both the highly homologous dipeptidyl peptidase IV (DPP4) and integrins. Although seprase has been shown to have gelatinase activity in membrane bound form, a truncated form of seprase that has been cleaved from the membrane exhibits higher gelatinase activity as compared to its integral membrane counterpart. The gelatinase activity of seprase increases with further proteolytic processing, which allows substrates greater access to the catalytic pocket. While seprase is known to be an important player in tumor cell invasion, the regulatory mechanism for seprase induction has yet to be elucidated.

SEPRASE

Structure

Seprase is a type II transmembrane, serine endopeptidase and was first described as a gelatinase of the invasive LOX human melanoma cell line.[1] It is highly conserved between humans and mice, and homologs are found across a diversity of species.[2-4] The 760aa, 97kDa monomer homodimerizes to form a 170kDa competent gelatinase via the formation of a pocket containing a catalytic triad of non-classical order comprised of Ser624, Asp702, and His734.[5,6] Seprase contains six major domains, two of which are quite short in that both the cytoplasmic and transmembrane domains together are only 26aa, and the remaining stalk, glycosylation rich, cysteine rich, and catalytic domains

make up the remaining 734aa.[5] The N-terminal cytoplasmic domain is very short and, therefore, is unlikely to have a role in signal transduction. The bulk of this protein is extracellular and its cell surface placement brings it in contact with the molecules of the extracellular matrix (ECM). Seven glycosylation sites fall within the glycosylation rich domain, and, in similar proteases, glycosylation is thought to play a role in the ability of this protease to homodimerize.[7] There are conflicting reports of the glycosylation requirement (see DPP4 section) and even the crystal structure did not clarify this domain's role in the formation of the tertiary and quarternary structure, nor was its role in enzymatic activity revealed.[8] The elucidation of the crystal structure of the extracellular domain did determine the homodimer to be 60Å x 80Å x 125Å, and the dimensions of the catalytic pore created by the homodimerized subunits were shown to have a length of ~27Å and a diameter of ~14Å; however, there is a side aperture that has a wider ~24Å opening through which a molecule could gain access to the hydrophilic catalytic triad.[8]

Enzymatic activity

Initially discovered due to its association with LOX cells and its ability to degrade gelatin and contribute to the invasive phenotype of this cell line, seprase is also involved in transient processes like endothelial cell migration during tubule morphogenesis in type I collagen (TIC).[1,9] High levels of seprase expression have also been found in metastatic colorectal carcinoma, gastric carcinoma, and others.[10-12] It is important to note that while the LOX cell line constitutively expresses high levels of seprase, the vast majority of tumor cells require an induction to up-regulate this protease beyond basal levels. Seprase is up-regulated in myeloma cells when cultured in the presence of bone marrow mesenchymal stem cells, but not in myeloma cells alone.[13] Processes involving tissue remodeling also appear to be influenced by seprase such as tail resorption in *Xenopus laevis* during development as well as during fibroblast remodeling in cases of idiopathic pulmonary fibrosis.[3,14] In contrast, seprase null mice had no overt phenotype and restricted expression was shown during early murine development so it does not appear to play an essential role in embryonic tissues where TIC is less abundant.[15]

Importantly, previous investigations of the gelatinolytic and DP activity of seprase isolated the dimers via detergent extraction. While effective for separating

proteins from a membrane, detergents also have the ability to loosen dimeric associations; therefore, exposure to detergent not only eliminates the steric hindrance created by the membrane, but also broadens the catalytic pocket of seprase thereby facilitating access to the active site resulting in artificially inflated gelatinase activity. The crystal structure of seprase as solved by Aergeerts and colleagues, indeed, shows a ~24Å opening through which entrance to the catalytic site can be gained as well as an additional opening of ~14Å in diameter that is ~27Å long.[8] Dimensions of access need to be considered when understanding the gelatin substrate upon which seprase acts *in vivo*.

TIC was initially described as three coiled strands of gelatin that wind around each other in a regular pattern, which is ensured by the placement of glycines every third residue, and the hydroxyprolines participated in the formation of a coiled coil structure.[16,17] A distinction was made between the structure of hydrated collagen and dried forms in that hydration causes the lattice to expand due to the placement of water within the triple helix. Hydration coupled with physiological temperatures supports the notion that, in solutions, once TIC is secreted from cells, it uncoils and is quite loosely associated until needed to form fibers.[18] When the three fibers are coiled into a triple helix, it is estimated to be approximately 10 nm, and once wound together with other triple helices, TIC can form a ~300 nm molecule.[19] Originally developed as a wound healing/skin graft application, it was noted that fibroblasts grown in hydrated collagen and are able to reorganize and condense the protein to form tight disks.[20] Rate of formation of the disk increased with cell number, but was inversely related to collagen concentration.[20] This was later applied to muscle cell collagen remodeling and seems to be a potentially useful tool to measure the tissue remodeling capability of tumor cells, specifically those within a collagen rich environment.[21]

Although seprase was originally touted as a membrane bound protease, recently, shortened forms of seprase, created through cleavage of the transmembrane dimers from the cell membrane, were detected in melanoma, invasive carcinomas of the colon, stomach, breast, and ovary and additionally in a number of ovarian carcinoma ascites samples.[22,23] These shortened forms appeared be processed even further while still maintaining the proteolytically active dimer formation and actually increasing their activity by heightening the accessibility of the substrate to the catalytic site.[22]

Gelatinolytically active, soluble forms of seprase also exist in bovine serum and most recently were identified in human blood samples.[24,25] Although some have suggested that a soluble form of seprase may induce unbridled destruction of tissues, it is important to note that seprase expression is transient and inducible, likely requiring numerous other molecules to be activated and appropriately localized to a substrate.

DPP4

Like seprase, DPP4 is as type II transmembrane serine protease that requires homodimerization and the creation of a catalytic pocket comprised of Ser-Asp-His non-classical triad for enzymatic activity. Because of its broad localization, DPP4 has correspondingly extensive substrate specificity. It is known to act on denatured collagen types III, V, I, II and IV (in order of decreasing activity),[26] the sympathetic neurotransmitter neuropeptide Y,[27] RANTES[28] and others. In an effort to make clear this distinction, many groups have utilized amino-terminal, Z-blocked, short peptides to measure and define the endopeptidase activity of seprase.[8,24,25,29] While there is high homology between the catalytic regions of DPP4 and seprase, it has been shown that the catalytic pocket of DPP4 is far more acidic and negatively charged, and therefore more hospitable to the amino terminus of potential substrates as compared to that of seprase. The Arg657 residue seems solely responsible for the unwelcoming environment in that when this residue was mutated to Asp there was a ~60 fold increase in its exopeptidase activity with a coincident ~350 fold decrease in its ability to cleave Z-blocked amino terminal short peptides.[8]

Beyond the DP activity of the catalytic triad, additional proteolytic capability was determined through a series of point mutations across a highly conserved portion of DPP4, and it was discovered that two glutamic acid residues (205 and 206) were also necessary for catalytic activity and may play a physical role in substrate specificity in that they bind to a positively charged residue on the substrate and exclude those that did not match.[30] A further series of mutation experiments were conducted to elicit a biochemical, but not structural, change to the catalytic pocket and found that the oxyanion hole created by the hydrogen binding between Tyr 547 and Tyr 631 is just as necessary for catalytic

activity as the triad.[31] The effects of structural changes induced by carefully mutating each one of nine glycosylation sites on the DPP4 extracellular domain were tested in an effort to influence the homodimerization capability of DPP4 and, although it was thought that glycosylation was necessary for dimerization and catalytic activity, these mutations did not have any influence on DPP4 activity.[32] While seprase and DPP4 are similar in many regards, this structural sensitivity has not been studied exhaustively, and it has yet to be determined if this type of glycosylation is necessary for seprase activity.

In cell models of gene expression, DPP4 and seprase, when in catalytically mutant forms, are also thought to act as tumor suppressors. The proteolytically inactive proteins suppressed the transformation of cell lines and inhibited cell line-derived tumor growth that is supported by stromal cells.[33-35] However, knockout animals did not show any change in susceptibility of tumor development, which suggests against a role in tumor suppression.[36] Further support for this idea was shown through the detection of seprase and DPP4 in various solid tumors at greater levels than that found in normal tissues.[10-12,22,37-40]

INHIBITORS OF THE SERINE PROTEASES

Because of their respective roles in tumor cell invasion and metastasis, it has long been recognized that DPP4 and seprase are prime targets for pharmacological inhibitors.[41,42] If Ser624 of the catalytic triad is mutated, the DP and gelatinase activity of seprase is abolished.[43,44] The importance of this amino acid was exploited by Adams and colleagues when they devised a small molecule inhibitor, PT-100 (Val-boro-Pro) directed against seprase, which specifically bound Ser624, and elicited anti-tumor effects via a T-cell immune response in the mouse model.[45] Other efforts have yielded a promising new molecule Gly-Pro^{P}(OPh)$_2$ that appears to be highly effective against DPP4, effective against seprase, and was able to dose dependently and irreversibly inhibit the tremendous invasive capability of LOX cells.[46]

The nearly ubiquitous DPP4 is involved in a wide variety of systems; therefore, diverse disciplines are evaluating the efficacy of inhibitors to this serine protease including type II diabetes,[47,48] neuromodulation,[49] and hematopoesis[50] applications. Due

to its known roles in type II diabetes, HIV infection, and ECM degradation during tumor cell invasion, a group of investigations have focused on identifying the bonds formed between DPP4 and its substrate via crystal structure resolution in an effort to design appropriate small molecule inhibitors. Substrates that are non-cleavable such as nonapeptide-Tat,[51] the slowly hydrolyzed diprotin A,[52,53] as well as the actual small molecule inhibitor valine-pyrrolidide,[54] have all been used to examine the enzyme-substrate interaction.

METASTATIC CELL ADHESION AND INVASION

To metastasize, tumor cells must adhere to and then digest the ECM, which facilitates invasion into the blood or lymph to complete the intravasation process. These cells must survive a gauntlet in the blood vessel, from withstanding shear forces to evading various proteins and cell types. If the cell endures the challenges presented within the vasculature, it is faced with adhering to vascular endothelial cells and subsequently confronted with the extravasation process. Cells that fail to arrest within the vasculature are cleared from the circulatory system. Tumor cell adhesion and invasion require both the adhesive and receptor qualities of the integrins as well as ECM digestion and remodeling capabilities of the proteases.

Adhesion

The integrins mediate metastatic tumor cell arrest, in part, via reestablishment of integrin-matrix interaction. In their function as adhesion molecules, they are able to link internal cell signaling to the outside environment comprised of other cells, the ECM, and various ligands. Because of the large number of type I transmembrane α and β subunits that can pair in a non-covalent association, the permutations formed can contribute to highly specific extracellular substrate binding as well as signal transduction.[55] Signal transduction through the integrins is rapid as is required in the case of platelet activation via collagen-integrin interaction; specifically, αvβ3 plays an important role in this process and assist others such as leukocytes and platelets in the arrest of tumor cells when circulatory flow was simulated.[56,57]

Extracellular matrix interaction with integrins is able to set off an intracellular cascade of regulatory events that direct subsequent protein localization. For example, in a study involving a colon cancer cell line that expresses high levels of Src, β1 integrin is known to play a role in signaling and mediating cell invasion in that β1 integrin is usually distributed evenly around the perimeter of this cell type; however, elevated Src levels cause it to form clustered regions in contact with the substrate and, when bound to collagen, β1 integrin regulates focal adhesion kinase (FAK), which itself has no receptor function, but due to its association with β1 integrin undergoes tyrosine phosphorlyation via Src while, concurrently, this high Src kinase activity disrupts E-cadherin, which plays a role in cell-cell adhesion.[58] When adding an antibody against β1 integrin, it inhibited the formation of these foci consisting of integrins, FAK and others, so it appears that interruption of the β1 integrin-collagen connection prevents this signaling pathway and, by extension, helps to maintain cell-cell contacts and thwarts the formation of focal adhesions, which have been shown to play an integral role in tumor cell invasion and metastasis.[58] Also, FAK was found to join the E-cadherin adhesion complex when the β1 integrin surface receptor is in the presence of TIC and initiate an intracellular signal that increases the tyrosine phosophorylation of β-catenin via Src kinase.[59] β-catenin undergoes nuclear translocation which effects β-catenin-Lef/Tcf target genes, among others, and this coincides with the disassociation of the E-cadherin complex.[59]

Invasion

Integrins, comprised of α and β transmembrane proteins, are heterodimeric receptors that are induced by ECM molecules to undergo a conformational change that triggers a intracellular cascade of signaling events that can ultimately lead to transcriptional activation. In malignant tumor cells, the integrins must be regulated in a switch-like manner so that they are first activated and able to associate with other tumor cells in a primary lesion setting, then deactivated so that attachment is released to enable metastasis and, finally, reactivated once again to facilitate invasion and formation of secondary lesions all while avoiding apoptotic triggers.

What initiates this cycle of events or contributes to regulation of each stage has not been entirely elucidated; however, great strides have been made towards

understanding this critical process. The α3β1 integrin was shown to be present on hepatocellular carcinoma invasive cells, but not on non-invasive cells, and this can be manipulated by the powerful cytokine, TGF-β1, in that it is able to effect a change in non-invasive hepatocellular carcinoma cells through the induction of α3 integrin transcription thereby inducing an invasive phenotype, all of which is supported by correlations to patient samples.[60] Interactions between a functional α3β1 integrin and the mesenchymal ECM has also been shown to initiate cell movement and position change in both tumor invasion settings of multicellular aggregates as well as tissue morphogenesis.[61] The multicellular aggregates release their cell-cell interactions facilitated by E-cadherin junctional complexes when presented with a substrate of ECM molecules such as TIC, fibronectin or laminin 1. E-cadherin helps to form the cell-cell junction in the aggregates, but when the cells are plated in a monolayer, E-cadherin expression falls off dramatically.

It is evident that the release of adhesion junctions is not solely responsible for tumor cell invasion and a variety of proteases are required to digest surrounding extracellular matrices to provide a path for metastatic tumor cells. This is supported by a study of maspin, the serine protease inhibitor, showing it to be a tumor suppressor because it is down-regulated in carcinoma cells and also is able to inhibit tumor growth, cell migration, cell invasion, and angiogenesis through its maintenance of ECM-cell contact via its physical and functional association with β1 integrin.[62] There are other players involved with these physical associations as well as signaling regulations, so research into this area will fill a critical void in our understanding of cell adhesion and tumor cell invasion.

ECM-PROTEASE SIGNALLING INTERACTIONS

We have already suggested that seprase is a transient and inducible protease and it has also been found that seprase associates with α3β1 integrin on the invadopodia of actively invading tumor cells[63] or fibroblasts during wound healing,[64] yet the regulatory connection between seprase and integrins remains unclear. While seprase has a short cytoplasmic tail comprised of only 6 amino acids and is unlikely to have a signal

transduction role, the β1 integrin that it is associated with has a large cytoplasmic region which is known for its ability to regulate intracellular signaling pathways.[65] Also, not only is α3β1 localized to areas of gelatin degradation, but may act as a receptor to facilitate ingestion of gelatin and accompanies the gelatin into the lysosome.[66] Antibodies developed against the α3 integrin subunit stimulate malignant human breast cancer cell phagocytosis of gelatin in a dose dependent manner while anti-β1 mAb's, although stimulatory, had a more moderate effect, and antibodies developed against a variety of α subunits did not promote phagocytosis at all.[66] This suggests that these antibodies may mimic the ligand that is required for phagocytosis. It is important that these integrin receptors surround the cells so that the extracellular environment can be sensed and an appropriate signaling response can be elicited.

In actively invading tumor cells it appears that available gelatin induces the production of gelatinases such as seprase. An investigation focused on LOX cells, which are unique in that they constitutively express very high levels of seprase, found that dimerized α3β1 integrin can not only colocalize, but directly associate with the seprase dimer at the invadopodia; however, this only occurs in the presence of TIC, but not when the cells are plated on a plastic surface.[63] Because the vast majority of cells do not express this high level of seprase, it is possible that LOX cells are an anomaly and in cells that do not otherwise express it, seprase can be induced via interaction of α3β1 integrin with TIC. Additionally, a study examining gene expression via cDNA microarray of aggressive and dedifferentiated forms of papillary thyroid carcinomas determined that, among others, both seprase and TIC (α2) expression was markedly increased as compared to the classic, well-differentiated version of this carcinoma; however, DPP4 was highly expressed in both aggressive and non aggressive forms.[67] Furthermore, the binding of α3β1 integrin to TIC, which stimulates an intracellular signaling pathway, could effectively link TIC to the invadopodia and cytoskeletal elements while seprase is being produced and directed to the area of TIC binding. This hypothesis is supported by other similar cases, specifically the conscription of MMP-2 by αvβ3 integrin in the CS-1 melanoma cell line[68] and colocalization of β1 integrin with FAK.[69]

EXPERIMENTAL MODELS

Visualizing formation of metastases

An inherent problem to the study of secondary lesions and micrometastases is that they are, by definition, small. The easily-palpable primary tumor can be readily visualized, measured and excised; however, the micrometastatic lesions can be slightly more challenging. Boons to this area of investigation, such as whole body fluorescence imaging[70-72] and positron emission tomography,[73] have become important tools enabling researchers to visualize *in vivo* cancer progression in xenograft models of many types of malignancies such as prostate cancer progression,[72] glioblastoma,[74] osteosarcoma,[71] breast carcinoma,[75] and others via cell labeling techniques.

The mouse model has been a great tool in the evaluation of therapies attempting to inhibit tumor progression, metastasis, and secondary nodule formation. Non-invasive techniques utilizing MRI and fluorescence imaging have allowed for longitudinal studies to be conducted using the same cohort of mice across the length of the study thereby eliminating the need for termination at time points. This is not only useful for the consistency in following a single animal throughout the study, but also increases the efficiency and financial feasibility of conducting studies involving multiple anticancer therapies and combinatorial permutations thereof. An example of this kind of technique was applied to a human ovarian carcinoma/nude mouse xenograft model and the efficacy of cisplatin to combat disease progression induced by SKOV-3 and NMP-1 cells delivered via intraperitoneal injection. Animals were serially imaged using T_2 MRI's of the abdomen in an effort to predict survival of treated mice compared to the control group.[76] In the mouse model system, however, and especially in the xenograft application, cell lines that stably express a unique marker, such as GFP or RFP, will be particularly suitable for longitudinal studies looking to visualize disease progression from its inception and primary tumor formation through metastasis and in the final stages of secondary lesion formation. This will also provide the needed internal marker to subsequently separate the wheat from the chaff via fluorescence assisted cell sorting and yield a homogeneous population of tumor cells from which to derive a genetic signature without confounding input from stromal cells or other cells or molecules found in the

tumor and surrounding area. The pros and cons of several experimental systems for modeling cancer development and metastasis have been thoroughly reviewed.[77]

In vivo model of the seprase-TIC connection

An ideal model to investigate the interaction between seprase and TIC *in vivo* might involve late stage ovarian carcinoma ascites development and peritoneal membrane lesion formation. Peritoneal mesothelial cells produce TIC and this is up-regulated *in vitro* in response to conditions that imitate ascitic fluid accumulation and cell proliferation similar to that found in late stages of ovarian cancer.[78] As previously mentioned, in solution and at physiological temperatures, once a cell produces and excretes the collagen it is no longer in triple helix form, but rather it is in an unwound gelatin state.[18] The generation of available substrate may create a stimulatory environment for seprase production within the ovarian carcinoma cells. Subsequent gelatinase acitivity may not only occur on the substrates found in solution, but may also act on the open surfaces of the omentum and facilitate adhesion and invasion of ascites cells.

Support for this interaction in ovarian cancer is shown in several investigations of ascites development and the behavior of multicellular spheroids. Spheroids, the tight aggregates of ovarian carcinoma cells derived from ovarian cancer patient ascites samples, are an adherent population of cells when exposed to different components of the ECM, specifically fibronectin and TIC, while monoclonal antibodies directed against the β1 integrin subunit interfered with this interaction.[79] This same group investigated the disaggregation, adhesion and invasion of spheroids in patient samples as well and found that type IV collagen and TIC increased the incidence of spheroid disaggregation.[80]

An investigation into the molecular mechanism of peritoneal dissemination and secondary lesion formation outlines other possible players in connection with seprase activation via TIC binding to α3β1. The study involved the HEY and OVCA 433 ovarian carcinoma cell lines and showed that endothelin-1 binds to the endothelin A receptor thereby up-regulating both α3β1 and α2β1 integrins, which bind to TIC.[81] In addition, TIC binding to β1 integrin stimulates integrin-linked kinase mRNA production and activity, which, in turn, acts to phosphorylate targets such as AKT and glycogen

synthatase kinase-3β whose downstream effectors include the activation of MMP-2 and MMP-9 as well as the uPA system.[81] β1 integrin has also been linked to FAK in a signaling complex with vSrc, which together promotes cell invasion.[69]

A novel role for the DPP4/seprase complex has been elucidated after the examination of endothelial-derived human invasive breast ductal carcinoma cells lining newly formed capillaries revealed the DPP4/seprase complex, but it was not localized to the major established vessels suggesting a role in the early stage of angiogenesis *in vivo*.[82] This same study went on to demonstrate, through the use of a monoclonal antibody against its gelatin binding domain that inhibited the receptor site, but not the catalytic domain, the integral role of the DPP4/seprase complex in localized gelatin degradation and wound closure within the context of the matrix metalloproteinase, plasminogen activator, and type II transmembrane serine protease systems.

Unlike the almost ubiquitous expression of DPP4, the transient nature of seprase expression and specific localization pattern clearly indicates a precise regulatory mechanism. The trigger and the signaling pathway that induces the up-regulation of seprase and preferentially shuttles it to the invasion and migration fronts of invading cells is not entirely clear. It does appear that there is a complex series of events and multiple players involved in this process, the characterization of which could contribute greatly to the treatment of ovarian tumor progression, metastasis, and secondary lesion formation.

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CHAPTER 2

Elevation of seprase expression and promotion of an invasive phenotype by collagenous matrices in ovarian tumor cells

INTRODUCTION

Despite efforts to hone existing tools and develop new diagnostics, ovarian cancer continues to elude early detection and remains one of the deadliest forms of gynecological cancer.[1,2] Epithelial ovarian cancer progresses by increased proliferative and ECM invasive activities of tumor cells, invasion of the submesothelial membrane, and formation of secondary lesions on the peritoneum.[3,4] Because early detection remains elusive, to prolong patient survival, treatments must inhibit tumor cell invasion to prevent colonization at secondary sites. Although the inhibition of proteases that promote tumor cell invasion and remodeling of the ECM are good targets for combating lesion formation, better still is preventing the initial induction of these proteases.

Seprase is found on actively invading tumor cells, but not inactive cells and is a key player in ECM remodeling during tumor cell invasion in a number of cancer types; however, its role in secondary lesion formation in ovarian cancer is not entirely clear. Type I collagen (TIC) is comprised of three strands of gelatin each of which contain adjoining glycine and proline residues.[5] It is this bond that is the known substrate for seprase.[6] Seprase, also known as fibroblast activation protein α, was first described in the LOX human malignant melanoma cell line and is a type II transmembrane, 760aa glycoprotein whose 97kDa monomer homodimerizes to form a catalytically active complex of 170kDa.[7-9] It is likely that activation, which involves the seprase dimer being shorn from the cell membrane to allow greater accessibility of its TICg substrate to the catalytic pore, is required prior to gelatinase activity.[10,11] These shortened and soluble forms of seprase have been detected in bovine serum, blood, and invasive carcinomas of the colon, stomach, breast and ovary, suggesting that seprase may not only act on gelatin, but on anti-fibrinolyis inhibitors as well.[10,12,13]

Seprase is known to be transiently expressed in a malignant tumor, but the mechanism of induction is not well characterized. In biologically relevant systems, seprase is not constitutively present on the surface of inactive cells, but rather is produced only in active cells. Because the peritoneal cavity is a TIC rich microenvironment, we hypothesized that seprase expression may be triggered by its TIC gelatin (TICg) substrate in ovarian carcinoma cells, which leads to promotion of lesion formation. In this study we not only aimed to elucidate the trigger of seprase induction, but characterize its contribution to lesion formation in an ovarian cancer model as well. We used the SB247 cell line, derived from ascites of a patient with stage IIIB serous adenocarcinoma, and showed that the invasive propensity of the SB247 cells is triggered by TIC stimulation, but not other ECM molecules. Furthermore, seprase production is enhanced in both intensity and rapidity when cells are exposed to the gelatin form of TIC. A reduction in seprase expression via RNAi has revealed a corresponding reduction in tumor cell invasion and TICg remodeling capability and is corroborated with an *in vivo* reduction in metastatic lesion formation on the peritoneal membrane of a late stage ovarian cancer mouse model. Additionally, our experiments using the mAb C27 against β1 integrin suggests the induction of seprase is transduced through the clustering of β1 integrin subunits. Overall, we show that the SB247 cell line interaction with TIC induces seprase expression, which facilitates tumor cell adhesion and invasion into the mesothelial layer lining the peritoneal membranes and leads to lesion formation. This investigation supports the notion that seprase is a major player in lesion formation and that prevention of β1 integrin clustering on tumor cells may inhibit tumor cell invasion and therefore may prove to be a useful tool in the treatment of late stage ovarian cancer.

MATERIALS AND METHODS

Cell lines

The SB247 cell line was derived from an ascites sample obtained from a patient with ovarian serous adenocarcinoma. Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, New York) and RPMI 1640 (Gibco) supplemented with 10% calf serum (Gibco), 5% Nu-serum (Collaborative

Research, Inc., Bedford, MA), 2 mM L-glutamine (Gibco), 1 unit/ml penicillin and 10 µg/ml streptomycin (Gibco) together called CCC media at 37ºC under 5% CO_2 in a humidified incubator. The SB247 cell line was used to generate several monoclonal subpopulations by transfection via Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) with the pGUS vector with different RNAi oligonucleotides (Donghai Chen and Wen-Tien Chen, unpublished data). The oligonucelotide hairpins used were 1384 (forward: accGAAAGGTGCCAATATTACACaagcttgGTGTAATATTGGCACCTTTCtttttt-3'; reverse: 5'-catgaaaaaaGAAAGGTGCCAATATTACACcaagcttGTGTAATATTGGCACCTTT-3', or a no target control hairpin that targets no sequence in the human genome (forward 5'-accGATTCGCGGGACCGATCGTGaagcttgCACGATCGGTCCCGCGAATCttttttt-3'; reverse: 5'-catgaaaaaaGATTCGCGGGACCGATCGTGcaagcttCACGATCGGTCCCGCGAAT-3'. SB247 cells are resistant to multiple antibiotics, which made selection for stable transfectants difficult. Therefore, polyclonal populations were grown in CCC, trypsinized (Gibco) and resuspended in PBS (Gibco) for Fluorescence Assisted Cell Sorting (FACS) to select for the greatest GFP intensity for each cell line. A subpopulation of stably transfected GFP-expressing cells then underwent a limited dilution to isolate monoclonal populations. Furthermore, FACS analysis was used to determine strength of GFP expression of the monoclonal populations (Sup. Fig. 2*b*).

RT- PCR analysis

Seprase expression in cells was measured with RT-PCR. In all cases, RNA extraction was conducted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. Immediately after RNA extraction, samples were converted to cDNA using 1st Strand cDNA synthesis for RT-PCR (AMV, Roche Applied Science, Indianapolis, IN) according to manufacturer's protocol and then stored at -20ºC for later RT-PCR application. Briefly, the cDNA of each sample was amplified using each pair of primers for seprase (forward: 5'-TACCCAAAGGCTCCAGCTAA-3' and reverse: 5'-ACAGGACCGAAACATTCTGG-3') and the endogenous control β-actin (forward: 5'-AGATGACCCAGATCATGTTTGA-3' and reverse: 5'-GCACAGCTTCTCCTTAATGTCA-3') under the following amplification scheme: 10 min at 95ºC; 40 cycles of 30 sec at 94ºC, 30 sec at 60ºC and 1 min at 72ºC; 10 min at 72ºC. Quantitech™ SYBR Green PCR

Kit (Qiagen) was used to prepare PCR reactions according to manufacturer's specifications. Real-time RT-PCR was conducted on the Opticon II (MJ Research, Watertown, MA) and analysis was facilitated by OpticonMONITOR™ Analysis Software Version 2.02 (MJ Research). All samples were normalized to β-actin and fold-change was calculated over the base line set to 1.

Seprase RNAi in vivo model

The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee of Stony Brook University. Each monoclonal cell line derived from SB247 cells stably transfected with the pGUS plasmid (Donghai Chen and Wen-Tien Chen, unpublished), which contains a seprase RNAi target (1384 #1, 1384 #4) or the plasmid containing a hairpin with no target (NT). In a 0.2 ml volume of PBS, 10^6 cells were intraperitoneally (*i.p.*) injected into 6 individual female Balb/c $^{nu/nu}$ that were 4-6 weeks old (Charles River Laboratories, Wilmington, MA). Animals were monitored every four days from Day 4 through Day 36 using the multispectral Maestro™ *in vivo* imaging system (CRI, Woburn, MA) on mice sedated using isofluorane in an oxygen delivery system and sedation was maintained for the duration of imaging. Images were captured at 2 X 2 binning, 500-720 nm spectral emission range and 300 sec exposure to visualize the GFP labeled lesions forming in the abdominal cavity. All images were processed using the associated software for spectral analysis.

To recover ascites, a 1 ml syringe fixed to a 22-gauge needle was coated with a 10X solution of Anticoagulant Citrate Detxtrose (Baxter Fenwal®, Deerfield, IL,) and 200 units/ml Spectrum® Heparin Sodium solution so that the void volume of the syringe and the bore of the needle were filled with 50 µl of anti-coagulant mixture. Reverse *i.p.* was conducted on isofluorane anesthetized mice and recovered fluid was placed on ice until the sample could be processed. A portion of the ascites sample was examined for spheroid formation and the remaining ascites were centrifuged at 1000 rpm for 10 min to separate the cells from the supernatant. The supernatant was then applied to SDS-PAGE and Western Blot analysis to identify species of TIC present. In addition, the site of injection lesion and sites of secondary lesion formation on the peritoneal membrane were excised and fixed in 10% neutral buffered formalin and paraffin embedded for sectioning.

SDS-PAGE and Western Blotting of TIC in ascites

Ascites generated from 36 days post *i.p.* injection of 10^6 SB247 cells into 4-6 week old female Balb/c $^{nu/nu}$ mice (Charles River) were evaluated for TIC. Ascites were harvested and prepared as indicated above and the supernatant was run on a 4-15% Tris-HCl (BioRad, Hercules, CA) gel in non-reducing conditions and transferred to a nitrocellulose membrane. Briefly, 5% non-fat dry milk was diluted in PBS pH 7.4 and the membrane was blocked overnight at 4ºC. The polyclonal anti-human TIC primary antibody (Rockland, Gilbertsville, PA) was diluted 1:10,000 and the polyclonal antibody against CNBr induced fragments of TIC (Calbiochem, San Diego, CA) was diluted 1:5,000 and applied to the membrane for 1 hr at RT. After washing, the anti-rabbit IgG-HRP secondary antibody sc-2004 (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:5,000 and then applied for 1 hr at RT. Bands were then visualized with PerkinElmer™ Western Lighting reagent (PerkinElmer, Boston, MA) according to manufacturer's instruction. The triple helix and gelatin strands were compared to control dilutions of purified TIC (BD Biosciences, Bedford, MA) for molecular size.

Immunohistochemistry

Paraffin embedded sections were cut to 4 μm and rabbit polyclonal antibodies against GFP ab290 (abcam Inc., Cambridge, MA), against CNBr digested fragments of TIC (Calbiochem) and Ki67 (abcam) for proliferation were used for detection via conventional immunohistochemical techniques. Briefly, paraffin embedded sections on slides were warmed at 55ºC for 20 min and the paraffin was removed via two changes of xylene for 20 min each. Slides were rehydrated in an ethanol series and boiled in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) for 20 min. Using 1% H_2O_2, endogenous peroxidase activity was blocked and sections were blocked with 4% BSA in TT buffer (500 mM NaCl, 10 mM Trizma and 0.05% Tween-20 solution) for 20 min. Primary antibodies were added at the following dilutions: GFP 1:500, TIC_{FRAG} 1:100, Ki67 1:100 and were incubated overnight at 4ºC. Sections were then washed 4X with TT buffer and the biotinylated goat anti-rabbit IgG secondary antibody BA-1000 (Vector Laboratories) was applied at a 1:400 dilution and incubated for 40 min. ABC-HRP was prepared according to manufacturer's instruction and applied to each section for 40 min

after washing 3X with TT buffer. The DAB solution (Vector Laboratories SK-4100) was prepared according to manufacturer's instruction and applied to the sections. Sections were then lightly counterstained with Gill's Hematoxylin (Fischer Scientific, Fairlawn, NJ). Ki67 positive cells were counted as a percentage of all cells in three random fields.

Statistical analysis

Statistical analyses were performed using GraphPad Prism® Software (GraphPad Software, San Diego CA). Students T-test was used to determine statistical significance. Only for analysis of the gel contractility assay ANOVA corrected for repeated measures was used coupled with post-hoc analysis between the three cell types using Tukey's Multiple Comparison Test. If P values were less than 0.05, then findings were regarded as significant.

RESULTS

TICg increases seprase expression in ovarian tumor cells

SB247 cells in culture expressed low levels of seprase. A 3.8-fold increase in seprase RNA expression was produced in the SB247 cell line when cells were incubated for 24 hrs on a rehydrated TIC coated plate; however, Growth Factor Reduced Matrigel did not elicit the same response and levels of seprase remained comparable to the very low levels produced by cells plated on a plastic (Fig. 1*a*). In addition, already adherent SB247 cells exposed to 50 μg/ml of TICg in media for 1 hr yielded a seprase expression response of 2.5-fold higher than media alone and, after 4 hrs of exposure at that same concentration, seprase expression was four-fold greater than media alone (Fig. 1*b*). Furthermore, when the non-adherent ascites environment was simulated through culture in a bacterial dish, the SB247 cells expressed seprase in amounts 2.7- and 2.9-fold greater when stimulated with 30 and 40 μg/ml TICg, respectively, as compared to non-stimulated controls after incubation for 2 hrs (Fig. 1*c*). While unstimulated SB247 cells maintain very low levels of seprase expression, interaction with TIC, either as a substrate or in the

media, regardless of cell anchorage status, results in rapid stimulation of seprase production.

Seprase knockdown cell lines have a reduced ability to remodel and invade TICg

Three subclones were isolated and were plated on TICg to evaluate the expression of seprase RNA. The control subclone containing an RNAi hairpin with no target (NT) was compared to subclone 1384 #1 and a significant (P=0.036), 44.1% reduction in seprase expression was detected (Fig. 2*a*). Further, RNAi against seprase is most effective in subclone 1384 #4 in that it nearly prevents seprase expression and significantly (P=0.0009) reduces it to 0.3% of the NT control (Fig. 2*a*). Neither proliferation *in vitro*, nor the ability of these cells to form colonies in soft agar appears to be dependent on seprase RNA expression (Fig. 2*b*, Sup. Fig. 2*a*). However, a reduction in seprase expression in the 1384 #1 and 1384 #4 cell lines corresponded with a significant (P=0.0037 and P<0.0001, respectively) inhibition of these cells to invade through TICg *in vitro* (Fig. 2*c*). Also, when applied to a gel contractility assay, over the five day time course and across all three TICg concentrations (220 μg/ml, 360 μg/ml and 570 μg/ml) the NT cell line retained the greatest ability to contract the TICg disks, whereas the increasing inhibition of seprase induction in the 1384 #1 and 1384 #4 cell lines was coincident with a progressive decline in disk contraction (Fig. 2*d*-2*g*). In fact, statistical analysis revealed that there was a significant difference between the NT cell line and the 1384 #4 cell lines at all three concentrations; 220 μg/ml (P=0.0105), 360 μg/ml (P=0.0005) and 570 μg/ml (P=0.0034). In addition, all three cell lines were significantly different from one another at the 360 μg/ml concentration (P<0.05). Therefore, by diminishing seprase production, we have reduced invasion and ECM remodeling.

Seprase expression is associated with greater secondary lesion formation in vivo

Apparent by fluorescence microscopy and then confirmed by flow cytometry, we found that the NT subclone expressed the lowest levels of GFP as indicated by mean fluorescence index, 1384 #1 showed 1.86-fold higher levels, and 1384 #4 exhibited the highest GFP signal at 2.52 times greater than the NT cell line (Sup. Fig. 2*b*). For example, if a cluster of 1000 1384 #4 cells were required to elicit a strong enough GFP

signal to be visualized using the *in vivo* imaging equipment, an equivalent GFP signal would only be produced when 2,520 NT cells were clustered. Even though heightened levels of GFP in cells that exhibited the least seprase expression may have enabled visualization of smaller lesions in the two knockdown cell lines, overall quantification was still greatest in mice inoculated with the NT cell line that has the greatest seprase expression, but weakest GFP signal (Fig. 3*a*, 3*b*). The GFP positive cell clusters were first visualized on the abdominal surface using the *in vivo* imaging equipment, but localization and adherence to the peritoneal membrane was only verified upon necropsy. Dissection of the peritoneal membrane, followed by a PBS rinse allowed enumeration of individual lesions that were GFP positive and remained tightly adhered to the peritoneal membrane. Therefore only attached lesions were counted and free-floating tumor cell clusters in the ascites fluid were not quantified. Further, this method allowed us to distinguish lesions formed on the ventral surface of the peritoneal membrane from tumors localized to the spleen and pancreas regions (Fig. 3*a*, *). Overall, a significant reduction in tumor formation was revealed between the NT and 1384 #4 subclones (P=0.034) (Fig. 3*a*, 3*b*).

The *in vivo* imaging system used during the longitudinal study enabled us to see that tumors formed initially at the site of injection merely four days post injection and persisted over the course of the experiment, which confirmed delivery of tumor cells at the outset of the experiment (Fig. 3*a,* open arrows). Necropsy revealed that tumor cell clusters were also found within the folds of the intestinal mesentery, an extension of the peritoneum, and on the surface other internal organs in addition to the ventral peritoneal membrane surface (Fig. 3*a,* white arrows), which was the region used for comparison. Labeled GFP cells were found to cluster near the spleen and pancreas (Fig. 3*a,* *), but individual lesions were difficult to discern in this region. Moreover, cell proliferation determined by immunohistochemistry utilizing the polyclonal antibody against Ki67 revealed that 1384 #1 cells within the lesions proliferated at a significantly greater rate than both NT cells (P=0.0006) and 1348 #4 cells (P=0.001) and proliferation detected in NT generated lesions were significantly greater (P=0.024) than 1384 #4 (Fig. 3*c*). Despite a weaker GFP signal and differences in proliferation, the NT subclone, with the

greatest expression of seprase, yielded a greater number of peritoneal membrane lesions than either knockdown subclone.

The TIC composition in peritoneal membrane-associated ovarian tumor lesions

Because the seprase high-expressing NT and 1384 #1 tumor cells, but not seprase low-expressing 1384 #4 cells, were adherent to the peritoneal membrane, the TIC composition was examined in the tumor attached to the membrane via immunohistochemistry (Fig. 4*a*). NT cells, and, to a lesser degree 1384 #1 cells, thoroughly disrupted the mesothelial cell lining, are closely associated with peritoneal fibroblasts (Fig. 4*a*, black arrows). While fragmented forms of TIC (TIC_{FRAG}) can be found within the lesions formed on the peritoneum regardless of cell type, the intensity of stain is greatest within the NT lesions (Fig. 4*a*, white arrows). These data illustrate the heightened invasive phenotype and corresponding TIC fragmentation of cells with relatively high and moderate seprase expression. It is important to note that lesions created by the 1384 #4 cell line remained very loosely attached to the peritoneal membrane, so much so that the post necropsy rinse with PBS dislodged most of the tumor clusters. Routine histological processing of the few clusters that remained actually caused them to separate from the membrane, whereas similar samples from the other cell lines were not affected in the same way (Fig. 4).

To examine the species of TIC present in the ascites supernatant derived from the mouse xenograft model, we applied the supernatant to SDS-PAGE and subjected them to Western immunoblotting using both antibodies directed against intact and TIC fragments. These experiments revealed that the majority of TIC recovered from ascites supernatant of all three cell types has been digested to fragments in a range of 60-65 kDa (Fig. 4*b,* lined arrow). In addition, the 130 kDa band found in all three ascites supernatants corresponds to the presence of intact gelatin strands (Fig. 4*b,* open arrow). However, in ascites supernatant resulting from both 1384 #1 and 1384 #4 inoculation, additional bands are evident at ~200 kDa and ~145 kDa (Fig. 4*b,* filled arrows), which indicate the presence of gelatin dimers and fragmented gelatin dimers. Further, none of the ascites samples showed the presence of triple helices confirming that only gelatin forms are found within the ascites fluid.

β1 integrin stimulates seprase RNA expression

To examine the role of β1 integrin and seprase in TICg contraction, a gel contractility assay was used. The remodeling capability of the SB247 cells is inhibited when they are exposed to 200 μg/ml of mAb C27 against β1 integrin, but the control mAb C37, directed against cell surface glycoprotein-90, does not exhibit the same effect (Fig. 5*a*). After 120 hrs, the NT subclone reduced the average diameter of the TICg disk to 26.3% of its original size when exposed to mAb C37, but contraction was inhibited when mAb C27 was applied and only a 47.8% reduction was seen. The effect of mAb C27 on the NT subclone was similar to the effect of the 1384 #1 partial knockdown of seprase reflected by the 47.2% reduction of the original disk when incubated in the presence of the control mAb C37 antibody. In the same way, when mAb C27 was applied to the 1384 #1 it diminished TICg remodeling to 66.3%, which is comparable to the 1384 #4 subclone and the control antibody at 66.3% overall reduction. Finally, the 1384 #4 subclone, in the presence of mAb C27 was still capable of disk remodeling, but this was significantly lessened to an 81.1% reduction.

To examine the impact of available β1 integrin in seprase expression of adherent cells, the SB247 cells cultured overnight were exposed to a series of dilutions of mAb C27. At 300 μg/ml seprase expression was induced 1.86-fold, whereas a concentration 600-800 μg/ml of mAb C27 was necessary to induce 3.31-fold elevation in seprase expression when the antibody was first bound to high-affinity binding plates (Figs. 5*b*, 5*c*). In addition, when incubated in non-adherent conditions similar to that of an ascites environment, SB247 cells expressed elevated seprase levels that were 6.8-fold greater in response to the 300 μg/ml concentration of mAb C27 (Fig. 5*d*), suggesting the involvement of β1 integrin in seprase expression. In contrast, when mAb C27 was first papain digested into Fab fragments and then applied to the cells in non-adherent conditions, no such response was detected (Fig. 5*d*), suggesting cross-linking of β1 integrin is involved. Together, our data strongly suggest that seprase expression is mediated through β1 integrin clustering on the tumor cell surface.

DISCUSSION

Seprase is present on tumor cells actively invading the ECM and fibroblasts actively involved in wound closure, but is not expressed in inactive cells of the same type.[14] Because seprase expression is coincident with activity, it suggests that seprase is transiently expressed. Consequently, it is likely that seprase is induced by some component to which only active cells are exposed. Indeed, we found that seprase is induced by cell exposure to TIC and can be rapidly induced at low TICg concentrations regardless of cell adhesion status (Fig. 1).

While Matrigel™ contains other matrix proteins simulating the components of the basement membrane, it does not contain TIC. The elevation of seprase RNA expression in the SB247 cells through plating on rehydrated TICg as compared to cells plated on plastic or growth factor reduced Matrigel™ indicates a defined induction that requires not merely any substrate to which cells can adhere, but rather TICg in particular. Similar results were found when using the HT1080 cell line in the same application, suggesting the TIC mediated induction of seprase is not limited to ovarian carcinoma cells (data not shown). In addition, when the SB247 cell line was exposed to different dilutions of TIC under two distinct anchorage conditions, seprase expression was elevated within a well-defined concentration range for each condition. Together, these data suggest that seprase can be induced in both the ascites-like environment as well as apical exposure to TICg, and expression is dependent on both concentration of TICg and duration of exposure in that up-regulation occurs at low concentrations shortly after exposure is initiated.

Further, our data shows that seprase contributes to tumor cell adhesion and invasion via remodeling TIC, and a diminution in seprase results in a corresponding reduction in invasion and remodeling capability. The subclones with lower seprase expression corresponded to a diminished capacity to invade through TICg as well as a reduced ability to remodel TICg in a gel contractility assay (Fig. 2). The gel contractility assay has been used to examine fibroblasts in a wound healing/ skin graft application and to melanoma cell lines.[15-17] We found the gel contractility assay to be a useful *in vitro* tool because the ability of tumor cells to remodel ECM proteins is directly linked with the capacity to adhere to and invade sites of secondary lesions.

The contribution of seprase to ECM remodeling is also reflected in formation of peritoneal membrane lesions in a mouse model of late stage ovarian cancer. Not only did fewer lesions form in mice inoculated with seprase knockdown cell lines, but invasion of the mesothelial layer and disruption of peritoneal membrane was reduced in lesions that did form (Fig. 3*a,* 3*b*, 4*a*). A significant reduction in secondary lesion formation was found between the cell lines with the highest and lowest seprase expression (Fig. 3*b*). The tumors that formed at the site of injection were used to confirm successful delivery of tumor cells. It is reasonable to find a tumor developing within the peritoneal cavity at the injection site due to mesothelial cells activation for wound healing purposes secreting matrix molecules that would either attract or catch tumor cells irrespective of seprase expression. Immunohistochemical analysis using the antibody against GFP permitted us to distinguish GFP positive tumor cells from surrounding fibroblasts and other cells associated with the peritoneal membrane lesions generated by the xenografts. Suppression of seprase within the tumor cells corresponds to a diminished ability to form lesions on the membrane (Figs. 3*a*, 4*a*). In addition, immunohistochemical analysis shows the knockdown cell lines to have reduced TIC fragmentation; however, TIC can be found within the lesions regardless of cell type, suggesting the involvement of other proteases such as matrix metalloproteinases (Fig. 4*a*). In support of the significance of the role of collagenases and gelatinases in the TIC-ovarian tumor cell interaction, an *in vitro* study of the adhesion and invasion of ovarian carcinoma cells to the peritoneal membrane showed that these cells more readily adhered to and invaded matrices consisting of TIC than those comprised of collagen IV, fibronectin, vitronectin and laminin.[18] Because the digestion of TIC and other ECM proteins facilitates the invasion of tumor cells and thus the anchorage of lesions to the membrane, inhibition of this mechanism has lead to a reduction in lesion formation.

Late stage ovarian cancer creates an ECM-rich microenvironment within the ascites fluid. The mesothelial cells lining the peritoneal membrane are normally triggered to produce collagen by an inflammatory response and the collagen produced is used to heal the wound when the peritoneum is disturbed. However, with malignancy during development of late stage ovarian cancer and ascites accumulation, activated mesothelial cells lining the peritoneal cavity are stimulated to produce abundant amounts of TIC that

is generally released into the fluid (Fig. 4*b*).[19,20] A previous study of ovarian carcinoma patient peritoneal effusions found that seprase positive samples correlate with diminished overall survival and suggest a predictive value for seprase detection in ascites samples.[21] Similar information may be gleaned from assessing the extent to which TICg has been cleaved and may potentially address the primary gelatinases responsible. Although our mouse model yielded many different species of TIC in the ascites fluid, gelatin dimers and dimer fragments were only found in ascites supernatants generated by inoculation of the two seprase knockdown cell lines, but not in fluid resultant from NT inoculation, which suggests that seprase may play a role in cleavage of gelatin dimers as well and the cleavage status of gelatin dimers may have some prognostic importance (Fig. 4*b*).

β1 integrin, a TIC receptor, co-localizes with seprase on the surface of actively invading tumor cells and human umbilical vein endothelial cells during angiogenesis.[22] In addition, integrin α3β1 has been implicated in promoting the invasion of hepatocellular carcinoma, melanoma and breast carcinoma cells through collagenous matrices.[23-25] Antibodies directed against β1 integrin have been shown to interrupt patient ovarian cancer ascites spheroids from binding to a TIC substrate.[3] In addition, a previous study using the same C27 mAb revealed that it inhibits the binding of endothelial cells to a TIC matrix and prevents cell migration during wound closure.[22] Our mAb C27, which binds to β1 integrin, appears to both induce seprase under a host of cell culture conditions and is able to inhibit gelatin remodeling of the SB247 cell line and subclones (Fig. 5). These seemingly disparate findings suggest that the production of seprase alone is not sufficient for collagen remodeling, but rather the TICg substrate must be bound to β1 integrin, an interaction which is disrupted by mAb C27. The seprase induction profile generated in the experiment where mAb C27 is anchored to a high affinity binding plate begins to suggest a model by which antibody induced crosslinking of β1 integrin subunits is responsible for the elevation of seprase expression (Sup. Fig. 4). Moreover, from the experiment involving the papain digestion of mAb C27, it is shown that, indeed, the antibody elicits a clustering effect on β1 integrin and it is that integrin clustering that induces seprase expression, while Fab fragments cannot (Fig. 5*d*). This integrin clustering effect appears to be a common mechanism for gelatinase production in tumor cells because not only has integrin interaction with the ECM been shown to promote the

production and activation of both soluble and membrane bound matrix metalloproteinases, but MMP-2, MMP-9, and MMP-14 have all been induced through exposure to antibodies or cross-linkers targeting β1 integrin.[26-30] Considered together, these data suggest that when tumor cells are stimulated via interaction with TICg, the expression of seprase contributes to remodeling and invading TIC matrices via β1 integrin.

It is likely that the TIC substrate that is bound to β1 integrin is first processed into TICg by members of the matrix metalloprotease family such as MMP-2 [31] and MMP-9, [32] both of which are known to cleave fibrillar TIC, and is subsequently acted on by gelatinases such as seprase. When the TICg has been digested by seprase, those fragments are released, and β1 integrin is prompted to interact with a new TIC strand, thereby advancing the protruding invadopodia into the substrate. As shown by seprase production via β1 integrin stimulation with mAb C27 and the concurrent inability to remodel TIC, it is likely that seprase does not interact with β1 integrin in the same way when lacking the TIC substrate. It is possible that mAb C27 moderately stimulates the production of seprase, but seprase is then unable to properly colocalize with β1 integrin, or perhaps is not properly activated by other proteases that are reliant on the integrin-substrate specific interaction.

Although small-molecule inhibitors are known effectively inhibit the proteolytic activity of different classes of proteases, the disruption of interactions between β1 integrin, seprase and ECM components may prove to be an additional tool against invasion.[33,34] The simultaneous inhibition of seprase proteolytic activity and prevention of β1 integrin interaction with the ECM could be useful to prevent formation of peritoneal tumor lesions in progressing cases of ovarian carcinoma. In this way tumor cell interaction with TIC would be hindered may prove a useful strategy in combating tumor progression in late stage ovarian cancer. It is clear that seprase plays a role in the ascites environment and it is likely to be a key player in metastatic lesion formation during late stage epithelial ovarian carcinoma. Finally, it is the transient and inducible nature of seprase that suggests seprase to be a specific target ideally suited for inhibition.

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ACKNOWLEDGEMENTS

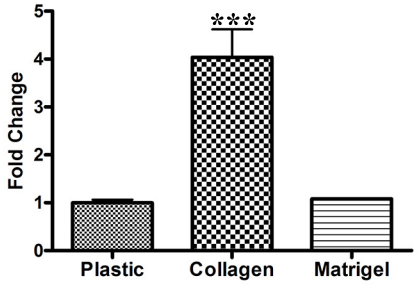
We thank Donghai Chen for the RNAi plasmid constructs and Howard Crawford for help with *in vivo* imaging and image processing.

Grant support

This research was supported by grants from the National Institutes of Health grants R01CA0039077 and R01EB002065 (to WTC).

Figure 1. Comparison of seprase RNA expression in cells treated with TIC. ***(a),*** A comparison of substrates. TIC (BD Biosciences) (1 mg/ml) diluted in DMEM and Growth Factor Reduced Matrigel™ Matrix (BD Biosciences) was coated onto tissue culture dishes, allowed to set and then dried overnight. Prior to use each of the dishes was washed twice with PBS and 5 X 10^5 SB247 cells were suspended in CCC, plated on the substrates, and incubated for 24 hours. Quantitative RT-PCR was used to assess seprase expression normalized to β-actin and was compared to levels in SB247 cells plated on plastic tissue culture dishes alone. *** indicates a significant difference of $P< 0.001$ as determined by Student's T-test and vertical lines indicate the SEM. ***(b)*** Comparison of TIC dilutions added to adherent cells. SB247 cells were plated and allowed to adhere to plastic tissue culture dishes in CCC over night. Cells adherent to plastic were then exposed to the indicated concentrations of TIC diluted in DMEM for l hour and 4 hours. All values were normalized to ß-actin and fold change is compared to cells in culture media containing no TIC at each time point. ***(c)*** Comparison of TIC dilutions under non-adherent conditions. Cells were suspended in solution of TIC dilutions $\leq$ 200 µg/ml and incubated in bacterial dishes for 2 hrs to prevent adherence and promote culture in suspension to simulate the ascites environment. Average ± SEM fold-change over seprase expressed by cells in media containing no TIC is indicated.

a


***
Fold Change
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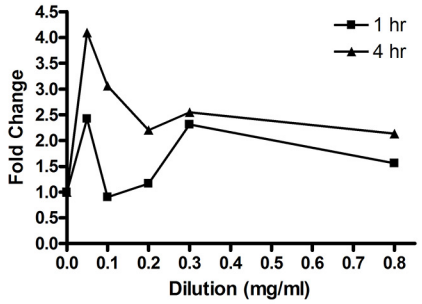
1
2
3
4
5
Plastic
Collagen
Matrigel

b


1 hr
4 hr
Fold Change
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1.5
2.0
2.5
3.0
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4.0
4.5
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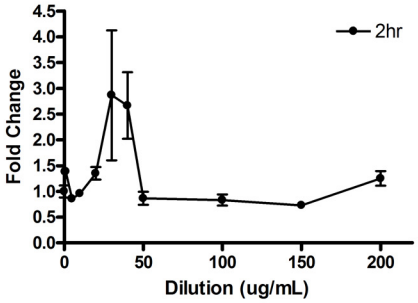
0.3
0.4
0.5
0.6
0.7
0.8
Dilution (mg/ml)

c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Fold Change
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1.5
2.0
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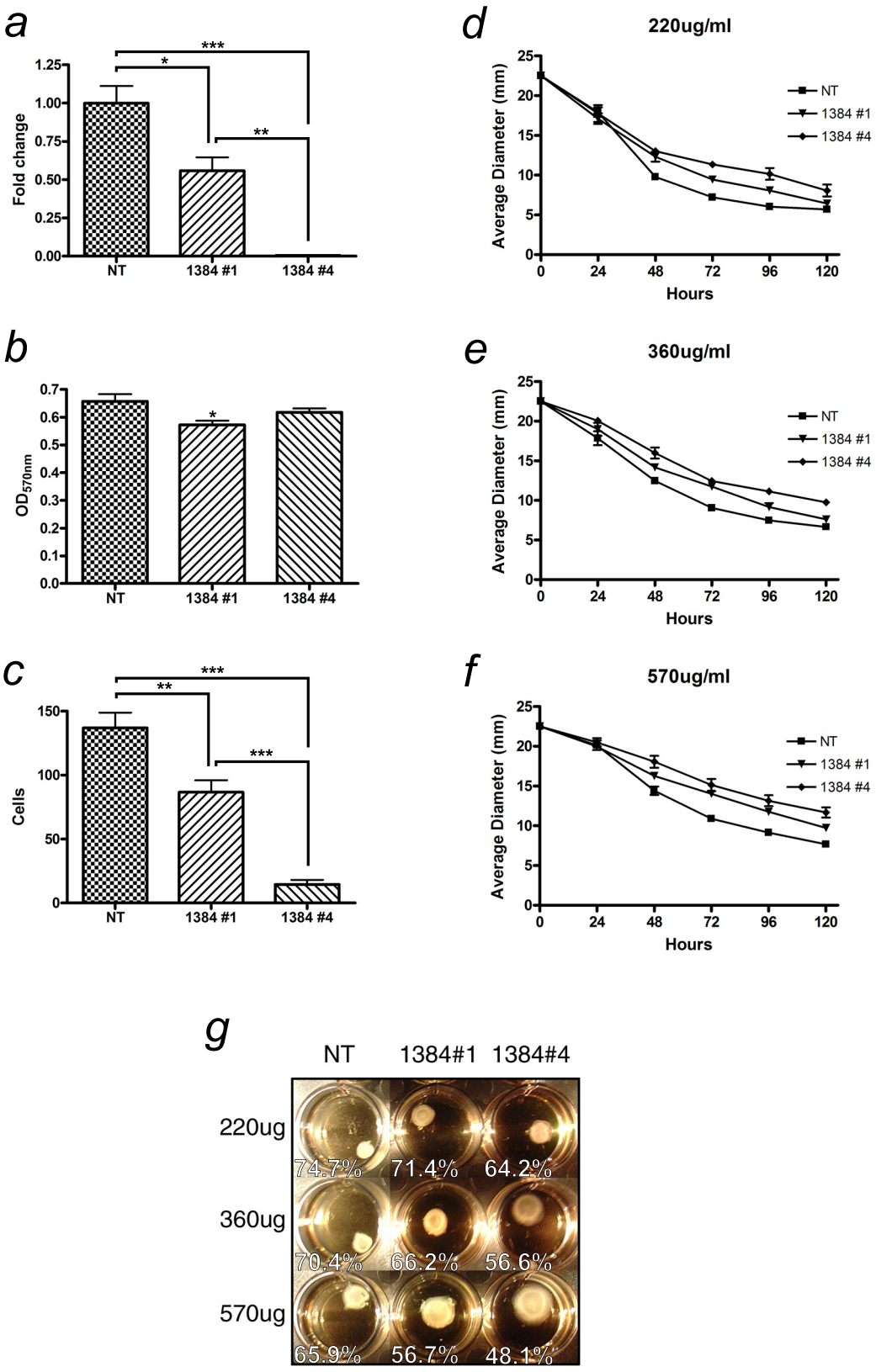
3.5
4.0
4.5
0
50
100
150
200
Dilution (ug/mL)

Figure 2. Characterization of monoclonal cells stably expressing seprase shRNA. ***(a)*** Quantitative PCR comparing seprase expression of SB247 monoclonal cells containing the RNAi vector with a no target hairpin (NT), or a hairpin targeting seprase (1384 #1, 1384 #4). Cells were plated overnight on dried 1 mg/ml TIC. ***(b)*** Proliferation of monoclonal cells. To determine that each of the monoclonal cell lines had similar metabolic rates, an MTT assay (ATCC, Manassas, VA) was conducted using 5 X 10^4 cells of each line suspended in CCC (phenol red-free) were plated in triplicate in a 96-well plate and incubated overnight at 37ºC under 5% CO_2. Control wells contained only phenol red-free media. To each well 10 µl of MTT Reagent was added and the plate was incubated for 2hrs at at 37ºC under 5% CO_2 followed by a 2hr incubation with a detergent reagent at RT. Absorbance was then measured at 570 nm. ***(c)*** Transwell migration of monoclonal cells to cross a TIC coated membrane. The 8 µm pore membranes of Millipore Multiscreen MIC 96-well plates (Millipore, Billerica, MA) were coated with TIC (20 µg/ml); control wells were uncoated. Each of the three cell lines were trypsinized, washed, suspended in DMEM and added to the upper chamber of the 96-well plate at 5×10^3 cells/well. CCC media containing 10% serum was added to the lower chamber. Cells were incubated for 48hrs. A cotton-tipped swab was then applied to each well to remove all non-migratory cells and only cells that had migrated through the membrane remained. The membrane was washed 2X with PBS, fixed with methanol, and stained with Hema 3 Stat Pack cell stain according to manufacturer instruction (Fisher Scientific, Kalamazoo, MI). Each membrane was then detached from the plate and air dried on a glass slide prior to mounting with Permount (Sigma-Aldrich, St. Louis, MO). Cells were counted at 400X magnification. Background values determined by counting cells that migrated in wells containing no TIC were subtracted from those values that reflected migration through TIC. Values presented are the means ± SEM of 9 filters. Significance was determined by a Student's t-test and is indicated as follows: *=$P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$. ***(d-g)*** Contraction of TICg by monoclonal cells. Gel contractility assay was performed to examine the ability of cells to reorganize and contract TICg. TIC diluted in DMEM to 220 µg/ml ***(d)***, 360 µg/ml ***(e)*** and 570 µg/ml ***(f)*** was added to the three SB274 subclones to a final cell concentration of 1×10^5 cells/ well. Cells and TIC dilutions were mixed and plated into 12-well tissue culture plates. After 1 hr at 37ºC under 5% CO_2 to insure gel formation, the perimeter of each disk was freed from the well via pipette tip and CCC was added. The diameters of the major and minor axes were taken daily and averaged. Each condition was evaluated in triplicate for three experiments and the vertical lines represent the error bars of the cumulative experiments. ***(g)*** Images of the disks contracted at Day 5. The inset numbers reflect the percent of average diameter reduction at the end of the five day incubation.


a
Fold change
1.25
1.00
0.75
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*
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NT
1384 #1
1384 #4
b
OD570nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
NT
1384 #1
1384 #4
c
Cells
150
100
50
0
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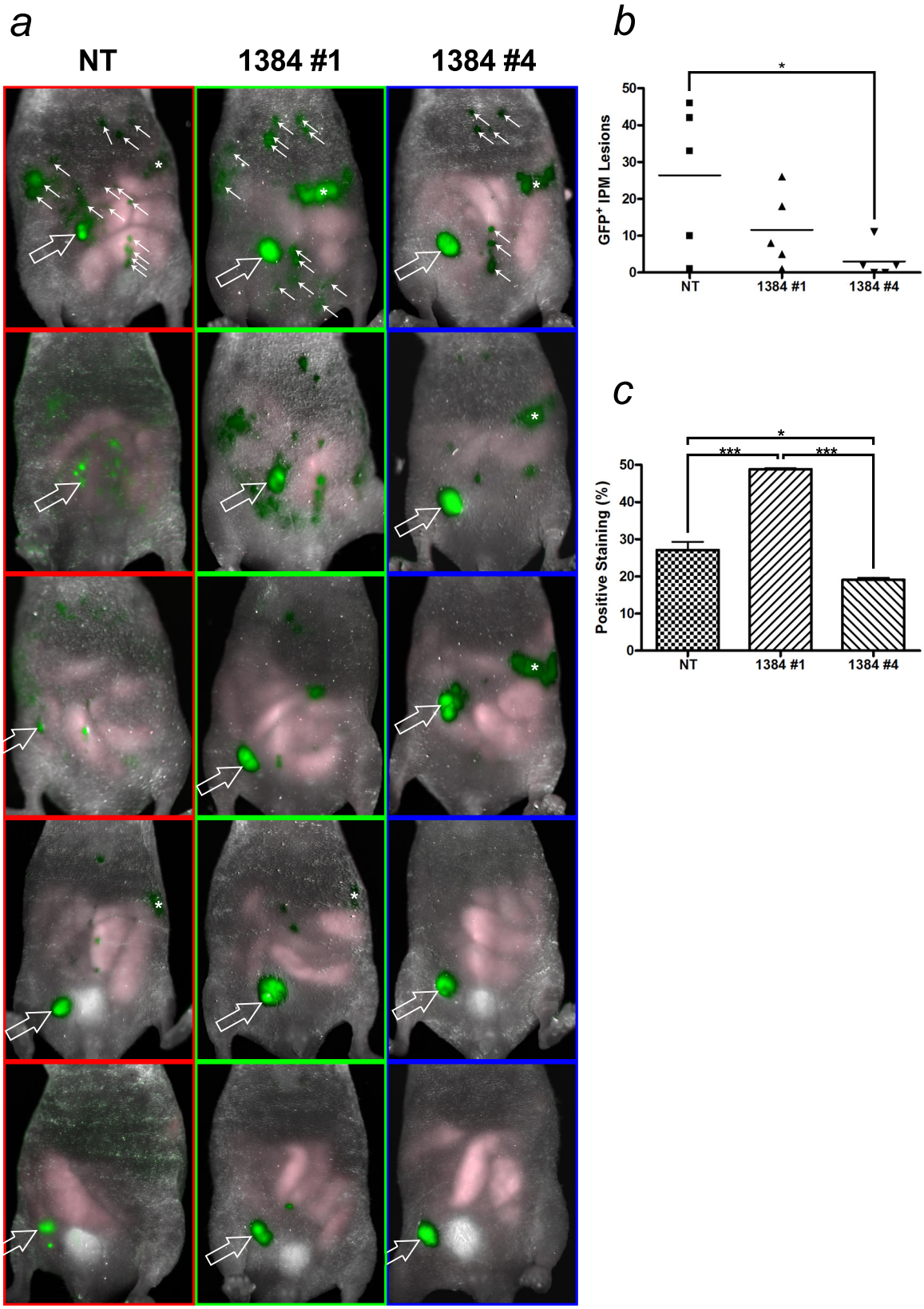
NT
1384 #1
1384 #4
d
220ug/ml
Average Diameter (mm)
25
20
15
10
5
0
0
24
48
72
96
120
Hours
NT
1384 #1
1384 #4
e
360ug/ml
Average Diameter (mm)
Hours
NT
1384 #1
1384 #4
f
570ug/ml
Average Diameter (mm)
Hours
NT
1384 #1
1384 #4
g
NT
1384#1
1384#4
220ug
360ug
570ug
74.7%
71.4%
64.2%
70.4%
66.2%
56.6%
65.9%
56.7%
48.1%

Figure 3. Seprase suppression corresponds to inhibition of peritoneal membrane lesion formation. Ovarian tumor progression was examined *in vivo* using *i.p.* inoculation of the SB247 cell line expressing high (NT), medium (1384 #1) and low (1384 #4) levels of seprase in nude mice. ***(a)*** Visualization of tumor cells created by GFP labeled subclones expressing different levels of seprase. A total of 15 Balb/c $^{nu/nu}$ mice were injected with 10^6 cells and their ventral sides were imaged every 4 days using the Maestro fluorescence imager. Images were captured at Day 24 and spectrally processed with the Maestro *In Vivo* Imaging software, for five mice per cell line. The green areas indicate regions of lesion development and the light pink regions are visualization of the intestines containing rodent food that fluoresces red. The bottom two mice in the NT and 1384 #4 groups as well as the bottom mouse in the 1384 #1 group all appear with white patches, which indicate a full bladder. The open white arrows point to the tumor that developed at the site of injection. The small white arrows indicate representative *i.p.* membrane lesions. The asterisks (*) indicate the lesions developed in the pancreas and spleen region, but are not attached to the ventral surface of the *i.p.* membrane as determined by fluorescence imaging during necropsy. ***(b)*** Enumeration of peritoneal tumor lesions of each of five mice for the three cell lines. Images were captured *in vivo* and mice were then sacrificed. During necropsy the peritoneal membrane was removed, rinsed with PBS and viewed under fluorescence to quantify adherent tumor clusters. ***(c)*** Quantification of Ki67 positive cells in peritoneal tumor lesions. Sections were prepared for immunohistochemistry and stained with the Ki67 proliferation marker. Positive cells were counted as a percent of the total cells in four random fields. Values presented are the means ± SEM and significance was determined by a Student's t-test and is indicated as follows: *=$P < 0.05$ and ***= $P < 0.001$.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NT
1384 #1
1384 #4
b
GFP+ IPM Lesions
50
40
30
20
10
0
*
NT
1384 #1
1384 #4
c
Positive Staining (%)
50
40
30
20
10
0
*
***
***
NT
1384 #1
1384 #4

Figure 4. Assessment of TIC_{FRAG} the lesion environment. ***(a)*** Immunohistochemistry of the peritoneal membrane-associated lesions resulting from *i.p.* inoculation of SB247 subclones expressing relatively high (NT) and medium (1384 #1) levels of seprase. We were unable to obtain comparable sections from animals inoculated with 1384 #4 cells due to the tumor clusters' loose association with the membrane which allowed the majority of the clusters to be rinsed from the membrane during a PBS rinse and those that remained were dislodged during routine histological processing. For control samples, only secondary antibody was applied to the sections, but they were counterstained in the same way. Positive staining is brown, negative regions appear white or pink and nuclei are stained blue due to Gill's hematoxylin counterstaining. Serial sections of formalin fixed paraffin embedded lesions show the degree to which GFP positive tumor cells invade the peritoneal membrane into surrounding muscle layers, is dependent upon seprase expression. In lesions derived from NT inoculation the membranes are highly disorganized and GFP positive tumor cell clusters (*) are found within the stromal matrix (black arrows); the mesothelial layer is indiscernible. There is a moderate disorganization of the 1384 #1 membrane. Fragmented TIC (TIC_{FRAG}) is found within both tumor lesion types (white arrows), but a more intense stain appears at the perimeter of NT lesions. Bar = 100um. ***(b)*** TICg species detected in ascites supernatant. Representative samples of ascites from NT, 1384 #1 and 1384 #4 applied to SDS-PAGE and subjected to Western immunoblotting with two antibodies against TIC, one that recognizes fragments of TIC and the other that identifies the intact triple helix or intact gelatin strands. The lined arrow indicated fragmented TICg, the open arrow points to an intact gelatin strand and the closed arrows denote higher molecular weight species of TIC. No intact triple helices were detected.

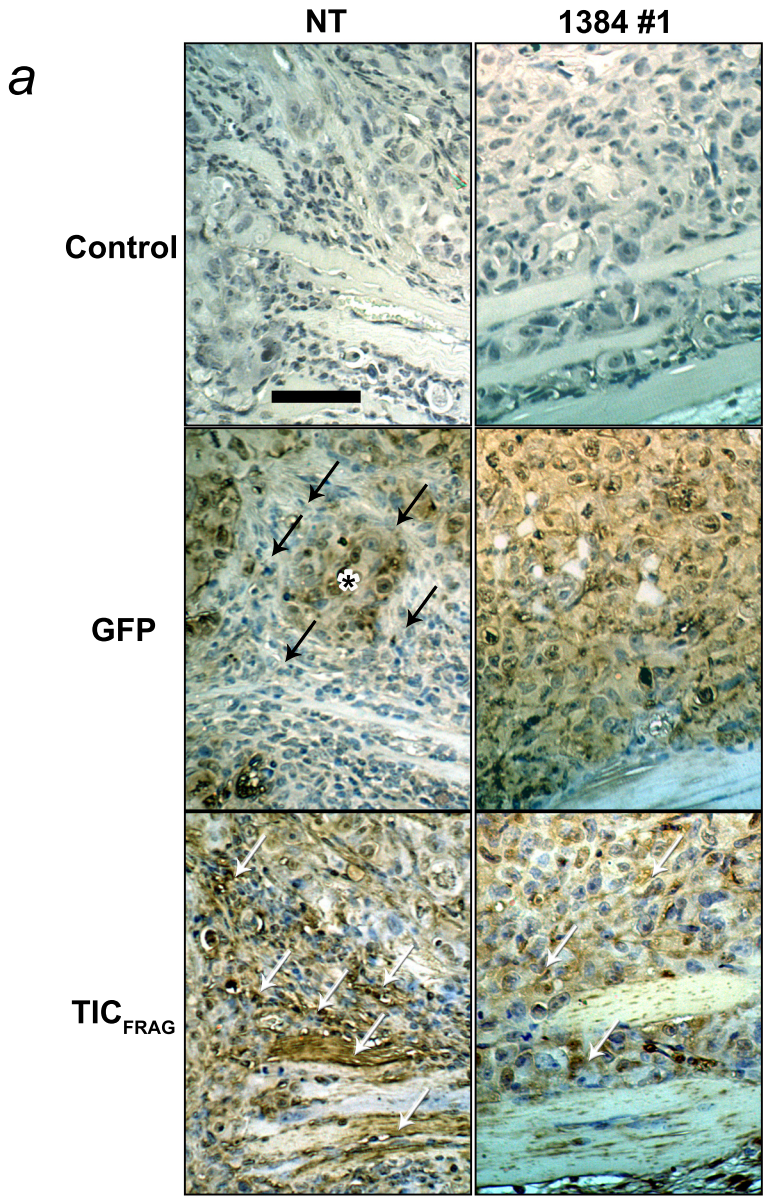
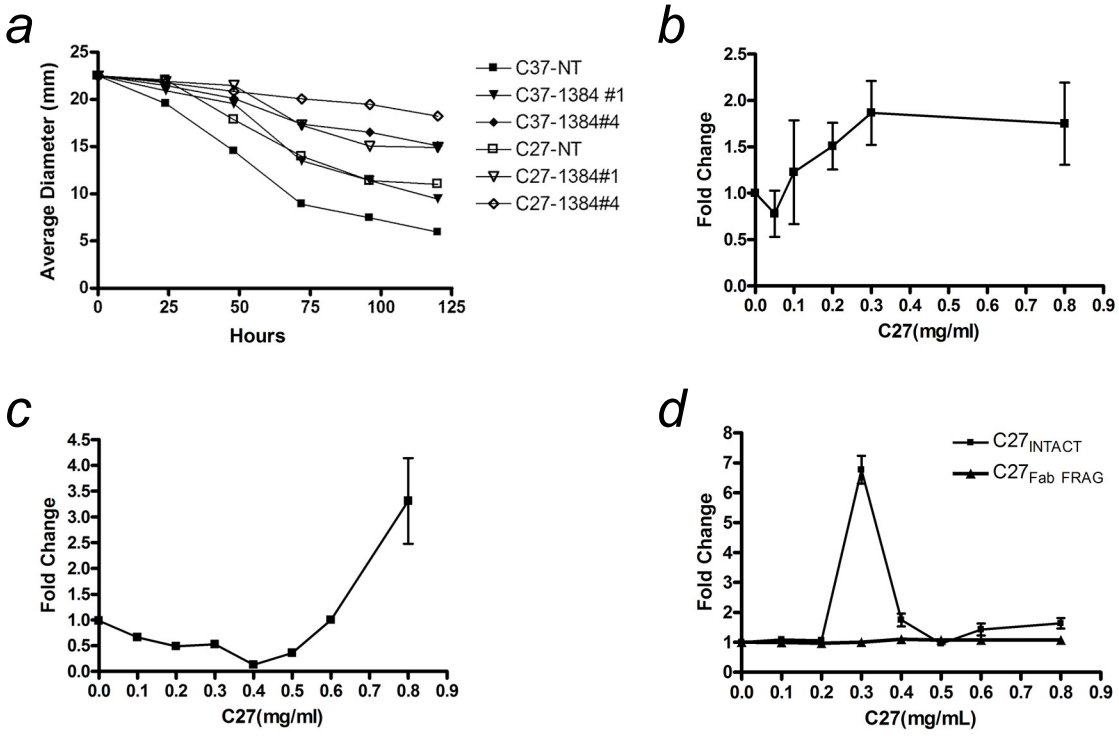
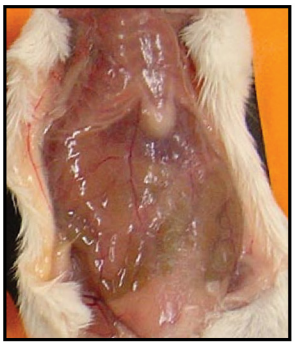

a
NT
1384 #1
Control
GFP
TIC FRAG
b
NT
1384 #1
1384 #4
~195-
~131-
~85-

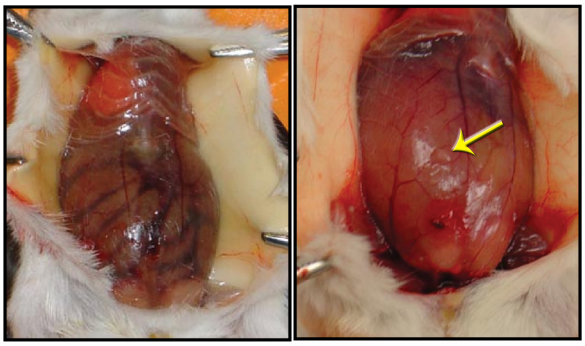
Figure 5. Inhibition of seprase function in gel contractility and induction of seprase expression in cells by anti- β1 integrin antibody. ***(a)*** Gel contractility by cells expressing high (NT), medium (1384 #1) and low (1384 #4) levels of seprase in the presence of mAb C27 against β1 integrin. To 360 μg/ml of TIC diluted in DMEM, 200 μg/ml mAb C27 or control mAb C37 was added and mixed with each of the three SB274 subclones to a final cell concentration of 1×10^5 cells/ well. Cells, TIC dilutions and mAbs were plated into 12-well tissue culture plates. After 1hr at 37ºC under 5% CO_2 to ensure gel formation, the perimeter of each disk was freed from the well via pipette tip and CCC was added. The diameters of the major and minor axes were taken daily and averaged. Each condition was evaluated in triplicate for three experiments and the vertical lines represent the error bars of the cumulative experiments. The influence of mAb C27, the mAb against β1 integrin (open symbols) is compared to a control mAb C37 against glycoprotein-90 (solid symbols) through the averages of daily measurements of the major and minor disk axes. ***(b-d),*** Seprase RNA expression of cells exposed to the mAb C27 under distinct adherence conditions. All measurements were normalized to β-actin. ***(b),*** Comparison of mAb dilutions added to adherent cells. Cells were plated and allowed to adhere to the dish overnight. Dilutions of mAb C27 were added to the culture media and incubated for 2hrs. RNA was then harvested, converted to cDNA and applied to quantitative PCR as described. ***(c)*** Comparison of cells added to pre-bound mAb C27 dilutions. Dilutions of mAb C27 were applied to CellBIND® (Corning, Lowell, MA) high-affinity binding plates and incubated overnight at 37ºC. SB247 cells were then plated and incubated for 2hrs at which time samples were processed for quantitative PCR. ***(d)*** Comparison of mAb C27 dilutions under non-adherent conditions. Cells were mixed with dilutions of mAb C27 and incubated for 2hrs on bacterial plates to maintain the cells in suspension and simulate an ascites environment. To determine if mAb C27 caused the crosslinking of β1 integrin subunits and clustering induced seprase expression, mAb C27 was digested with papain and the Fab portions were purified using Fab Preparation Kit (Pierce, Rockford, IL) according to manufacturers instruction and were applied to SB247 cells in suspension. All samples were then processed for and assessed by quantitative PCR.


a
Average Diameter (mm)
Hours
C37-NT
C37-1384 #1
C37-1384#4
C27-NT
C27-1384#1
C27-1384#4
b
Fold Change
C27(mg/ml)
c
Fold Change
C27(mg/ml)
d
Fold Change
C27(mg/mL)
C27INTACT
C27Fab FRAG

Supplemental Figure 1. Preliminary SCID Beige mouse model of late stage ovarian cancer. Female Fox Chase Scid Beige mice (Taconic Farms, Germantown, NY), which lack T-cells, B-cells and Natural Killer cells, were i.p. injected with 10^6 SB 247 cells diluted in a 0.2 ml volume of PBS. Ascites and peritoneal membrane lesions (yellow arrow) developed in mice injected with the SB247 cell line while control mice, injected only with PBS, developed no excess peritoneal fluid, nor did they develop any discernable lesions.



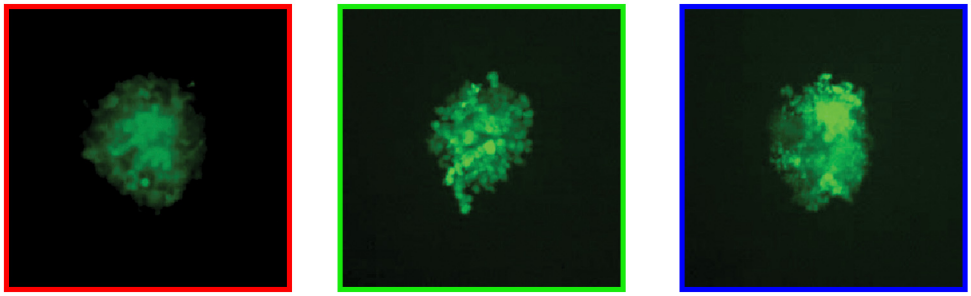
PBS



SB247

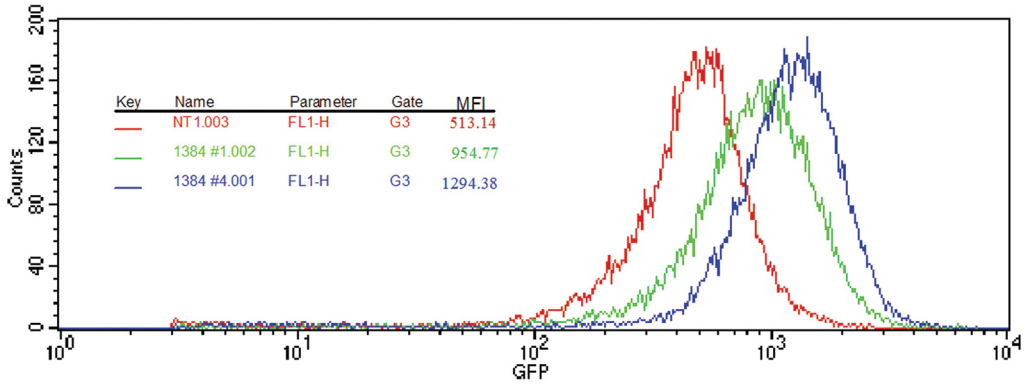
Supplemental Figure 2. Analysis of anchorage independent colony formation and GFP expression. ***(a)*** Each of the three cell lines were evaluated by soft agar assay to test for anchorage independent colony formation. The base layer applied to each well of a 6-well plate was comprised of Agar Noble (Difco™ BD, Sparks, MD) diluted to 0.5 % in 1X RPMI and 10% fetal calf serum (FCS) and was allowed to solidify. The top agarose was formed by mixing 0.35% UltraPure Agarose (Invitrogen) with 1X RPMI and 10% FCS. For each well 5 X 10^3 were mixed in top agarose and were plated to set over the base layer. Plates were incubated in a humidified 37ºC incubator for 12 days at which time colonies were visualized under fluorescence microscopy. Each of the three cell lines was able to form anchorage independent colonies. ***(b)*** Flow cytometry allowed for measurement of mean fluorescence index (MFI) of the three cell lines. Cells were trypsinized and resuspended in PBS. Each cell line was assessed for green fluorescence using FACSCalibur™ (BD, Franklin Lakes, NJ). The NT cell line is represented in red with the lowest MFI followed by the 1384 #1 cell line in green with a mid-range MFI and 1384 #4 that had the highest MFI.

a



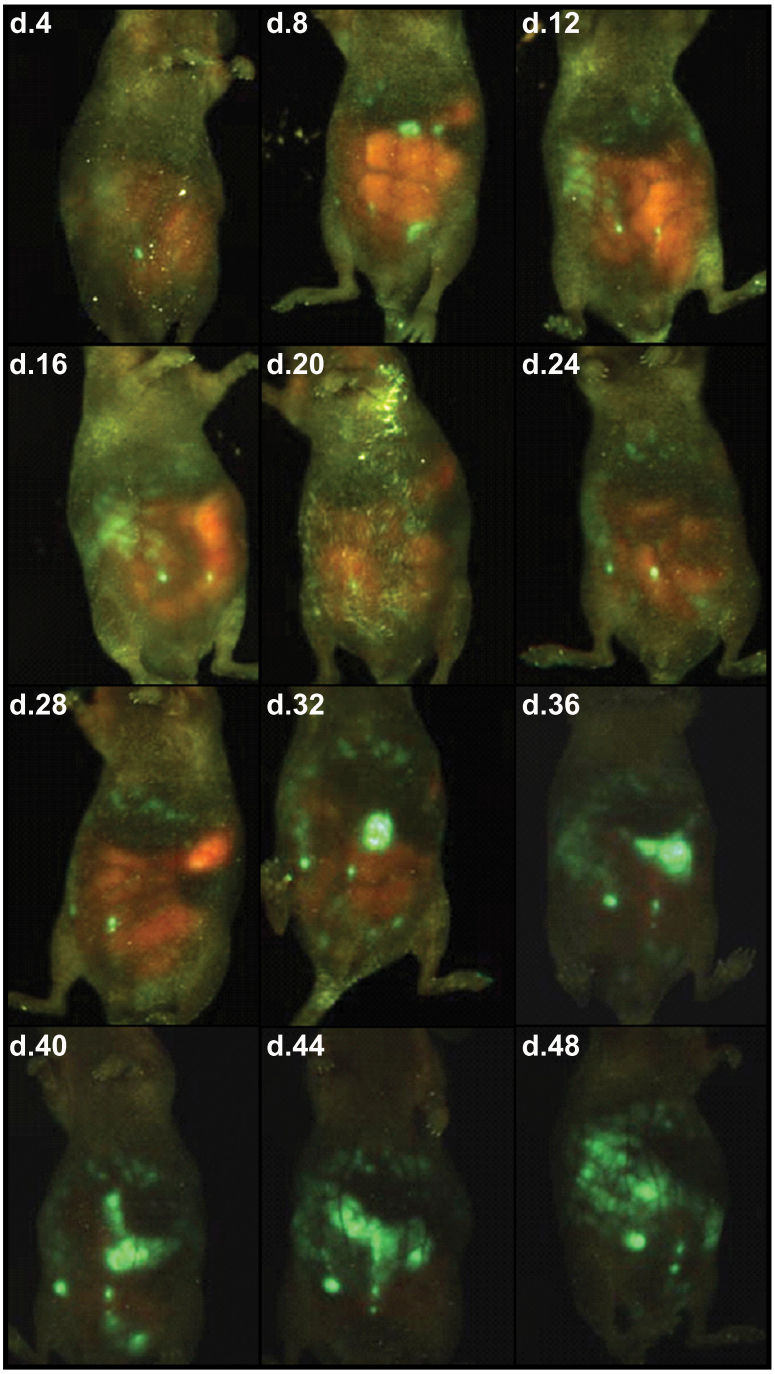
NT 1384 #1 1384 #4

b

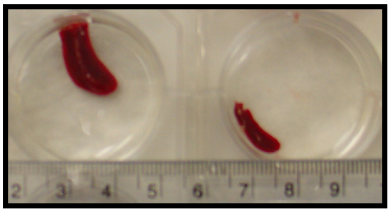


Supplemental Figure 3. Initial characterization of the Balb/c $^{nu/nu}$ mouse model. Many factors contributed to the change in animal model. Primarily the Balb/c $^{nu/nu}$ mouse contributed to a far more robust system in that, while it lacks both B-cells and T-cells it retains natural killer cell function. In addition, this mouse type is nude and therefore lacks fur that is autofluorescent. The fur of the Fox Chase SCID Beige mouse acted to obscure images taken during *in vivo* progression of lesion formation and necessitated the application of a depilatory agent (Nair®, Church & Dwight Company, Princeton, NJ) to the abdomen of each mouse prior to imaging. This called for a greater frequency of handling each animal as well as and increase in the use of anesthesia over the course of the experiment. Further, application of the depilatory agent every four days created irritations of the abdominal skin. To minimize the influence of these factors the Balb/c $^{nu/nu}$ mouse was called into service. ***(a)*** In this experiment SB247 cells (5 X 10^6) that were transfected with the pGUS vector alone without any hairpins were delivered i.p. into the mouse and imaged every four days from day 4 (d.4) through d.48. The experiment was terminated when the mouse appeared moribund and showed abdominal distension and much of the abdomen fluoresced green. ***(b)*** Necropsy revealed splenomegaly in the mouse injected with SB247 cells (left) as compared to the PBS control (right). ***(c)*** The ascites of the injected mouse were examined via fluorescence microscopy and spheroids of GFP positive tumor cells were detected, similar to that found in patient ascites samples.

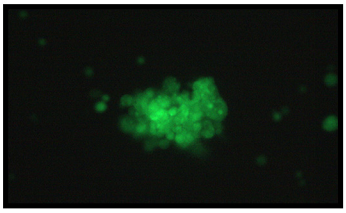
a



b

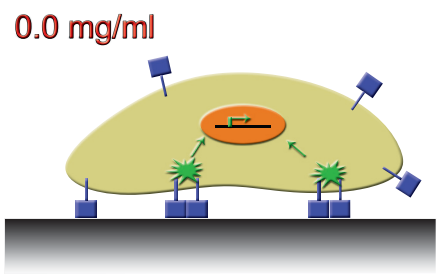


c



Supplemental Figure 4. Model of mAb C27 induced β1 integrin ligation and initiation of seprase transcription. ***(a)*** SB247 cell plated in the absence of mAb C27. When a cell is plated on a plastic dish some β1 integrin subunits (blue) can be in close enough proximity of one another to crosslink (green) and stimulate a basal level of seprase transcription (small green arrow within the orange nucleus). ***(b)*** With 0.2 mg/ml of mAb C27 (red) adhered to a high affinity binding dish and the cells plated on top, a moderate disruption of integrin crosslinking occurs and this corresponds to a partial reduction in stimulatory signaling for seprase transcription. ***(c)*** At a concentration of 0.4 mg/ml, mAb C27 engages individual β1 integrin subunits, but the antibody is not at a high enough concentration to engage the subunits within crosslinking distance and therefore seprase expression is nearly abolished. ***(d)*** At the higher concentration of 0.8 mg/ml, the antibody is able to engage multiple β1 integrins and induce widespread crosslinking thereby creating a strong signal to promote high levels of seprase transcription (large green arrow within the nucleus).

a



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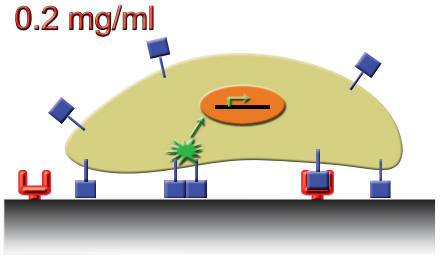

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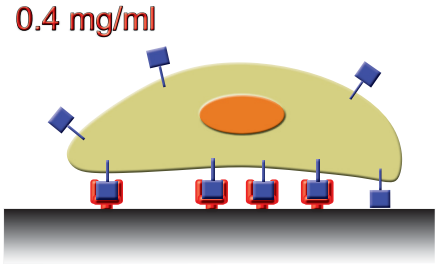

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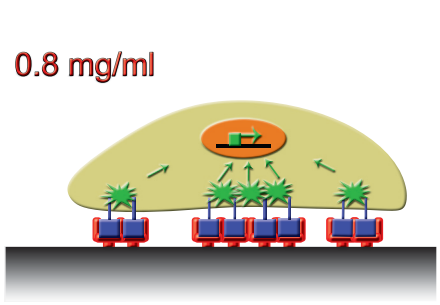

Supplementary Table 1. Initial immunocytochemical characterization of SB247. Cells were incubated on TIC coated slides overnight under CO_2 at 37ºC. Wells were then washed with two changes of 2% BSA in PBS and fixed with 7.4% paraformaldehyde. Between washing steps with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS and blocked for 2hrs in a 2% BSA/PBS blocking buffer. Primary antibodies were diluted 1:50 in blocking buffer and after a PBS wash were incubated for 2hrs at 4ºC. The wells were then washed 3X with PBS. Samples that were treated with FITC conjugated primary antibodies were stained with a 1:500 dilution of Hoescht (33342) and visualized directly by fluorescence microscopy after application on VectaShield mounting medium for fluorescence H-1000 (Vector Laboratories, Burlingame CA). It was verified that the SB247 cell line was indeed derived from the tumor cell population within the patient ascites sample and not mesothelial cells as evidenced by EMA positive staining and endothelial marker negative staining.

Antibody	Target	+/-	Company	Catalog Number
D8	Seprase	+	W-T Chen Laboratory	†
D28	Seprase	+	W-T Chen Laboratory	†
E26	DPP4	+	W-T Chen Laboratory	†
HER2	c-erb, B-2, Ab-17	+	NeoMarkers Fremont, CA	Ms-730-p1
EMA	Epithelial Marker Antigen	+	Dako Carpinteria, CA	F0797
CD31*	Endothelial Marker Ab-1 (JC/70A)	-	NeoMarkers Fremont, CA	Ms-353-S
CD34	Endothelial Marker α-HPCA-2	-	Becton-Dickenson Immunocytometry Systems San Jose, CA	348053
Hoechst (33342)	Nuclear Stain	+	Molecular Probes Eugene, OR	H3570

* Not FITC conjugated. Dako Cytomation LSA B2 system-AP Ref. K0674 Carpinteria, CA was used according to manufacturer's instruction.

† As described in [22].

CHAPTER 3

Future Directions

INTRODUCTION

While there is still no way to prevent cancer, and early detection prior to metastatic progression is in its infancy, the overarching goal of tumor cell biology is to inhibit invasion and metastasis. Primary tumors can be efficiently excised, but secondary lesions have proven to be more insidious. For this reason, many cancer therapies are directed against both tumor cell adhesion and invasion in an effort to slow metastatic disease.[1] In addition, there has been a recent push to understand the effects of the tumor cell microenvironment and its influence on the epithelial to mesenchymal transition that characterizes the change in tumor cell phenotype during malignant transformation.[2,3] This dissertation focused on the role of seprase in peritoneal membrane lesion formation in late stage ovarian cancer and, while this new data contributes to our understanding of this protease and suggests potential therapeutic avenues, there is still much more to learn.

SEPRASE IN THE CROSS-HAIRS

Seprase is known to play a key role in tumor cell invasion. Small molecule inhibitors directed specifically at seprase such as Gly-Pro^{P}(OPh)$_{2,}$ which inhibits invasiveness in LOX cells,[4,5] as well as more general dipeptidyl peptidase inhibitors[6] have been successful both *in vitro* and in mouse models. These types of molecules, in addition to antibodies directed against seprase,[7-9] may be of great use especially in an ovarian cancer setting because of the ease of *i.p.* delivery. However, there is an argument against *i.p.* delivery of antibodies in cases of metastatic ovarian cancer due to an inability to penetrate established lesions.[10] In the absence of proper diagnostics to catch the cancer early, prior to establishment of secondary lesions, the development of an inhibitor to metastasis that is specific to tumor cells and largely innocuous to normal cells, maybe of great use in a prophylactic application with minimal side effects. Furthermore, although small molecule inhibitors and antibodies may effectively inhibit the proteolytic activity of

the individual proteases, the disruption of interactions between β1 integrin, seprase and DPP4 may prove to be a more potent weapon against invasion. Perhaps antibodies that target these interaction sites will be a promising avenue to explore.

PRODUCTION AND ACTIVATION

Seprase is known to colocalize with a number of molecules including β1 integrin at the invadopodial membrane surface.[11] Because ovarian carcinoma cells preferentially adhere to and invade type I collagen (TIC) this interaction has become a priority in the investigation of late stage ovarian cancer.[12] The research presented here suggests a potential mechanism of seprase induction via clustering of the mechanoreceptor of TIC, β1 integrin, at the cell surface in an ovarian carcinoma model as a result of TIC interaction. Importantly, integrin clustering has come to the forefront in the progression of ovarian carcinoma peritoneal lesion formation and appears to be a common mechanism for protease production targeting TIC.

Piecing together the pathway that stimulates seprase expression may elucidate other potential mechanisms for seprase inhibition. Its transient and inducible nature that is restricted to active fibroblasts or actively invading tumor cells suggests seprase to be a precise target ideally suited for inhibition in that peripheral effects resulting from specific inhibition would be limited. However, seprase, in soluble form, has been found in bovine serum[13] and determined to play a role in fibrinolysis because it is identical to antiplasmin-cleaving enzyme in human plasma samples.[14] This speaks to potential broader ramifications of inhibiting seprase because, if it is involved in clotting cascades, then general inhibitors may have broad and misdirected effects. If seprase plays a more expansive role beyond wound healing and the tumor microenvironment, it will be important to determine if the partners of seprase are different in these two arenas so that inhibitors may be most specifically targeted to the interaction between these associated molecules as not to disrupt other functions of the protease. Moreover, it will be interesting to decipher the source of the soluble and hematogenous seprase and deduce how is seprase packaged and exported to circulation. Whether in recombinant or blood borne form, soluble seprase lacks both the transmembrane and cytoplasmic domains.

Ascertaining the mechanism for cleavage of these domains and determining if initial transmembrane status required for dimerization are both areas that have not yet been explored.

Although originally identified as an integral membrane protease, we now know seprase can be activated via cleavage from the membrane and that further proteolytic processing can heighten its gelatinase activity; however the identities of these proteolytic activators have only been narrowed by EDTA sensitivity.[15,16] Other molecules necessary for seprase activity are beginning to emerge due to their colocalization with seprase, although their individual contributions have not been thoroughly described. It is clear however, that during tumor cell invasion, seprase does not work alone. In addition to seprase, other cell surface proteases and receptors that localize to the invadopodia include but are not limited to: DPPIV,[17-19] MMP-2 and MT1-MMP,[20,21] and the urokinase plasminogen activator receptor.[22] Coincidently, β1 integrin clustering has been found to be responsible for the production of many of these same players such as pro-MMP-2 and subsequent activation into MMP-2 by MT1-MMP.[23,24] It is possible that MT1-MMP could act in the same way to cleave seprase from the membrane. In addition, MMP-9 is also induced via β1 integrin ligation and is thought to target E-cadherin for ectodomain shedding which may facilitate *i.p.* dissemination of ovarian tumor cells.[25] An investigation into the role of these MMP's as a sheddase for seprase may reveal additional elements in the machinery involved in the tight regulation of seprase production and activation.

PRE-PROCESSING of TIC INTO TICg

Seprase is a gelatinase, which is able to act on the partially denatured collagen triple helical fiber or fully denatured gelatin strand.[17,26] For this reason it is likely that not only does seprase require activation by a sheddase, but the collagen triple helix must be at least partially unwound upon prior to seprase gelatinolytic activity. Candidates for this role are found in the MMP family of proteases.

The ability of MMP-2 and MMP-9 to cleave triple helical collagen remains a topic of debate. Some researchers suggest that MMP-2, MMP-9, and seprase are entirely

unable to cleave intact triple helical collagen.[26] They maintain that these three gelatinases work in concert with MMP-1, which, *in vitro*, causes a localized unwinding of triple helical collagen even under chelating conditions where MMP-1 cannot exert its own proteolytic activity. This same group showed that only the unwinding or partial denaturation of TIC is necessary for soluble seprase to exhibit its collagenase activity *in vitro*. Another coterie argues that both MMP-2 and MMP-9 can cleave triple helical collagen.[27,28] Advanced modeling techniques have called into question the true structure of triple helical collagen and proposed that the regular fibrillar structure should be replaced by a model that considers localized variations in structure.[29] Further confounding this relationship, is the argument that, under physiological conditions in solution, TIC does not form a tightly wound and regular triple helix, but rather coils randomly.[30] This leaves open the possibility that localized irregularities in intact TIC may be acted upon by proteases that have been classified solely as gelatinases on the basis of their *in vitro* activity.

Additional support for the role of multiple proteases acting on TIC in the ascites environment is that the molecular weight of TIC fragments recovered from patient ascites, are not the ¼ and ¾ length of TIC generated from MMP-2[27] and MMP-9[28] cleavage, but rather it is a lower molecular weight smear (preliminary data). Future investigations should focus on the ascites of late stage ovarian cancer patients and assess the major proteolytic players. Many of these proteases are soluble and may be evaluated via analysis of the supernatant.

INTRACELLLULAR EVENTS FOR SEPRASE PRODUCTION

In light of our findings that seprase is produced as a result of β1 integrin subunit clustering, future investigations should aim to compare these closely related collagenase inductions and examine the timing as well as other players involved. Specifically, the SRC kinase pathway should be investigated in detail as it relates to seprase induction. Not only has the aggregation of β1 integrin subunits been found to stimulate production of MT1-MMP, MMP-2 and MMP-9, but further elucidation of this mechanism has revealed that SRC kinase signaling induces transcriptional activation via EGR1.[31] If

induction of all of these collagenases and gelatinases converge within a single pathway, it would provide the perfect target to shut down tumor cell invasion and lesion formation.

When new adhesions are formed, it is necessary to suppress the production and activation of proteases until the time when they are needed to degrade the ECM and facilitate advancement of the cell. For this reason, the cyclic pattern is tightly regulated and sensitive to the extracellular environment. Antibodies against β1 integrin have been used to show this receptors role in many facets of tumor cell adhesion and invasion. These antibodies have inhibited disaggregation of spheroids[32] and interfered with adherence to TIC substrates[33,34] and mesothelial cells.[35] Moreover, β1 integrin antibodies have induced production of MMP-2[36] and disrupted SRC's action on E-cadherin.[37] Taken together it is clear the β1 integrin has a centralized role in regulating cell adhesion and invasion. Further investigation into both the structural and regulatory role of β1 integrin's stimulatory effect on seprase production may result in better targets for inhibition.

Additional experiments that focus on the comparison of LOX cells, which constitutively express seprase, with cells that can be stimulated to produce seprase such as the SB247 cell line, may also lend insight into seprase regulation. Examination of promoter sequences may reveal binding sites of activators and repressors that tightly regulate the production of seprase. Further, an examination of the state of β1 integrin on the surface of LOX cells versus inducible cells may reveal the source of constitutive seprase expression to be that β1 integrin is perpetually in the "on" conformational state.

UTILITY OF *IN VIVO* MODELS OF OVARIAN CANCER

Until now, seprase has not been closely examined in its role in ovarian carcinoma and specifically its function in the formation of secondary lesions that often accompany late stage ovarian cancer. Our utilization of a stably-transfected, fluorescently-labeled cell line that is derived from a patient ascites sample and application to a mouse model system, is a solid start to investigating adhesion and invasion in late stage ovarian cancer. Not only does this type of longitudinal study contribute to consistency, but it also aids in delineating the contributions of both the tumor and stromal cells and the interactions

between the two within the xenograft model. The labeled cells also facilitate recovery for microarray applications that may prove to be a useful tool in the identification of the major players of seprase-mediated tumor cell invasion. There are likely key transcription factors and co-activators that can be identified from these microarray results, which may reveal multiple targets against which therapies can be developed. Through the use of an siRNA and inducible system[38] systematic knock down experiments can be conducted on individual candidates derived from those microarray experiments and followed by combinatorial permutations of gene suppression may reveal the cornerstone of ovarian tumor cell adhesion and invasion.

There are of course inherent problems with using a mouse to model human ovarian cancer and predict the utility of antibodies and small molecule inhibitors to disease progression. The most glaring issue is the difference in physiology, specifically the bursal membrane surrounding the ovary which is present in rodents, but absent in humans.[39] While this does not present a problem with the modeling of either early or late stage ovarian cancer, it does interfere with modeling the key transition from readily resected primary tumor development to the difficult to manage metastatic disease. Also, the commonly used immunodeficient mice, while readily accepting the tumor xenograft do not mount an immune response against antibodies and likely respond quite differently to small molecules thereby influencing therapeutic response and limiting their utility as models of human disease. Furthermore, even if human tumor cells are used, as was the case in our experiments, when metastatic lesions develop the stromal cells are of murine origin which create a disease progression context of dubious biological relevance. However, the use of companion animals such as dogs with naturally occurring cancers may provide an important resource to cancer researchers in that their physiology is more similar to humans than that of the mouse.[40,41]

There is a tremendous effort being made to understand and combat metastatic cancer. Studies focused on the composition of the tumor cell microenvironment and stimulatory signals received by tumor cells within the primary tumor prior to secondary lesion formation, are the key to preventing metastatic disease. Care should be taken with the models that are used in cancer research so that painstaking discoveries in this arena have the necessary biological relevance to human disease. Finally, investigations of the

unexplored topics presented here will further our comprehension of tumor cell adhesion and lesion formation and will contribute to development of precise therapeutic strategies against metastatic cancer progression.

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