The C-terminal 26-residue peptide of serpin A1 stimulates proliferation of breast and liver cancer cells: role of protein kinase C and CD47

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Abstract C26, the C-terminal 26 residue peptide of serpin A1, significantly increased cell proliferation in cultures of hepatoma cells, but not in porcine kidney epithelial cells, human skin fibroblasts or keratinocytes. The mitogenic activity of C26 was preferentially inhibited with a protein kinase C (PKC) inhibitor, an antibody against CD47 and CD47 short interfering RNA. The mutant C26-K19R,N22M, imitating a thrombospondin-like cell adhesion motif, increased the mitogenic activity in both Hep G2 cells and MCF-7 breast cancer cells. Phosphorylation of C26 at T24 (a putative PKC phosphorylation site) resulted in a 1.9–2.5 increase in mitogenic activity over C26 in MCF-7 cells.

Keywords: α1-Antitrypsin; β1-Integrin; CD36; Epidermal growth factor; Integrin-associated protein

1. Introduction

Serpin A1, also known as α1-antitrypsin or α1-proteinase inhibitor, is the best known and most abundant member of the serpin family of proteinase inhibitors and plays a key role in the control of homeostasis by neutralizing the deleterious effects of neutrophil elastase. This inhibition of serine proteinases may explain the action of serpin A1 in the suppression of cell growth in breast cancer cells, because it prevents the proteolytic release of membrane-bound transforming growth factor-α [1]. In most instances, serpin A1 is actually a growth factor in a variety of cells from epithelial, fibroblastic and hematopoietic origin [2–5]. We found that the mutation M351E in a recombinant serpin A1 fusion protein increased the anti-elastase activity without changing at all the mitogenic activity associated with the inhibitor [5]. This observation suggested that the mitogenic activity resides at a position different from the classical serpin active site loop. The only section of serpin A1 known to have biological activity independent of the active site loop is the C-terminal end of the molecule. Proteolytic cleavage of serpin A1 results in the release of a peptide comprising the last 36 amino acids (C36). This peptide has chemotactic activity [6], induces monocyte activation with a concomitant increase of proinflammatory cytokines [7], is a specific transcriptional downregulator of enzymes involved in bile acid synthesis in vitro and in vivo [8] and stimulates thymidine incorporation into ME 1477 melanoma cells [9]. We assumed that at least part of these biological activities resides within the last 26 amino acids of the peptide. In fact, the C-terminal 26 residue peptide contains the β-sheet structure identified in the three dimensional serpin A1-trypsin complex [10], as well as the binding site of the serpin–enzyme complex to its putative receptor in Hep G2 cells [6]. In the following study, we demonstrate that C26 is a mitogen for Hep G2 and MCF-7 cells and identify two additional sections of the molecule which could play a key role on cell proliferation.

2. Materials and methods

2.1. Materials

All cell lines used in this study were from the American Tissue Type Culture Collection, with the exception of human skin fibroblasts (Cambrex) and human skin keratinocytes (Invitrogen). Cell culture media were obtained from Invitrogen and Sigma. Epidermal growth factor (EGF) was from R & D systems. Serpin A1 and thrombospondin 1 were from Calbiochem. Alamar Blue was from Biosource. Albumin- and azide-free monoclonal antibodies against CD36 (clone 1A7), CD47 (clone B6H12.2) and β1-integrin (P5D2) were from Neomarkers (CD36, CD47) and Santa Cruz Biotechnology (β1-integrin). Myristoylated FARKGALRQ, a specific PKC inhibitor [11], was obtained from Sigma. Pre-designed double-stranded CD47 siRNA (ID#2811), GAPDH siRNA and siPORT amine were purchased from Ambion.

2.2. Synthetic peptides

Synthetic peptides C26, C26p, C26t (Fig. 1) and RFYGGMWK were obtained from the Sheldon Biotechnology Centre (McGill University, Montreal) and Alpha Diagnostic International. The peptides were purified by HPLC and the molecular weight was confirmed by mass spectrometry. The structure of C26 was further confirmed by amino acid sequence analysis (Sheldon Biotechnology Centre). The peptide 4N1-1 (RFYVVMWK) was obtained from Bachem. Performic acid oxidation of C26 was done by mixing 1 vol of hydrogen peroxide with 9 vol of 98% formic acid for 1 h. The formed performic acid was used to oxidize the dried C26 peptide for 2 h on ice.

2.3. Cell proliferation

Cell proliferation was assessed with a spectrophotometric assay using Alamar Blue, as previously described [12]. Briefly, cells were seeded into 96-well plates at a concentration of 3000 (Hep G2), 8000 (MCF-7) or 5000 (all other cultures) cells/well in 200 μl medium supplemented with 10% fetal bovine serum (keratinocytes were cultured in keratinocyte serum-free medium, Invitrogen). After 16 h, the monolayers

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Abbreviations: C26, PFVFLMIEQNTKSPLFMGKVVNPTQK; C26p, C26 phosphorylated at T24; C26t, PFVFLMIEQNTKSPLFMGRVVMPTQK; PKC, protein kinase C; EGF, epidermal growth factor; 4N1-1, RFYVVMWK; GAPDH, glycerylaldehyde-3-phosphate dehydrogenase; siRNA, short interfering RNA

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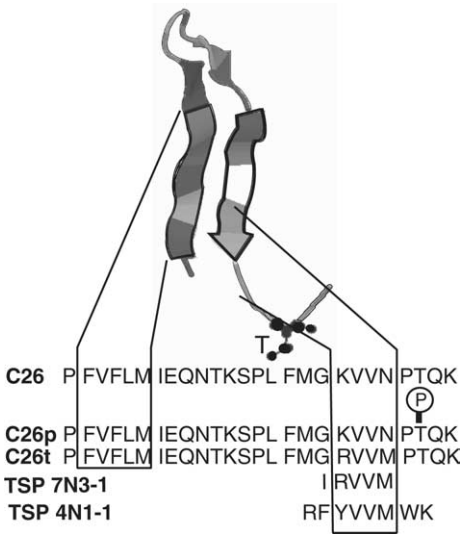

Fig. 1. Comparison of synthetic peptides based on the C-terminal structure of serpin A1. C26, amino acid sequence of the last 26 amino acids of serpin A1, showing in boxes the positions of FVFLM, the motif interacting with the serpin-complex receptor and KVVN, a sequence homologous to a thrombospondin cell adhesion domain. The 3D structure was designed with the RasMol program. C26p, C26 phosphorylated at threonine 24, a putative PKC phosphorylation site. TSP 7N3-1, the thrombospondin cell adhesion motif taken as a model for C26t, a modified C26 containing four of the five amino acids of TSP 7N3-1. TSP 4N1-1, the best studied peptide of thrombospondin, known to interact with CD47, as described in Section 3.

were washed two times with phosphate buffered saline containing 10 mM HEPES (pH 7.5) and incubated for 24 h with a serum-free medium to quiesce the cells. We used the same incubation medium for Hep G2 and MCF-7 cells. It was RPMI 1640 supplemented with 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 300 μg/ml fatty acid free, tissue culture tested bovine serum albumin (Sigma), 5 μg/ml bovine transferrin (ICN) and 10 μg/ml gentamicin. The cells were incubated with 100 μl of the peptide solutions prepared in this serum-free medium. 48 h later, the state of cell proliferation was measured as the increase in absorbance after an 8 h incubation with 10% (v/v) Alamar Blue in the serum-free medium. For siRNA experiments, the serum free medium was as indicated above but contained only 100 μg/ml albumin and no gentamicin. 4000 Hep G2 cells were plated per well. The liposome–siRNA complexes were prepared as indicated by the manufacturer (Ambion) and added to the cells only during the 24 h-quiescence period (0.75 μl siPORT amine per 100 μl medium per well). Cell proliferation was measured as indicated above.

3. Results and discussion

Although the biologically active, naturally occurring C36 terminal peptide of serpin A1 has been the subject of numerous studies, it is reasonable to assume that the active part of the peptide is localized at the terminal end comprising the β-sheet domain of the serpin A1–trypsin complex [10]. This structure was identified with the automated protein homology-modeling server "Swiss-Model" [13,14] of C26 and is depicted in Fig. 1. The C26 peptide includes the sequence FVFLM, originally identified by Joslin et al. [6] as the binding site to the serpin–enzyme complex receptor. After uptake, C36 interacts directly with transcription factors regulating bile acid synthesis. Mutations of the FVFLM site destroy this interaction [8]. Analysis of C26 with the Scanprosite program of Expasy (ca.expasy.org) revealed a protein kinase phosphorylation site at threonine 24. This finding would be irrelevant in a secreted protein such as Serpin A1. However, this site could be accessible for phosphorylation after uptake of the C-terminal peptide.

We investigated first the mitogenic activity of C26 (Fig. 2). The highest concentration tested (20 μM) is within the normal concentrations of serpin A1 in plasma. Under pathological conditions, such as acute leukemia, this concentration (including serpin A1 and serpin A1–elastase complexes) is substantially increased [15]. Fig. 2A shows that the peptide was able to stimulate cell proliferation in Hep G2 cells, one of the best-known target cells of C36. The degree of stimulation after a 48 h incubation in serum-free medium was substantial (55% over controls), because the well known Hep G2 mitogen EGF (20 nM) stimulated cell proliferation by only $42.2 \pm 14\%$ over controls under identical conditions (mean ± S.E.M., $P < 0.05$, $n = 8$). It is unlikely that blocking the inhibitory activity of endogenous serpin A1 may cause the C26-mediated increase in cell numbers. First, this serpin, at a concentration of 20 nM, known to significantly increase cell proliferation of HL60 cultures [5]was not inhibitory in Hep G2 cells ($1.5 \pm 5.6\%$ stimulation over controls, $n = 5$). Furthermore, the amount of Serpin A1 produced by Hep G2 cells in the serum-free conditions described here was very low (0.3 nM), as measured with a serpin A1 ELISA [16]. The mitogenic activity of C26

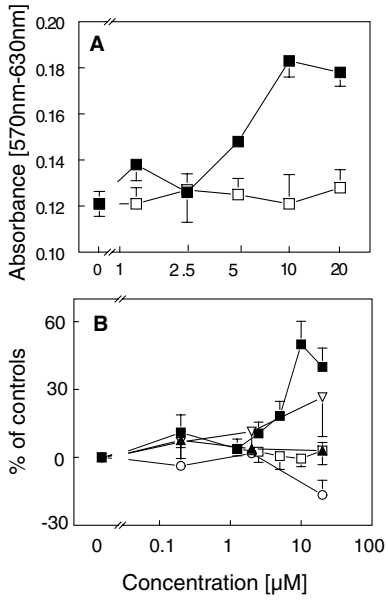

Fig. 2. Role of C26 in cell proliferation. (A) Comparison of the effects of C26 on cell proliferation in Hep G2 cultures before (closed squares) and after (open squares) treatment with performic acid, using the Alamar Blue technique as described in Section 2.3. Results from one representative experiment showing mean ± S.E.M. ($n = 4$). (B) Summary of all experiments using different cell lines. The results are means ± S.E.M. expressed as percent stimulation over control cell cultures. The squares correspond to the incubation of Hep G2 with C26 ($n = 11$) and oxidized C26 ($n = 5$). Open triangles, cultures of human skin keratinocytes ($n = 10$–13). Closed triangles, Porcine kidney epithelial cells ($n = 6$). Circles, human skin fibroblasts ($n = 8$–9). The stimulation of cell proliferation in Hep G2 cells was significant according to analysis of variance and Student–Newman–Keuls multiple comparison test: $P < 0.05$ (5 μM), $P < 0.001$ (10 μM), $P < 0.001$ (20 μM). The inhibition observed with 20 μM C26 in skin fibroblasts was significant ($P < 0.05$, paired t test).

was lost after performic acid oxidation, probably due to the presence of two methionines at the two opposite sites of the hairpin structure of C26, one of them within the putative binding site of the serpin–enzyme complex receptor. The mitogenic activity was rather specific for hepatoma cells, because no significant increase of cell proliferation could be seen in human skin fibroblasts or porcine kidney epithelial cells (Fig. 2B). The increase of keratinocytes observed in the presence of 20 μM C26 was non-significant. Surprisingly, the decrease in cell numbers in cultures of skin fibroblasts at the same concentration was significant ($P < 0.05$, paired t test, $n = 8$). These experiments suggest that the mitogenic action of C26 is particularly relevant in cancerous epithelial cells, with no significant changes in normal epithelial cells and a marginal, but significant decrease in skin fibroblasts at the highest concentration tested.

We investigated the possible involvement of PKC on the action of C26 on cell proliferation. Hep G2 cells were incubated with 20 nM EGF or 20 μM C26 in the presence of increasing concentrations of myristoylated FARKGALRG, a specific PKC inhibitor. The cells were incubated as usual for 48 h and proliferation was measured with the Alamar Blue technique (Fig. 3A). The results are expressed as percent inhibition as compared with the action of the growth factors alone (= 100%). The mitogenic activity of C26 was significantly more sensitive to the action of the inhibitor than that observed with EGF. These results suggest that PKC plays a more important role in the mitogenic action of the peptide than that observed in EGF signaling. This could have something to do with the PKC phosphorylation site at T24 in C26, as will be discussed later.

Uptake of the serpin A1 terminal peptide alone or as part of the serpin–enzyme complex has been studied in different cell lines, including hepatoma cells and monocytes. Three membrane receptors have been implicated in this process: the serpin–enzyme complex receptor, the LDL receptor and the multifunctional CD36 scavenger receptor [6,17,18]. We investigated the involvement of CD36 (also known as the thrombospondin receptor) by incubating the cells with and without C26 in the presence of a specific blocking antibody against CD36 (Fig. 3B, circles). As a control, we used an antibody against CD47, known to recognize a different peptide motif of thrombospondins (Fig. 3B, squares). Surprisingly, the CD36 antibody did not have any effect on the action of C26 on cell proliferation, whereas the antibody against CD47 significantly reduced the proliferating effects of C26. These results suggest that CD47 (also known as the integrin-associated protein, IAP) is involved in the action of the peptide. It could be argued that the CD47 antibody could act not only as a function blocker but also as a ligand. Therefore, we tested the action of another antibody which blocks β1-integrins, membrane receptors mediating the action of thrombospondin in MCF-7 cells [19] and modulating the action of CD47 in smooth muscle cells [20]. The inhibition of C26-mediated cell proliferation by the antibody against β1-integrin (73 ± 8%) as compared to controls (95 ± 10) was significant (Wilcoxon test, $P < 0.04$, $n = 6$) at an antibody concentration of 0.02 μg/ml. A more direct evidence of the involvement of CD47 in the action of C26 can be obtained by a sequence-specific, postranscripional gene silencing of CD47 with the method of RNA interference [21] (Fig. 3C). Hep G2 cells were transfected with small interference RNA (siRNA) for silencing GAPDH (a well-established control siRNA) and CD47 (designed using an algorithm developed by Cenix Bioscience-Ambion). The liposome mixture was removed and the cells were incubated for 48 h with C26 followed by a 24 h incubation with Alamar blue. The C26-mediated stimulation of cell proliferation was

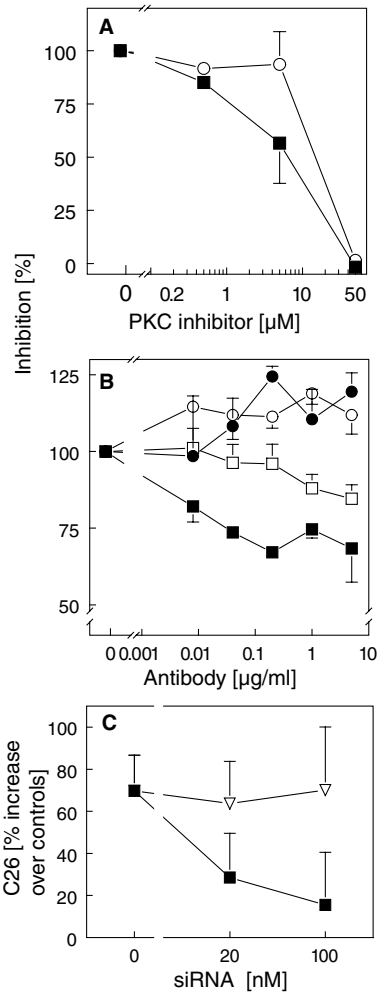

Fig. 3. Effect of protein kinase inhibitor, antibodies against CD36 and CD47 and small interference RNA for CD47 on the C26-mediated stimulation of cell proliferation. (A) Hep G2 cells were treated with 20 μM C26 (closed squares) or 20 nM EGF (circles) and increasing concentrations of the PKC inhibitor described in Section 2.1. Mean ± S.E.M. ($n = 7$). The decrease observed in the presence of 5 μM PKC inhibitor was significantly lower in cells treated with C26 as compared with those treated with EGF ($P < 0.025$, paired t test). (B) Representative experiments indicating the effect of increasing concentrations of antibodies against CD36 (circles) or CD47 (squares) in control cell cultures (white) and cultures in the presence of C26 (black). Mean ± S.E.M. of triplicate determinations. Further experiments indicated that the inhibition of C26-mediated cell proliferation by the antibody against CD47 as compared to controls was significant ($P < 0.05$) at the concentrations of 0.04, 0.2 and 1 μg/ml ($n = 4$, analysis of variance and Student–Newman–Keuls multiple comparisons test). (C) Hep G2 cells were transfected with increasing concentrations of CD47 siRNA (squares) and GAPDH siRNA as a control siRNA (triangles). One day after transfection C26 (20 μM) was added. Cell proliferation was measured by adding Alamar Blue after 48 h and continuing the incubation for 24 h. The difference between the reduction of the C26-mediated increase in cell proliferation using 100 nM CD47 siRNA as compared with that observed with 100 nM GAPDH siRNA was significant ($P = 0.031$, Wilcoxon test, $n = 6$).

significantly inhibited with 100 nM CD47–siRNA as compared with the effects seen with 100 nM GAPDH–siRNA ($P = 0.031$, Wilcoxon test, $n = 6$).

An analysis of the amino acid sequence of C26 revealed the presence of a KVVN motif, similar to the thrombospondin C-terminal cell adhesion motifs identified by Kosfeld and Frazier [22] with the synthetic peptides 7N3-1 and 4N1-1, shown in Fig. 1. The recently elucidated 3D structure of the C-terminal domain of thrombospondin 1 [23] indicates that both VVM peptides are also associated with β-sheet domains. In order to confirm the involvement of CD47 and the PKC phosphorylation site on the action of C26, we synthesized two C26 analogs: C26p, containing a phosphate group at T24 and C26t, in which K19 and N22 are substituted by R19 and M22. This creates the motif RVVM, containing four of the five amino acids of 7N3-1. Although 4N1-1 has been the peptide of choice for studies in connection with the integrin-associated protein CD47, we preferred to mimic the 7N3-1 motif, which required the change of only two amino acids.

Until now, a possible role of the serpin A1 terminal peptide on cell proliferation or thymidine incorporation has been observed only in cancer cell lines, such as the hepatoma cell line Hep G2, as described in this paper, and the melanoma cell line ME 1477 [9]. We thought that the breast cancer cell line MCF-7 could be a target for C26, because it shares many of the properties of Hep G2 cells. In fact, both cell lines are carcinomas (epithelial cell origin) and can synthesize large amounts of serpin A1 [1,24,25]. Therefore, C26, C26p, C26t and the commercially available thrombospondin-peptide 4N1-1 were added to cultures of Hep G2 and MCF-7 and the changes in cell proliferation were measured as indicated in Section 2.3. The three C26 peptides significantly stimulated cell growth in both Hep G2 and MCF-7 cells. In general, the modified peptides C26t and C26p had a higher mitogenic activity than C26, but the effects were quite different in the two cell lines. C21t, the peptide with the thrombospondin motif, significantly increased cell proliferation as compared with C26 in Hep G2 cells (5 μM, $P < 0.01$) and MCF-7 cells (20 μM, $P < 0.05$). The action of C26t was more effective in Hep G2 cells, corresponding to a shift of the dose–response curve towards low concentrations. This was not the case with the phosphorylated peptide, which did not significantly increase cell proliferation as compared with the effects of C26 (Fig. 4A). On the contrary, the mitogenic action of the phosphorylated peptide C26p was 1.9–2.5 times higher than that of C26 in MCF-7 cells (10 μM, $P < 0.001$; 20 μM, $P < 0.001$). In Hep G2 cells, the thrombospondin peptide 4N1-1 had no significant effect on cell proliferation. These results suggest that the attachment of the thrombospondin-like motif to the C26 β-hairpin plays a key role in the mitogenic activity, because it can not be efficiently replaced with the small thrombospondin peptide. We have also found that thrombospondin 1 (2 nM), under conditions which stimulate skin fibroblast cell proliferation (Congote et al., in preparation), did not have any significant effect in Hep G2 cells ($1 \pm 4.6\%$ stimulation over controls, $n = 7$). Furthermore, we were not able to detect any endogenous thrombospondin 1 in the medium conditioned by Hep G2 cells using an ELISA (limit of detection = 0.7nM). In MCF-7 cells, 4N1-1 by itself had a low, but significant mitogenic activity in this cell line. This could not be observed with a control peptide RFYGGMWK (20 μM, results not shown). It is unlikely that CD47 is directly involved in peptide uptake by internalization. This is the function of the other serpin A1-C36 receptors described above. However, CD47 could have a synergistic effect with the other receptors. The best example of this synergistic function of CD47 is the CD47-mediated enhancement of IGF-I receptor signaling in smooth muscle cells [26]. In this system, the VVM peptides of thrombospondin delay the de-phosphorylation of IGF-I receptors, enhancing downstream signaling.

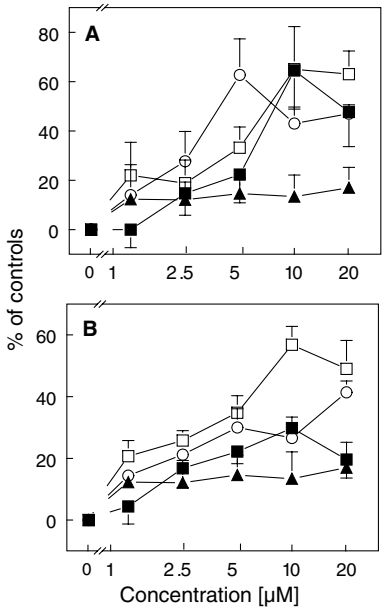

Fig. 4. Comparison of the effects of C26, C26p, C26t and 4N1-1 on cell proliferation. Hep G2 cells (A) and MCF-7 cells (B) were treated with increasing concentrations of the peptides and cell proliferation was measured as indicated in Section 2.3. The results are expressed as mean ± S.E.M. of six experiments in Hep G2 cells (except for the incubations of 4N1-1, $n = 5$) and of 5 experiments with MCF-7 cells. C26, closed squares. Phosphorylated C26 (C26p), open squares. C26 with a thrombospondin-like motif (C26t), circles. 4N1-1, triangles. Analysis of variance and Student–Newman–Keuls multiple comparisons test indicated that in Hep G2 cells, the stimulation of cell proliferation by C26, C26p and C26t was significant ($P < 0.001$ for 20 μM, $P < 0.001$ for 10 μM C26 or C26p, $P < 0.001$ for 5 μM C26t, $P < 0.01$ for 10 μM C26t and $P < 0.05$ for 5 μM C26p). In Hep G2 cells, C26t had a significantly higher stimulation than that observed with C26 at 5 μM ($P < 0.01$) or 5 μM C26p ($P < 0.05$). In Hep G2 cells, the differences between C26 and C26p were non-significant and the stimulation caused by 4N1-1 was non-significant. In MCF-7 cells (B), the stimulation of cell proliferation by C26, C26p and C26t was significant ($P < 0.001$ for all concentrations (2.5–20 μM), except for 2.5, 5 and 20 μM C26 ($P < 0.01$) and 2.5 μM C26t, $P < 0.01$). C26t had a significantly higher stimulation than that observed with C26 at 20 μM ($P < 0.05$). At 10 and 20 μM,C26p produced a significantly higher cell proliferation than that observed with C26 ($P < 0.001$). The stimulation observed with 4N1-1 in MCF-7 cells was significant (5 μM and 10 μM, $P < 0.05$; 20 μM, $P < 0.01$).

In summary, we have shown that the mitogenic activity of the C-terminal section of serpin A1 is localized within the last 26 amino acids. The activity of the peptide is sensitive to PKC inhibitors and T24 phosphorylation resulted in a significant increase of activity in MCF-7 cells. The mitogenic activity is increased by mutations mimicking a thrombospondin adhesion motif and inhibited with antibodies against the thrombospondin-binding membrane protein CD47.

Serpin A1 plays a key role in the neutralization of neutrophil elastase. Problems arise at the moment when the balance serpin-elastase is destroyed. Tumor growth and metastasis in-

volving breast cancer cells could be enhanced at sites of high proteolytic activity, in which there is an excess of serine proteases over serpin A1. This would lead to release of transforming growth factor-β [1], conversion of all available serpin A1 to serpin–enzyme complexes, binding and uptake of the C-terminal peptide and stimulation of cell proliferation.

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