

# Seprase Promotes Rapid Tumor Growth and Increased Microvessel Density in a Mouse Model of Human Breast Cancer

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## ABSTRACT

Seprase is a cell surface serine protease that is expressed to high levels by invading human breast carcinoma cells. To investigate the role of seprase in breast cancer, MDA MB-231 human mammary adenocarcinoma cells were engineered to express active seprase to high levels. All cells grow rapidly in cell culture. But differences are discovered when the cells are tested for tumorigenicity, growth, and microvessel density by implantation into the mammary fat pads of female severe combined immunodeficient mice. Control transfectants that do not express seprase grow slowly whereas cells that express seprase to high levels form fast-growing tumors that are highly vascular. Microvessel density is elevated in tumors of two different lines of seprase transfectants to  $146 \pm 67.4$  and  $144 \pm 33.42$  vessels/mm<sup>2</sup> as compared with  $50.5 \pm 12.9$  vessels/mm<sup>2</sup> for tumors of control-transfected cells that do not express seprase. Seprase-expressing cells are better able to attract blood vessels and exhibit rapid tumor growth.

## INTRODUCTION

Breast cancer is characterized by unregulated growth and angiogenesis. These disease processes are mediated in part by the aberrantly high cell surface protease activities of breast cancer cells. Seprase, also called fibroblast activation protein- $\alpha$ , is a cell surface protease that is overexpressed in breast cancer (1, 2) and other cancers such as melanoma (3). Moreover, tumor tissues from breast cancer patients have aberrantly high levels of seprase activity (4). But the role of seprase in the pathobiology of cancer is not well understood. Seprase has long been implicated in promoting tumor cell invasion (5–7). Indeed, investigation of melanoma cell lines revealed that those with high levels of seprase were most invasive (3, 5, 8). Moreover, seprase-expressing melanoma cells degrade extracellular matrix more extensively than cells with low levels of seprase (5). However, the role of seprase in cell-mediated matrix degradation is not clear because inhibitor studies suggest that matrix metalloproteinases are responsible for the bulk of matrix degradation (9, 10). Moreover, recent results also indicate that seprase can promote tumor growth (11, 12).

Seprase is a type II integral membrane protein that is a member of the S9b family of postprolyl peptidases (7, 13). As such it can cleave the Pro-Xaa peptide bond at the NH<sub>2</sub>-terminus. This ability to cleave the prolyl peptide bond is limited to relatively few enzymes, the best studied of which is another S9b protease, dipeptidyl peptidase IV (DPPIV). Indeed, seprase and DPPIV are closely related, having identical domain structure, 50% overall amino acid sequence homology, and 70% amino acid sequence homology in the catalytic domain (14). Seprase and DPPIV are serine proteases that have the catalytic domain arranged in the nonclassical order. Seprase and DPPIV are multifunctional proteases that possess gelatinase activity as well as

amino dipeptidase activity (3–6, 15, 16). The considerable overlap of substrate specificity between seprase and DPPIV indicates that clues to seprase function can come from what is known about DPPIV. Notably, DPPIV can change the receptor selectivity and specificity of bioactive peptides (13).

Here, the behavior of breast cancer cells engineered to overexpress seprase was investigated. We demonstrate that seprase promotes growth and increased microvessel density in tumors of breast cancer cells. Together these findings indicate that seprase expression stimulates breast carcinoma cells to grow in part by activating the stromal response to tumors.

## MATERIALS AND METHODS

**Seprase cDNA and Transfection.** A wild-type, full-length seprase cDNA was prepared by reverse transcription-PCR from MDA MB-436 human breast cancer cells as described earlier (11). The full-length and wild-type sequence was confirmed by sequence analysis of both strands of the seprase cDNA. Cells were stable-transfected with pcDNA 3.1 with or without seprase cDNA using Lipofectin reagent as recommended by the supplier (Life Technologies, Inc.). After 2 days, culture media were replaced with 400  $\mu$ g/ $\mu$ l G418, 5% fetal bovine serum, 1% penicillin-streptomycin, 1% insulin, and  $\alpha$ -MEM for selection.

**Cells.** MDA MB-231 human breast adenocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Transfecting MDA MB-231 cells produced two lines that overexpress seprase. The WTY-1 and WTY-6 seprase-expressing lines were isolated by fluorescence-activated cell sorting (FACS) of mixed populations that survived G418 selection. Cells positive for surface expression of seprase were identified with monoclonal antibody (mAb) F19 to seprase. FACS confirmed lack of seprase expression in the corresponding control transfectants. The control transfectants of MDA MB-231 cells (Neo), WTY-1, and WTY-6 lines are polyclonal but stable for expression of seprase over multiple passages.

**Flow Cytometry.** Flow cytometric analysis determined levels of cell surface seprase expression. Adherent Neo, WTY-1, and WTY-6 cells were grown to 70–90% confluence in 75 cm<sup>2</sup> flasks in serum containing culture media. Cells were washed with PBS and then incubated with trypsin-EDTA for 2 min at 4°C to detach cells and were washed once with PBS. Gentle scraping was used to ensure cell detachment. Hybridoma cells producing the F19 mAb directed against fibroblast activation protein- $\alpha$ /seprase were purchased from the American Type Culture Collection. Cells were incubated with 1 ml of F19 mAb containing hybridoma growth media or serum-containing culture media (control) on ice for 45 min. Cells were washed once with PBS and then incubated with a secondary FITC-conjugated goat antimouse IgG (Amersham Pharmacia, Piscataway, NJ) on ice for 25 min. Cells were washed with PBS and suspended in 400  $\mu$ l of PBS. Cells were analyzed using the Becton-Dickinson FACScan, FACStar, with turbo sort operated by Ashley Whitlow in the Department of Microbiology and Immunology (University of Arkansas for Medical Sciences, Little Rock, AR).

**Cytospin.** Cells ( $10^4$ /cytospin) were centrifuged onto a glass microscope slide, air dried, and then fixed in methanol at  $-20^\circ\text{C}$ . Cells were treated with 3% H<sub>2</sub>O<sub>2</sub> for 15 min then washed with PBS and blocked with 5% (w/v) nonfat dry milk in PBS. After washing with PBS, the cells were incubated with undiluted F19 hybridoma supernatant overnight at 4°C. The cells were washed with PBS and then incubated with biotinylated goat antimouse IgG (1:500; Vector, Burlingame, CA) for 30 min at room 22°C. The cells were washed with PBS and treated with avidin-biotin complex method kit (Vector), washed with PBS, and exposed to 3,3'-diaminobenzidine (Vector). The cells were counterstained with hematoxylin and coverslipped with Permount (Fisher).

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**Preparation of Cell Extracts Enriched for Seprase.** The procedure is modified from that reported previously (5, 11). Cells were grown to 90% confluence in two 75 cm<sup>2</sup> flasks. Media were removed, the cells washed three times with PBS (10 ml each wash), and then extracted with 5 ml of 2.5% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl (pH 7.5) at 4°C for 15 min with agitation. Adherent cell material was suspended by scraping, and the lysate was gently homogenized with 20 strokes of pestle A in a 15-ml dounce homogenizer on ice. Care was taken to avoid excessive foaming. The homogenate was transferred to a 15-ml conical culture tube and cleared by centrifugation for 10 min at 3000 × *g* at 4°C. Protein determinations were performed using the bicinchoninic acid assay (Pierce), and total protein used to isolate seprase was normalized. The supernatant was transferred to a fresh tube and incubated with 100 μl of packed wheat germ agglutinin agarose (Vector) with agitation at 4°C overnight. The wheat germ agglutinin agarose beads were collected by centrifugation, and the supernatant was removed. Seprase was eluted from the beads by suspending them in 50 μl of Laemmli SDS-PAGE sample buffer containing DTT and 10% *N*-acetylglucosamine, incubating on ice for 30 min. The mixture was transferred to a 1.5-ml microcentrifuge tube and centrifuged for 2 min at 10,000 × *g*. The supernatant was loaded directly onto zymograms or SDS-PAGE.

Gelatin zymography was performed as described previously (9, 17) and was used to evaluate seprase activity in cell extracts. Zymogram gels consisted of 10% (w/v) SDS polyacrylamide and 1 mg/ml gelatin.

**Immunoblots.** Seprase samples were loaded onto a 4–12% SDS-PAGE gel and subjected to electrophoresis. The proteins were transferred to a nitrocellulose membrane (0.45 μm) on a Hoefer semi-dry transfer unit at 100 mA for 45 min at 4°C. Free binding sites on the nitrocellulose were blocked with 5% nonfat dry milk, 0.1% Tween 20, and Tris-buffered saline for at least 1 h. The F19 antibody was applied to the membrane with no dilution for overnight at 4°C with agitation. Excess antibody was removed by washing the membrane six times with 0.1% Tween 20 in Tris-buffered saline. The secondary antibody was a horseradish peroxidase-conjugated goat antimouse IgG (Perkin-Elmer) diluted 1:2500 in blocking buffer for 30 min at room temperature with agitation. After six washes, the reactive bands were detected using a chemiluminescence detection system (enhanced chemiluminescence kit, Amersham).

**Cell Proliferation Assay.** Cells were seeded (5 × 10<sup>3</sup> cells/well) in triplicate in 96-well plates. Plates were then incubated for 0, 24, and 48 h in serum-containing or serum-free culture media at 37°C, 5% CO<sub>2</sub>. Cell number was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and reading the absorbance at 540 nm as described by others (18, 19). Growth data were analyzed using ANOVA between groups.

**Tumor Biology.** Cells (2 × 10<sup>6</sup>/site) of rapidly growing cells were suspended in serum-free medium and injected s.c. (100 μl/site) into the mammary fat pads of 4–6 week-old female severe-combined immunodeficient mice. Cells were implanted into two axillary and two abdominal fat pads of each animal. Seven animals were used to evaluate each cell line. Mice were checked every other day for palpable tumor masses. When tumors appeared, the height,

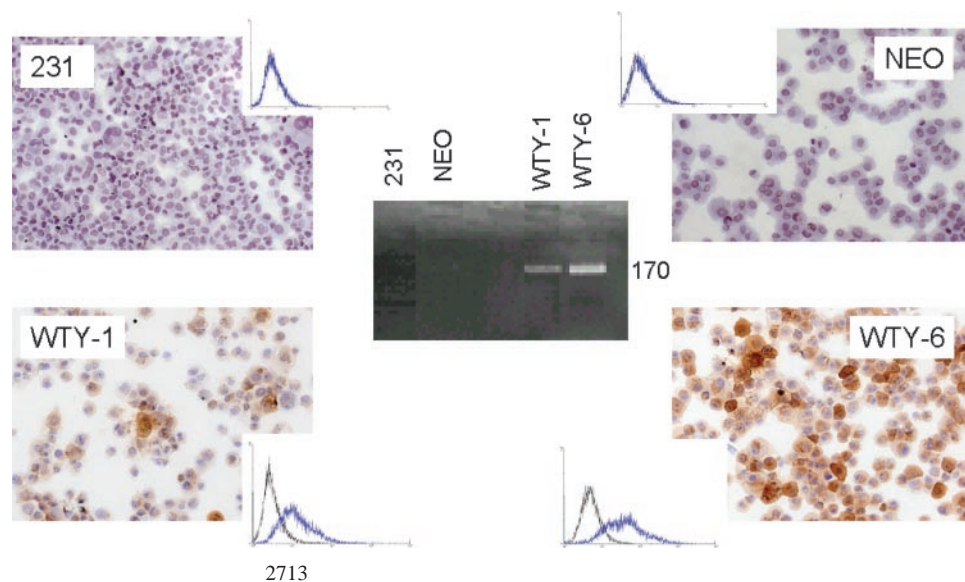
length, and width of the tumors were measured with a fine caliper. Although the animals were living, tumor volumes were determined by multiplying the height × length × width of the tumors. When any tumor reached 1.3 cm in any dimension, all animals in that group were anesthetized and euthanized by Institutional Animal Care and Use Committee approved procedures. The mice were subjected to complete necropsy. All tumor tissue was harvested, measured, weighed, and then several types of sample were prepared. Small tumors were cut in half, and one portion was fixed in paraformaldehyde and prepared for paraffin embedding and histological stains. The other portion was snap embedded in ornithine carbamyl transferase and snap frozen in liquid nitrogen to prepare frozen sections. The largest tumor from each animal was immediately prepared for FACS to determine seprase expression. To prepare for FACS, the tumor tissue was cut into fine pieces with a sharp razorblade and forced through a fine nylon mesh to separate the cells in 10 ml of PBS. The aggregates of cells were removed by centrifugation, and the cells in the supernatant were stained with mAb F19 and prepared for FACS as described above. Erythrocytes were lysed with ammonium chloride before analysis.

**Immunohistochemistry.** For microvessel determinations, paraformaldehyde-fixed and paraffin-embedded sections of primary tumors prepared in the Experimental Histology Core facility in the Department of Pathology (University of Arkansas for Medical Sciences) were deparaffinized and rehydrated. Epitope retrieval was accomplished by steaming slides for 20 min in citrate buffer [(pH 6.0) Zymed]. Endogenous peroxidase activity was quenched with 3% H<sub>2</sub>O<sub>2</sub> for 15 min at 22°C. Nonspecific protein-binding sites were blocked with 5% (w/v) nonfat dry milk in PBS. The sections were stained overnight with rat antimouse CD34 mAb (Hycult Biotechnology) at 1:20 dilution at 4°C. The sections were washed with PBS and stained with biotinylated rabbit antirat IgG (Vector) for 30 min at 22°C. Subsequent detection procedures are the same as described above for cytospin immunohistochemistry. The sections were observed and images captured using a Zeiss Axioskop 2 mot plus microscope and the Zeiss AxioCam MRc digital camera in the Digital Microscopy Laboratory at the Arkansas Cancer Research Center operated by Richard C. Kurten, PhD with the assistance of Ryan Vestal and Brian Shank.

## RESULTS

To investigate the role of seprase in tumor biology, MDA MB-231 human breast cancer cells that do not express seprase (11, 14) were engineered for stable expression of seprase. The seprase-expressing cells in two different subpopulations, called WTY-1 and WTY-6, were enriched by at least two rounds of FACS using F19 monoclonal antibody to seprase (20, 21). Thus, the control transfectants (Neo) and the seprase-expressing cells are polyclonal, mixed populations that maintain stable seprase expression phenotypes. FACS with F19 reveals cell surface expression of seprase in WTY-1 and WTY-6 cells that is not found in the parental MDA MB-231 or control-transfected

Fig. 1. Cytospins of cells (MDA MB-231, parental MDA MB-231 cells; Neo, control transfectants of MDA MB-231 cells; WTY-1, MDA MB-231 cells transfected with wild-type seprase cDNA; WTY-6, another line of MDA MB-231 cells transfected with wild-type seprase cDNA). Cells stained with F19 monoclonal antibody to seprase appear brown because of the 3,3'-diaminobenzidine reaction product whereas the blue hematoxylin detects all cells. Insets, corresponding FACS with F19 (blue trace) relative to control (black trace). Center, gelatin zymogram showing *M<sub>r</sub>* 170,000 gelatinase activity in extracts of WTY-1 and WTY-6 cells but not in extracts of parental and control transfectants. *M<sub>r</sub>* is indicated on the right × 10<sup>-3</sup>.



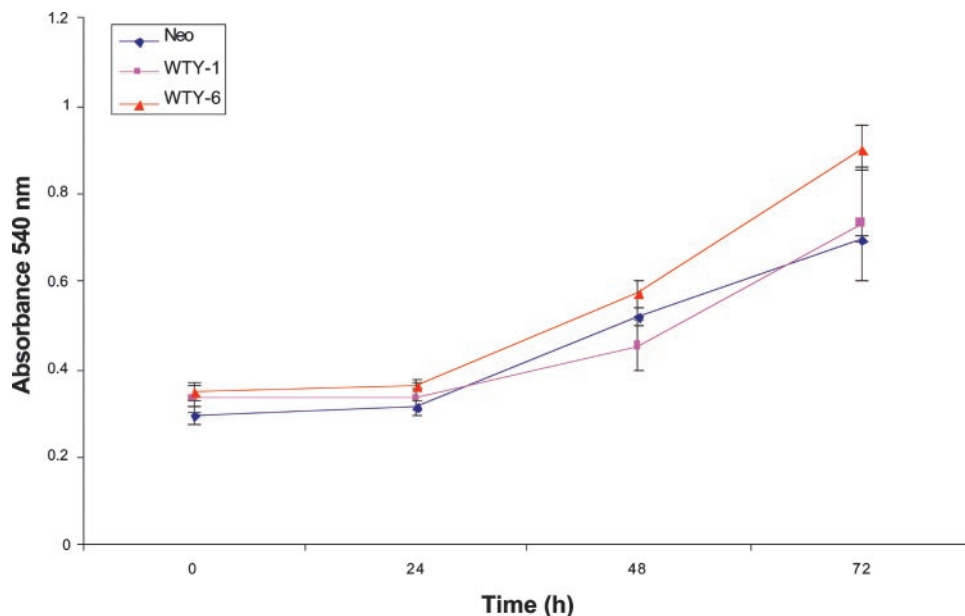


Fig. 2. Growth curves of control transfectants of MDA MB-231 cells (Neo), WTY-1, and WTY-6 cells in culture as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Neo cells (Fig. 1, insets, FACS, F19 blue trace, control black trace). FACS of the transfectants revealed that 70% of WTY-1 cells and 90% of WTY-6 cells are positive for cell surface seprase. Immunohistochemistry using F19 confirmed that parental MDA MB-231 cells and vector-only control transfectants (Neo) of these cells do not express seprase, but the WTY-1 and WTY-6 transfectants express seprase (Fig. 1; seprase-specific brown 3,3'-diaminobenzidine stain, blue hematoxylin counterstain). Notably, the cytopins reveal that a large fraction of the expressed seprase is intracellular as has been observed in carcinoma cells of breast cancer patients (Ref. 2; Fig. 1). Cell extracts were prepared to investigate the proper assembly and protease activity of seprase. Seprase was enriched using wheat germ agglutinin agarose, and the extracts were loaded to equal protein on gelatin zymograms. The  $M_r$  170,000 seprase activity is detected in WTY-1 and WTY-6 extracts but not in extracts of parental MDA MB-231 or Neo cells indicating that the seprase expressed by the transfectants properly assembles into the  $M_r$  170,000 form and that it is proteolytically active (Fig. 1, middle panel).

The growth properties of the Neo, WTY-1, and WTY-6 transfectants of MDA MB-231 cells were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (18, 19). Cells ( $3.34 \times 10^3$ /well) were seeded into the wells of 96-well culture plates in 100  $\mu$ l of growth media. At the specified times, the plates were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and absorbance at 540 nm was measured. Neo, WTY-1, and WTY-6 cells grew at similar rates over 72 h (Fig. 2). Thus in culture, seprase expression did not appear to confer a growth advantage.

**Expression of Active Seprase Increases Tumor Growth.** Distinct differences between the cells were observed for tumor growth *in vivo*. The Neo, WTY-1, and WTY-6 transfectants were used to investigate the role of seprase in tumorigenesis, tumor growth, and tumor microvessel density. Cells were injected into the mammary fat pads of female severe combined immunodeficient mice. Groups of seven mice were used for each cell line and  $2 \times 10^6$  cells/site were injected into two axillary and two abdominal mammary fat pads. Tumors of seprase-expressing breast cancer cells grew more rapidly than control transfectants that did not express seprase (Fig. 3; Table 1). The volume of each tumor was determined by measuring its length, width, and height. The average tumor volume for all tumors in

each group was determined daily and plotted until any tumor reached the predetermined maximum allowable size of 1.3 cm in any dimension (Fig. 3). The WTY-1 transfectants grew most rapidly, yielding palpable tumors 11 days postinoculation, and the largest tumor in the group reached the maximum size after 25 days (Fig. 3). The WTY-6 transfectants appeared to grow more slowly (Fig. 3). Palpable tumors were detected after 19 days, and the largest tumor reached the size cutoff at 38 days. Both seprase-expressing groups grew more rapidly than Neo transfectants that had very small but palpable tumors detected after 19 days that did not enlarge appreciably over 41 days, and no tumor of Neo cells reached the cutoff size in this timeframe. This slow growth of tumors of Neo cells was comparable with that of tumors of parental MDA MB 231 cells (not shown), and tumors of both cell types grew somewhat more slowly in severe combined immunodeficient mice than reported previously (22). Interestingly, all tumors, including the small tumors of the Neo cells showed evidence of invasion into surrounding tissues, particularly muscle (not shown). This is consistent with previous reports of the invasiveness of MDA MB-231 cells in animal models (22) and suggests that the Neo cells were not impaired by the transfection and selection procedures. Seprase expression confers a growth advantage on tumor cells growing *in vivo* that is not apparent in cells growing in culture (compare Figs.

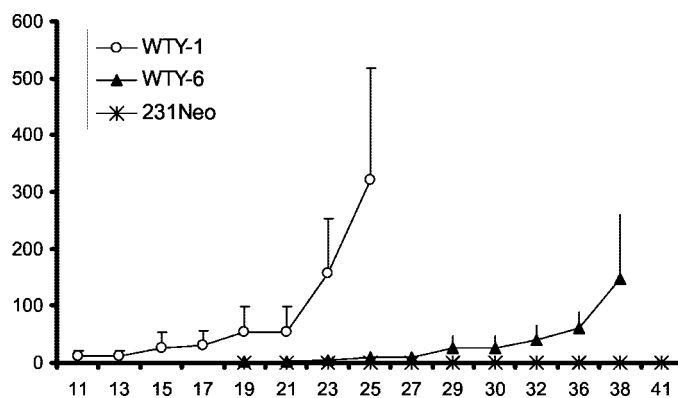


Fig. 3. Growth of tumors from WTY-1 cells ( $\circ$ ), WTY-6 cells ( $\blacktriangle$ ), and control transfectants of MDA MB-231 cells (Neo cells; asterisks). Tumor volumes were measured using calipers as described in the text.

Table 1 Growth and angiogenesis for tumors of breast cancer cell lines in SCID<sup>a</sup> mice

Cell line	Tumor take (tumors/injection sites)	Average weight of tumors (g)	Percentage of seprase positive tumor cells (FACS)	Metastasis (positive animals/total animals)	Microvessel density (avg. vessels/mm <sup>2</sup> )
Neo	19/28 (67.9%)	0.066 ± 0.038	2.37	No (0/7)	50.5 ± 12.9
WTY-1	23/28 (82 %)	0.96 ± 0.38 <sup>b</sup>	63	Yes (3/7)	146.25 ± 67.4 <sup>c</sup>
WTY-6	28/28 (100 %)	0.90 ± 0.26 <sup>b</sup>	72.63	Yes (5/7)	144 ± 33.42 <sup>c</sup>

<sup>a</sup> SCID, severe combined immunodeficient; FACS, fluorescence-activated cell sorter; Neo, control transfectants of MDA MB-231 cells.

<sup>b</sup> *t* Test: Neo/WTY-1,  $P = 0.0012$ ; Neo/WTY-6,  $P = 0.0002$  (significance indicated by  $P \leq 0.017$  after Bonferroni correction).

<sup>c</sup> *t* Test: Neo/WTY-1,  $P = 0.005$ ; Neo/WTY-6,  $P = 0.0003$  (significance indicated by  $P \leq 0.017$  after Bonferroni correction).

2 and 3). This is consistent with the hypothesis that seprase acts on the microenvironment of the tumor to facilitate growth.

The levels of seprase in the cells of the tumors were confirmed by FACS and by immunohistochemistry of frozen sections (Fig. 4). A large chunk of one tumor from each of the seven animals in the three groups was placed on ice immediately after harvest. The tumors were chopped into small pieces with a razor and forced through a fine mesh to separate the cells and prepared for FACS. Representative FACS results and immunohistochemical stains are shown (Fig. 4). FACS revealed that the expression of seprase remained very low in the Neo control transfectants (2.37% cells positive) and remained elevated in the WTY-1 (63% positive cells) and WTY-6 tumors (73% positive cells). These findings were confirmed by manually counting the seprase-positive cells in random fields of the sections.

#### Expression of Active Seprase Increases Microvessel Density.

For tumors to grow they must be able to integrate successfully with normal host tissues at the primary tumor site and at metastatic sites. Tumors must attract a blood supply to sustain growth. Therefore, the microvessel densities in tumors formed by cells expressing seprase (WTY-1 and WTY-6) and cells with low seprase levels (Neo) were determined. One tumor from each of the seven animals in the three groups was prepared for immunohistochemistry with antibodies to mouse CD34. Microvessel density was determined as described previously (23). Microvessel density was elevated in tumors expressing high levels of active seprase relative to control transfectants with low

seprase (Fig. 5). For seprase-expressing cells, the average microvessel density was 146 (WTY-1,  $n = 7$  mice) and 144 vessels/mm<sup>2</sup> (WTY-6,  $n = 6$  mice) but only 50.5 vessels/mm<sup>2</sup> (Neo,  $n = 6$  mice) for control transfectants (Table 1). These findings indicate that seprase-expressing cells are best able to induce the host stromal cells to produce microvessels.

## DISCUSSION

The results show that expression of seprase by breast cancer cells stimulates tumor growth by evoking a robust stromal response. Seprase-expressing cells grow in culture at rates similar to that of the control transfectants that do not express seprase, but when the cells are implanted into the mammary fat pads then seprase-expressing cells have a growth advantage. One aspect of the growth advantage is the greatly increased microvessel density in tumors of seprase-expressing cells compared with those of cells that do not express seprase. The ability to attract new blood vessels to the tumor is critical for sustained tumor growth. Thus, endothelial cells are often stimulated to produce new vessels by tumor cells and seprase apparently participates in this tumor-stroma "cross-talk." Recently, seprase and DPPIV were directly implicated in angiogenesis through their identification on endothelial cells by homology cloning and gene-specific reverse transcription-PCR screening for serine proteases (24). Seprase might activate angiogenesis-promoting peptides like DPPIV. For example,

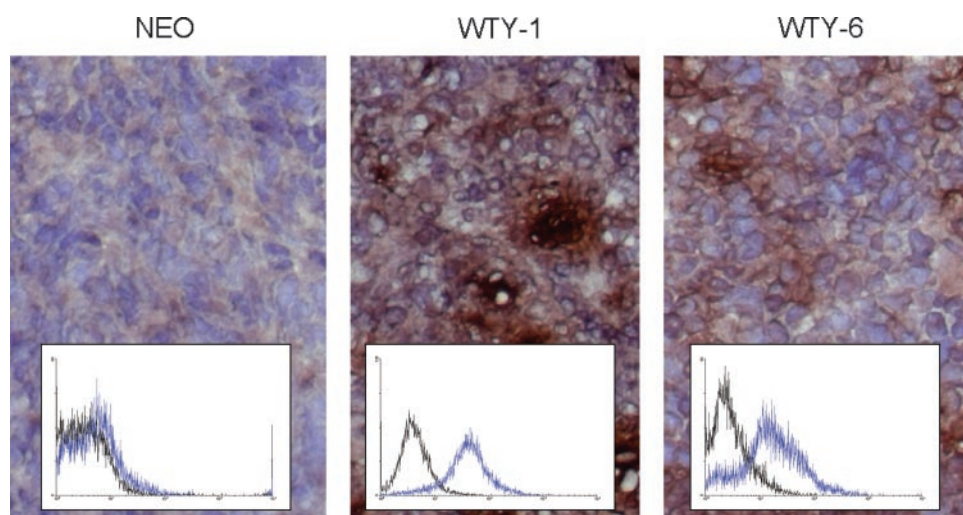


Fig. 4. Seprase expression in tumors of breast cancer cells was determined by immunohistochemistry (brown 3,3'-diaminobenzidine stain) and flow cytometry (insets). Neo, control transfectants of MDA MB-231.

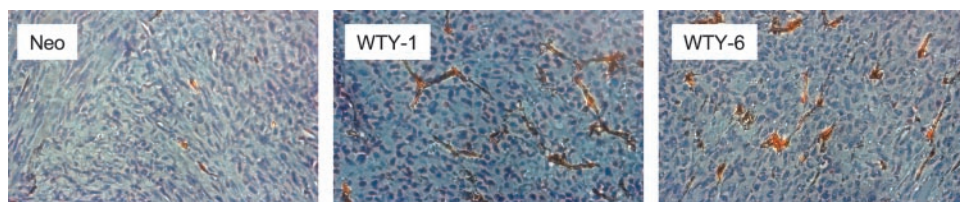


Fig. 5. Microvessel density of tumors of seprase-negative control transfectants of MDA MB-231 cells (Neo cells) and seprase-positive WTY-1 and WTY-6 cells as determined by immunohistochemistry with antibodies to CD34. Vessels are visualized as brown stained tubes and tumor cells are detected with hematoxylin counterstain.

removal of the NH<sub>2</sub>-terminal Tyr-Pro from neuropeptide Y (NPY) by DPPIV changes its receptor selectivity to make it proangiogenic (25, 26). NPY is a sympathetic cotransmitter that is released during nerve activation and ischemia (25, 26). Intact NPY causes vasoconstriction and smooth muscle cell proliferation primarily through interaction with the Y1 receptor. Cleavage of NPY by DPPIV results in loss of the NH<sub>2</sub>-terminal two amino acids forming NPY3–36 and terminates its interaction with the Y1 receptor. Instead, interaction of NPY3–36 with non-Y1 receptors, notably Y2 receptor is promoted. The Y2 receptor has proangiogenic functions and apparently promotes endothelial cell migration and tube formation in Matrigel (27, 28).

Seprase apparently has an important role in human breast cancer, but the precise nature of its function is unclear. All groups agree that seprase is induced and expressed to aberrantly high levels in human breast cancer (1, 2, 29). We investigated 27 cases of infiltrating ductal carcinoma of the breast and found that the carcinoma cells as well as the stromal cells express seprase (2). The finding of carcinoma cell expression of seprase is supported by the constitutive expression of seprase in several human breast cancer cell lines and by its expression in the malignant cells of a number of other carcinomas including melanoma and gastric cancer (11, 14, 30). On the other hand, two other studies of human breast cancer, including one survey of 112 cases, have indicated that seprase is detected primarily in the reactive stromal fibroblasts, suggesting that reactive fibroblasts are the main source of seprase in breast cancer (21, 29). Thus, there is debate about which cells express seprase in tumors of human breast cancer. However, regardless of its source, the presence of high levels of seprase in malignant breast and other epithelial tumors and its absence in normal adult tissues has prompted investigation of seprase as a target for therapies designed to kill tumor cells (31, 32).

A report by Ariga *et al.* (29) found that high seprase expression in stromal cells correlated with better prognosis and prolonged disease-free survival of patients with breast cancer. This finding is surprising because it is at odds with this study and other reports linking seprase expression by tumor cells to increased invasion and growth (5, 11, 12). The mechanisms underlying rates of tumor growth, disease progression, and ultimately patient outcomes are complex and dependent on many factors of which seprase expression is one. Further work is needed to elucidate the mechanisms by which seprase affects tumor growth to reconcile these apparently opposing observations.

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