AD_____

AWARD NUMBER: W81XWH-07-1-0424

TITLE: Annexin II Dependent Mechanism of Breast Cancer Progression

PRINCIPAL INVESTIGATOR: Mahesh C. Sharma, Ph.D.

CONTRACTING ORGANIZATION: Drexel University College of Medicine
Philadelphia, PA 19102

REPORT DATE: June 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 June 2009	Annual	1 June 2008 – 31 May 2009

4. TITLE AND SUBTITLE

Annexin II Dependent Mechanism of Breast Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0424

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Mahesh C. Sharma, Ph.D.

E-Mail: mahesh.sharma@drexelmed.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Drexel University College of Medicine
Philadelphia, PA 19102

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anti-cancer agents. Invasive and metastatic cancers of the breast are distinguished by their propensity of newly formed blood vessels (neoangiogenesis). Neoangiogenesis is a significant independent prognostic indicator in early stage breast cancer (1). Delineating the molecular mechanism(s) of neoangiogenesis may provide new insights into the biology of breast cancer progression and metastasis and may provide novel prognostic and therapeutic tools. Recently, the plasminogen (PLG)/plasmin (PL) system was demonstrated to play an important role in breast cancer progression and metastasis. Experimental studies in animal models combined with extensive clinicopathological data provide a compelling case indicating that proteins of PLG/ PL pathways play a key role in breast cancer progression and metastasis(2). In this context, enzymes of the PLG/PL pathway have been reported to have prognostic value in breast cancer and are associated with poor prognosis both for overall and disease free survival(2). In fact these molecules have been associated with a high rate of relapse for patients with breast cancer Preliminary studies in animal model demonstrated that PLG gene deficient mice (PLG-/-) display inhibition of tumor invasion, lymph node metastasis and angiogenesis supporting the idea that PL is required for angiogenesis, tumor growth and metastasis(3). Despite established role in tumor angiogenesis, growth and metastasis it is still unclear how

15. SUBJECT TERMS
Annexin II, angiogenesis, breast cancer, plasmin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	33	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction:

It has been recognized for decades that growth and development of breast cancer is dependent on angiogenesis (1). Weidener *et al* reported that microvessel density (either count or grade serves as a measure of tumor angiogenesis) in invasive breast carcinoma is associated with metastasis and, thus, may be a prognostic indicator (2,3). Increase in tumor microvasculature not only allows for rapid growth of tumors but may also provide the means for tumor cells to enter and exit the circulation during hematogenous tumor spread. In addition, endothelial cells (EC) may play a significant role in tumor progression by providing invading tumor cells with essential molecules necessary for extracellular matrix (ECM) degradation such as proteolytic enzymes [4]. Therefore, tumor angiogenesis plays an active and critical role in tumor progression and metastasis. Regulation of angiogenesis is a fundamental mechanism to control of tumor progression [1]. Using this novel approach, Folkman and colleagues identified angiostatin (AS), an internal fragment of plasminogen (PLG) spanning kringle 1-4 region, as one of the most powerful angiogenesis inhibitors [5]. These investigators further demonstrated 95% regression of human breast cancer by AS treatment in xenograft mice model without toxicity [6]. Later other investigators also demonstrated impressive anti-human breast cancer activity by AS gene therapy [7].

Recently, AS therapy has been reported to inhibit breast cancer induced bone metastasis [8]. Angiostatin was the first anti-angiogenic protein to enter therapeutic cancer clinical trials. Despite the potential anti-breast cancer therapeutic value of AS, it's clinical utility is hampered by limited availability of the recombinant bioactive AS. Human pharmacokinetics, particularly a short half-life in circulation [6] makes drug delivery challenging. Since discovery of AS, various mechanism(s) of action for AS have been suggested, including from our laboratory. Despite the identification of multiple receptors for AS [9-11], current knowledge of how AS inhibits breast cancer growth and metastasis is still unclear. If mechanism of action for AS is identified, more candidate drugs can be developed to target receptor(s). To delineate the AS's molecular mechanism we identified, purified and characterized a potential receptor for AS from EC surface [12, 13]. Using proteomics approach we have identified this protein as annexin II [12] and proposed a likely mechanism in angiogenesis. To explore its mechanism in breast cancer we have identified expression of AS's receptor annexin II in invasive human breast cancer cell line (MDA-MB231) in vitro and human ductal carcinoma in vivo [14]. Annexin II is one of the most abundant EC surface fibrinolytic receptors for PLG [15].

It is capable of converting inactive enzyme PLG to highly active protease plasmin (PL) almost 300 fold [16]. Consistent with previous observations we found that invasive MDA-MB231 cells expressing high levels of annexin II were also capable of converting PLG to PL with high efficiency. This is in contrast to poorly invasive cell line (MCF-7), which failed to convert PLG to PL (see preliminary data). Pericellular plasmin-mediated degradation of extracellular matrix (ECM) has been reported to induce tumor cell invasion, metastasis and tumor progression [17- 19]. In addition, plasmin librates matrix bound pro growth factors bFGF and VEGF during proteolysis of ECM [20]. Furthermore, plasmin mediated proteolytic processing of growth factors is required to induce cell proliferation and tumor progression [21]. Thus, annexin II may play a pivotal role in the **pro-anti-angiogenic switch mechanism** through precise regulation of

PLG and growth factors activation. Recent studies on PLG knockout mice (PLG-/-) reported an absolute requirement for plasmin in cancer invasion, angiogenesis and tumor progression [22-24]. This suggests that invasive breast cancer cells generate plasmin with a prominent role in ECM degradation, invasion for tumor progression and metastasis to distant sites. In this context, plasmin inhibitors have been tested in clinical setting as well as in xenograft mouse model of cancer and showed promising results [25-27]. In our laboratory we have made a direct attempt to

block in vivo annexin II mediated plasmin generation in mouse model of Lewis Lung Carcinoma

(LLC) and found remarkable inhibition of tumor growth by monoclonal antibody mediated

blocking of annexin II [28]. Bone is very common metastatic site for breast cancer. Emerging studies suggest that AS treatment inhibits MDA-MB231 induced bone metastasis through direct anti-osteoclastic activity [8]. It is interesting to note that annexin II increases osteoclast formation and bone resorbtion [29, 30]. These reports further support the link between annexin II and breast cancer metastasis and also strongly support our findings. It is likely that AS inhibiting breast cancer progression and metastasis by blocking annexin II functions in invasive breast cancer as we propose. Targeting this component of fibrinolytic system (PLG/PL) has yielded

exciting results in the war against cancer [31, 32]. It remains to be seen whether targeting fibrinolytic receptor annexin II will have clinical efficacy yet to be answered. Another central function of annexin II in the cell is its role in signal transduction mechanism. Annexin II is a calcium and phospholipid binding protein and major in vivo substrate for protein tyrosine kinase and PKC [33, 34]. It binds to the cytoskeleton protein actin and helps to organize into dynamic meshwork of actin fibers. Recent reports suggest that AS treatment induces the rise in intracellular calcium ($[Ca^{++}]_i$) through the PI-3 kinase signaling pathways [35], which requires reorganization of the actin cytoskeleton. Reports suggest that contact between breast cancer cells and EC induces an immediate and transient increase in intracellular $[Ca^{2+}]$ [36] indicating that signal transduction pathways are involved in these interactions. It is conceivable that targeted disruption of annexin II by AS treatment may disorganize actin microfilament architecture, affecting cellular physiology such as cell-cell interaction, migration and proliferation [37]. Targeted disruption of actin microfilament assembly has been demonstrated in invasive (MDA-MB231) breast cancer cell death and morphological changes in cell shape [38]. Annexin II has a limited tissue distribution and is not typically expressed in normal and mature organs such as liver and brain (Sharma et al, unpublished observations). However, its expression in liver cancer and brain tumor are highly up regulated [28, 39-41]. We found that quiescent EC do not express annexin II but exposure to growth factors up-regulates annexin II expression suggesting its possible role in cell proliferation, angiogenesis and tumor progression. Recently we reported that anti-annexin II antibody perturbs cell growth and induces EC cell apoptosis in a dose dependent manner; disrupting blood vessel formation in vitro [42]. These data suggest that disruption of the cell surface exposed annexin II may play a pivotal role in signal transduction mechanism.

One of the attributes that metastatic cells must develop is the ability to degrade the ECM in order to initiate tumor progression and induce metastatic spread. To accomplish this, metastatic cells may activate annexin II fibrinolytic activity to generate plasmin, which in turn cleaves basement membrane constituents to clear the path for cellular invasion and migration.This is one of the prerequisite steps of angiogenic and metastatic processes. It is likely that AS binding to annexin II acts as antagonist and may disable the plasmin generation capacity of the cell and potentially inhibits invasion [22] cell migration [43] and proliferation [44, 45].

Our novel studies in breast cancer indicate that annexin II mediated plasmin provides a model system with which to further probe the molecular mechanism underlying breast tumor progression. Annexin II protein expression appears to act as a tumor and metastasis promoter by cell surface mediated plasmin generation. Our preliminary data suggest that invasive breast cancer cells are equipped with the machinery necessary for degradation of ECM initiating angiogenesis and metastasis. It is not unreasonable to mention that AS mediated remarkable inhibition of breast cancer and metastasis [8] seen by Folkman and colleagues [6] may be due to blocking of annexin II and its mediated signaling. Results obtained from this study will establish the role of fibrinolytic receptor annexin II in angiogenesis, metastasis and breast tumor progression and may lead to design of effective breast cancer therapeutics.

BODY

We have successfully completed task 1 of this idea award as proposed. Part of the results of task1 has been reported in 2008 annual report. In this annual report we are reporting the results of the task1 (c) and d The results are summarized below.

Task 1 : To delineate the molecular mechanism by which breast cancer cells surface annexin II generate plasmin, which in turn facilitates ECM degradation, cellular invasion and migration leading to tumor growth and metastasis.

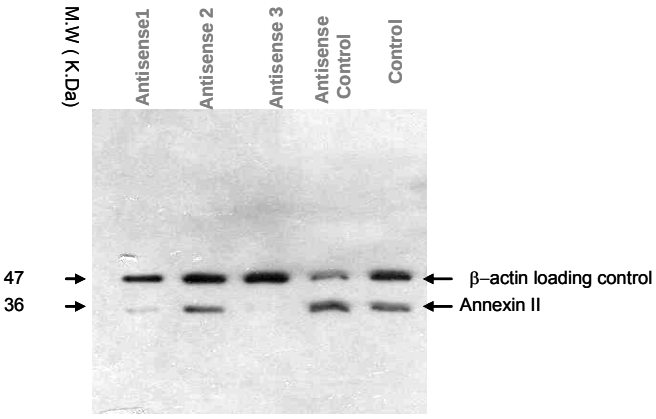
c) Study the role of annexin II in plasminogen activation using annexin II silenced cells (12-15 months)
d) Evaluate the therapeutic efficacy of monoclonal antibody to annexin II in mouse model of breast cancer.

RESULTS

Annexin II gene silencing and its role in plasminogen activation:

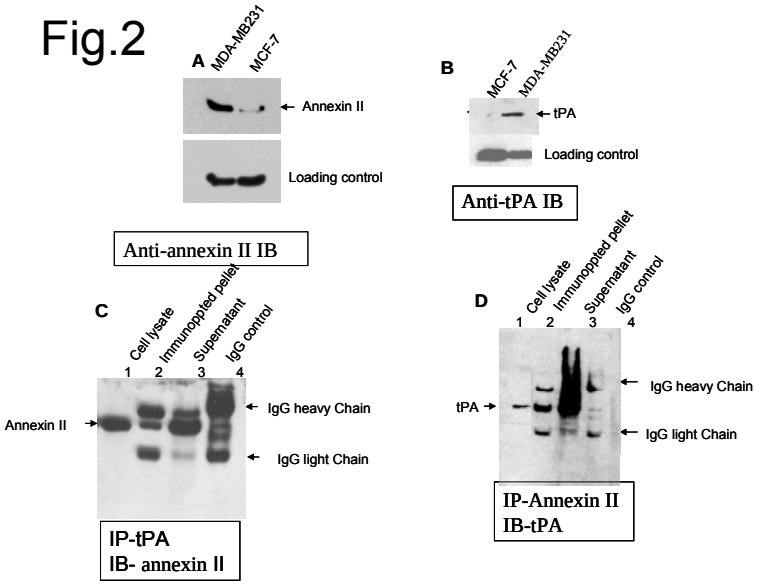
We have successfully suppressed annexin II gene in invasive breast cancer cells MDA-MB231. Results have been reported in 2008 annual report using RNAi approach. We have further shown that annexin II does not involved in MDA-MB231cell proliferation however; interestingly, we observed that annexin II suppressed breast cancer cells are less migratory as compared to wild type cells. We hypothesized that cell surface annexin II may regulate plasminogen activation which in turn may degrade extracellular matrix (ECM) and facilitates cell migration. We have tested this hypothesis using annexin II silenced cells.

Fig.1 : Silencing of annexin II gene using antisense strategy.



Role of annexin II in plasminogen activation: To investigate whether breast cancer cell surface annexin II is involved in plasmin generation, we used annexin II suppressed cells.

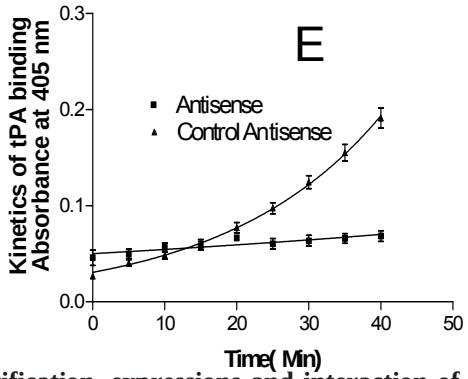
tPA binding to cell surface annexin II: tPA is a well established ligand for cell surface annexin II. Interaction of tPA with annexin II regulates plasmin production. This mechanism of ANX II/tPA induced plasmin generation in breast cancer has long been overlooked. To investigate whether tPA interaction with breast cancer cell surface annexin II is involved in plasmin generation. We investigated the expression of tPA and annexin II in human breast cancer cell lines MDA-MB231 (invasive and ER^-) and MCF-7 (non invasive and ER^+). Immunoblot analysis clearly demonstrates little or no expression of annexin II and tPA in MCF-7 cells. High expression of annexin II and tPA was observed in invasive MDA-MB231cells (Fig.2, panels A and B). Our results suggest that the expression of these proteins may be involved in the invasiveness of breast cancer. Since plasmin is implicated in the invasive behavior of breast cancer cells, we tested whether these proteins interact in order to activate plasminogen to plasmin. Our results identify that tPA and annexin II are specifically synthesized by invasive breast cancer cells. Next we investigated whether the biochemical interaction of tPA and ANX II is able to produce plasmin under physiological conditions. To test this, we investigated the requirement of annexin II for tPA binding using the gene silencing approach. Antisense ODN were used to suppress annexin II in MDA-MB231 cells (Fig.1). Using this approach we were able to suppress > 90% of annexin II expression (Fig.1). These annexin II silenced cells were used to investigate their ability to bind tPA. Cells were incubated with recombinant tPA for 30 minutes. Unbound tPA was removed by washing cells with PBS. These tPA bound cells were incubated with plasminogen to determine their ability to activate plasminogen to plasmin. Our results indicates that annexin II suppressed cells failed to bind tPA and were unable to activate plasminogen to plasmin suggesting that annexin II on the cell surface of breast cancer cell is critical for tPA binding and its dependent plasmin generation (Fig. 2 E and G). These results were further supported by the fact that the MCF-7 cells lacking annexin II expression failed to bind tPA and were therefore unable to activate plasminogen to plasmin (Fig. 2F). Contrary to this MDA-MB231 cells overexpressing annexin II bound to the tPA and efficiently converted plasminogen to plasmin (Fig.2F). Taken together, our in vitro data and ex vivo experiments clearly indicates that cell surface annexin II is required

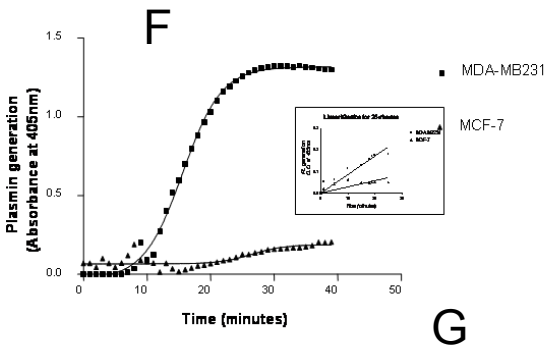


for tPA binding on breast cancer cells for efficient plasmin generation. Cells not incubated with tPA were used as a control in parallel experiments to rule out uPA dependent, as well as endogenous plasminogen activity. Absorbance from the control was subtracted from tPA dependent plasminogen activation activity.

Our data suggest that tPA binds to live cells surface possibly to annexin II. To confirm this we used biochemical techniques to provide evidence indeed ANX II interacts with tPA. Protein-protein interaction analysis determined by immunoprecipitation followed by Western blot analysis clearly shows that annexin II interacts with tPA (Fig.2 panels C and D).

Fig.2.





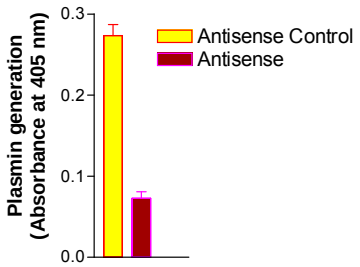


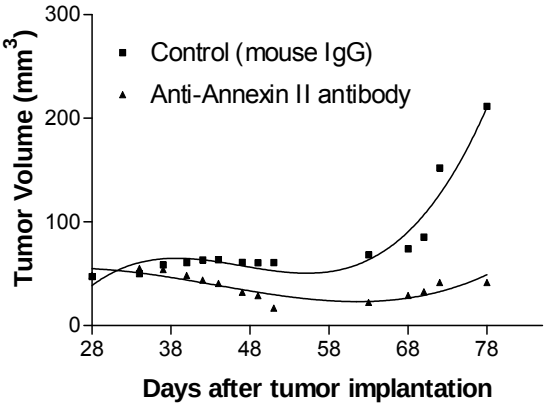
Fig.2. Identification, expressions and interaction of annexin II and tPA in human breast cancer cell lines: Breast cancer cell lysates (20µg) were separated by SDS-PAGE and protein expression was analyzed by immunoblot analysis**.** Figure displays selective expression of annexin II and tPA in invasive breast cancer cell line MDA-MB231 and poorly invasive cell line MCF-7 (Panels A and B). MDA-MB231 cell lysate was immunoprecipitated (IP) with anti-tPA antibodies and immune complex was electrophoresed and immunoblotted with anti-annexin II (Panel C, Lane 1 positive control, lane 2 IP pellet, lane 3 supernatant and lane 4 is IgG negative control). Panel D: cell lysate was immunoprecipitated (IP) with anti-annexin II antibodies and immune complex was electrophoresed and immunoblotted with anti-tPA (Lane 1 positive control, lane 2 IP pellet, lane 3 supernatant and lane 4 is IgG negative control). Co-immunoprecipitated pellet in lane 2 of both gels indicates interaction of tPA and annexin II.

Fig.2. E RNAi mediated suppression of annexin II gene inhibits cell surface tPA bindings and plasminogen activation. To confirm involvement of annexin II in plasmin generation and its dependent cell migration, annexin II gene was silenced. Three different antisenses were designed and synthesized as 16-mer dithionated oligonucleotide to silence annexin II gene. Immunoblot analysis suggests that two out of three antisenses were highly potent and almost completely silenced annexin II expression (Fig.1). β–actin was used as loading control to confirm equal protein loading. Quantitative analysis of band density shows more than 90% inhibition of annexin II expression as compared toβ–actin loading control (Fig.1). Annexin II silenced cells failed to bind tPA (Panel E)therefore significantly inhibited plasminogen activation (G). MCF-7 cells lacks annexin II expression were unable to activate plasminogen while MDA-MB231 overexpress annexin II were able to activate plasminogen efficiently (F).

Task 1. d) Evaluate the therapeutic efficacy of monoclonal antibody to annexin II in mouse model of breast cancer (15-20 months).

Cancer cell migration plays a pivotal role in neoangiogenesis (new blood vessel formation), tumor invasion, and metastasis (1). Numbers of published articles, including our own, have shown that plasmin destroys BM and degrades ECM which in turn facilitates cellular migration which is a requirement for tumors to become angiogenic and invasive (2-5). In this task we used monoclonal antibody to annexin II to block cell surface annexin II. We tested therapeutic efficacy of monoclonal antibody to annexin II.

The in vitro data presented in this report indicate that annexin II may be involved in tPA dependent plasmin generation in ER-ve invasive breast cancer cells. The excessive plasmin generation has been reported to play critical role in angiogenesis, tumor progression and metastasis specifically in breast cancer. Therefore, specific inhibitors of plasmin have been tested and showed promising results in vitro as



well as in mouse models. Since our data strongly suggest that cell surface annexin II is involved in plasmin generation in breast cancer, we investigated whether specific blocking of annexin II would inhibit human breast cancer growth by blocking plasmin generation. To investigate this, we used human breast cancer cells and implanted in nude mice. When palpalable tumor appeared, one group of animal was treated with monoclonal antibody to annexin II and other group was treated with equal dose of mouse IgG and considered as control. After treatment, tumor was measured and data were analyzed by GraphPad prizm software. Indeed, significant inhibition of human breast cancer growth was observed by a single dose of anti-annexin II antibody treatment (10 mg/kg of body weight) given intravenously. In contrast, exponential tumor growth was observed in IgG-treated control group. Fig. 3A shows a plot of relative tumor volume. Representative control and antibody-treated nude mice are showing tumor volume after antibody therapy (Fig. 3A). Histopathological examination of tumors is underway to determine tumor characteristic. Also we are evaluating the effect of anti-annexin II antibody on breast cancer metastasis. These findings will be reported in final report.

Fig. 3. Anti-annexin II monoclonal antibody therapy of nude mice bearing human breast cancer. Nude mice were implanted with invasive human breast tumor cells (MDA-MB231). Tumor bearing mice were treated with either anti-annexin II monoclonal antibody or mouse IgG (control) (10 mg/kg of body weight through tail vein) when palpalable tumors appeared. Tumor growth was monitored after a single dose of antibody treatment; the line graph represents relative tumor volume from five different animals (A). Photograph of representative animals from each group are shown in panel B.

Key achievements

We have successfully completed the tasks as we have planned in statement of work. Followings are key achievements.

Task 1

c) Annexin II gene silencing data clearly indicates that breast cancer cell surface annexin II is involved in plasmin generation

d) Anti-annexin II antibody appeared to inhibit human breast cancer growth in xenograft model.

Reportable outcomes:

Results will be presented in annual meeting of College of American Pathologist (CAP) meeting October 11-14, 2009 in Washington, DC. Abstract is accepted for publication in ***Archives of Pathology and Lab. Medicine*** Full manuscript has been submitted to Cancer Research *for* Review

Conclusions:

Based on our results we concluded that

1) Cell surface annexin II is crucial for tPA binding and its dependent plasmin generation

2) Annexin II may be a therapeutic target to inhibit invasive breast cancer.

References:

1. Folkman, J. Fighting cancer by attacking its blood supply. Sci Am, *275:* 150-154, 1996.
2. Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. N., Moore, D. H., Meli, S., and Gasparini, G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst, *84:* 1875-1887, 1992.
3. Weidner, N. and Folkman, J. Tumoral vascularity as a prognostic factor in cancer. Important Adv Oncol 167-190, 1996.
4. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med, *1:* 27-31, 1995.
5. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Cao, Y., Moses, M., Lane, W. S., Sage, E. H., and Folkman, J. Angiostatin: a circulating endothelial cell inhibitor that suppresses angiogenesis and tumor growth. Cold Spring Harb Symp Quant Biol, *59:* 471-482, 1994.
6. O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J. Angiostatin induces and sustains dormancy of human primary tumors in mice. Nat Med, *2:* 689-692, 1996.
7. Griscelli, F., Li, H., Bennaceur-Griscelli, A., Soria, J., Opolon, P., Soria, C., Perricaudet, M., Yeh, P., and Lu, H. Angiostatin gene transfer: inhibition of tumor growth in vivo by blockage of endothelial cell proliferation associated with a mitosis arrest. Proc Natl Acad Sci U S A, *95:* 6367-6372, 1998.
8. Peyruchaud, O., Serre, C. M., NicAmhlaoibh, R., Fournier, P., and Clezardin, P. Angiostatin inhibits bone metastasis formation in nude mice through a direct anti-osteoclastic activity. J Biol Chem, *278:* 45826-45832, 2003.
9. Troyanovsky, B., Levchenko, T., Mansson, G., Matvijenko, O., and Holmgren, L. Angiomotin: an angiostatin binding protein that regulates endothelial cell migration and tube formation. J Cell Biol, *152:* 1247-1254, 2001.
10. Tarui, T., Miles, L. A., and Takada, Y. Specific interaction of angiostatin with integrin alpha(v)beta(3) in endothelial cells. J Biol Chem, *276:* 39562-39568, 2001.
11. Moser, T. L., Stack, M. S., Asplin, I., Enghild, J. J., Hojrup, P., Everitt, L., Hubchak, S., Schnaper, H. W., and Pizzo, S. V. Angiostatin binds ATP synthase on the surface of human endothelial cells. Proc Natl Acad Sci U S A, *96:* 2811-2816, 1999.
12. Tuszynski, G. P., Sharma, M., Rothman, V. L., and Sharma, M. C. Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. Microvasc Res, *64:* 448-462, 2002.
13. Sharma, M. C., Sharma, M. R., and Berger, D. H. Identification of angiostatin binding protein in endothelial cells. J of Surgical Research, *86*, 1999.
14. Tuszynski, G. P., Sharma, M. R., Rothman, V. L., and Sharma, M. C. Over- expression of annexin II in invasive breast cancer cells in vitro and ductal carcinoma in vivo. Proc. Am. Assoc. Cancer Research 43, A#129, 2002.
15. Hajjar, K. A. and Menell, J. S. Annexin II: a novel mediator of cell surface plasmin generation. Ann N Y Acad Sci, *811:* 337-349, 1997.
16. Kassam, G., Le, B. H., Choi, K. S., Kang, H. M., Fitzpatrick, S. L., Louie, P., and Waisman, D. M. The p11 subunit of the annexin II tetramer plays a key role in the stimulation of t-PA-dependent plasminogen activation. Biochemistry, *37:* 16958-16966, 1998.

17. blasi, F. a. S., M.P Proteases and cancer invasion: from belief to certainty. Biochimica et Biophysica Acta, *1423:* R35-R44, 1998.
18. Blei, F., Wilson, E. L., Mignatti, P., and Rifkin, D. B. Mechanism of action of angiostatic steroids: suppression of plasminogen activator activity via stimulation of plasminogen activator inhibitor synthesis. J Cell Physiol, *155:* 568-578, 1993.
19. Bajou, K., Masson, V., Gerard, R. D., Schmitt, P. M., Albert, V., Praus, M., Lund, L. R., Frandsen, T. L., Brunner, N., Dano, K., Fusenig, N. E., Weidle, U., Carmeliet, G., Loskutoff, D., Collen, D., Carmeliet, P., Foidart, J. M., and Noel, A. The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic strategies. J Cell Biol, *152:* 777-784, 2001.
20. Rifkin, D. B., Mazzieri, R., Munger, J. S., Noguera, I., and Sung, J. Proteolytic control of growth factor availability. Apmis, *107:* 80-85, 1999.
21. McColl, B. K., Baldwin, M. E., Roufail, S., Freeman, C., Moritz, R. L., Simpson, R. J., Alitalo, K., Stacker, S. A., and Achen, M. G. Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. J Exp Med, *198:* 863-868, 2003.
22. Bajou, K., Devy, L., Masson, V., Albert, V., Frankenne, F., Noel, A., and Foidart, J. M. [Role of plasminogen activator inhibitor type 1 in tumor angiogenesis]. Therapie, *56:* 465-472, 2001.
23. Brodsky, S., Chen, J., Lee, A., Akassoglou, K., Norman, J., and Goligorsky, M. S. Plasmin-dependent and -independent effects of plasminogen activators and inhibitor-1 on ex vivo angiogenesis. Am J Physiol Heart Circ Physiol, *281:* H1784-1792, 2001.
24. Albo, D., Rothman, V. L., Roberts, D. D., and Tuszynski, G. P. Tumour cell thrombospondin-1 regulates tumour cell adhesion and invasion through the urokinase plasminogen activator receptor. Br J Cancer, *83:* 298-306, 2000.
25. Szende, B., Okada, Y., Tsuda, Y., Horvath, A., Bokonyi, G., Okamoto, S., Wanaka, K., and Keri, G. A novel plasmin-inhibitor inhibits the growth of human tumor xenografts and decreases metastasis number. In Vivo, *16:* 281-286, 2002.
26. Soma, H., Sashida, T., Yoshida, M., Miyashita, T., and Nakamura, A. Treatment of advanced ovarian cancer with fibrinolytic inhibitor (tranexamic acid). Acta Obstet Gynecol Scand, *59:* 285-287, 1980.
27. Lentschener, C., Li, H., Franco, D., Mercier, F. J., Lu, H., and Soria.J Introperatively administered aprotinin increases one year survival after elective liver resectionfor colorectal metastasis. Proteolysis and Fibrinolysis, *13:* 39-45, 1999.
28. Sharma, M., Rothman, V. L., Tuszynski, G. P., Croul, S. a., and Sharma, M. C. Antibody-directed targeting of angiostatin's receptor annexin II inhibits Lewis Lung Carcinoma tumor growth via blocking of plasminogen activation: Possible biochemical mechanism of angiostatin's action. J Biol Chem, *submitted*, 2005.
29. Menaa, C., Devlin, R. D., Reddy, S. V., Gazitt, Y., Choi, S. J., and Roodman, G. D. Annexin II increases osteoclast formation by stimulating the proliferation of osteoclast precursors in human marrow cultures. J Clin Invest, *103:* 1605-1613, 1999.
30. Takahashi, S., Reddy, S. V., Chirgwin, J. M., Devlin, R., Haipek, C., Anderson, J., and Roodman, G. D. Cloning and identification of annexin II as an autocrine/paracrine factor that increases osteoclast formation and bone resorption. J Biol Chem, *269:* 28696-28701, 1994.
31. O'Reilly, M. S., Pirie-Shepherd, S., Lane, W. S., and Folkman, J. Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. Science, *285:* 1926-1928, 1999.
32. Rabbani, S. A. and Gladu, J. Urokinase receptor antibody can reduce tumor volume and detect the presence of occult tumor metastases in vivo. Cancer Res, *62:* 2390-2397, 2002.
33. Boyko, V., Mudrak, O., Svetlova, M., Negishi, Y., Ariga, H., and Tomilin, N. A major cellular substrate for protein kinases, annexin II, is a DNA-binding protein. FEBS Lett, *345:* 139-142, 1994.
34. Keutzer, J. C. and Hirschhorn, R. R. The growth-regulated gene 1B6 is identified as the heavy chain of calpactin I. Exp Cell Res, *188:* 153-159, 1990.

35. Jiang, L., Jha, V., Dhanabal, M., Sukhatme, V. P., and Alper, S. L. Intracellular Ca(2+) signaling in endothelial cells by the angiogenesis inhibitors endostatin and angiostatin. Am J Physiol Cell Physiol, *280:* C1140-1150, 2001.
36. Lewalle, J. M., Cataldo, D., Bajou, K., Lambert, C. A., and Foidart, J. M. Endothelial cell intracellular Ca2+ concentration is increased upon breast tumor cell contact and mediates tumor cell transendothelial migration. Clin Exp Metastasis, *16:* 21-29, 1998.
37. Hall, A. Rho GTPases and the actin cytoskeleton. Science, *279:* 509-514, 1998.
38. Mgbonyebi, O. P., Russo, J., and Russo, I. H. Roscovitine inhibits the proliferative activity of immortal and neoplastic human breast epithelial cells. Anticancer Res, *18:* 751-755, 1998.
39. Masaki, T., Tokuda, M., Fujimura, T., Ohnishi, M., Tai, Y., Miyamoto, K., Itano, T., Matsui, H., Watanabe, S., Sogawa, K., and et al. Involvement of annexin I and annexin II in hepatocyte proliferation: can annexins I and II be markers for proliferative hepatocytes? Hepatology, *20:* 425-435, 1994.
40. Roseman, B. J., Bollen, A., Hsu, J., Lamborn, K., and Israel, M. A. Annexin II marks astrocytic brain tumors of high histologic grade. Oncol Res, *6:* 561-567, 1994.
41. Reeves, S. A., Chavez-Kappel, C., Davis, R., Rosenblum, M., and Israel, M. A. Developmental regulation of annexin II (Lipocortin 2) in human brain and expression in high grade glioma. Cancer Res, *52:* 6871-6876, 1992.
42. Sharma, M. C., Meena R. Sharma,Vicki L. Rothman, George P. Tuszynski Targeted disruption of annexin II inhibits angiogenesis in vitro and tumor growth in vivo. Proc. Am. Assoc. Cancer Research 43, A# 894, 2002.
43. Tarui, T., Majumdar, M., Miles, L. A., Ruf, W., and Takada, Y. Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of angiostatin. J Biol Chem, *277:* 33564-33570, 2002.
44. Chiang, Y., Rizzino, A., Sibenaller, Z. A., Wold, M. S., and Vishwanath, J. K. Specific down-regulation of annexin II expression in human cells interferes with cell proliferation [In Process Citation]. Mol Cell Biochem, *199:* 139-147, 1999.
45. Vishwanatha, J. K., Chiang, Y., Kumble, K. D., Hollingsworth, M. A., and Pour, P. M. Enhanced expression of annexin II in human pancreatic carcinoma cells and primary pancreatic cancers. Carcinogenesis, *14:* 2575-2579, 1993.
46. Sharma, M. R., Tuszynski, G. P., and Sharma, M. C. Angiostatin-induced inhibition of endothelial cell proliferation/apoptosis is associated with the down-regulation of cell cycle regulatory protein cdk5. J Cell Biochem, *91:* 398-409, 2004.

Annexin II and tPA interaction on breast cancer cell surface is required for efficient plasmin generation which in turn facilitates cell migration

Miriam Smith[1], Chalon Saunders[2], Ari D. Brooks[1], Robert T. Ownbey[2], Meena Sharma[3] and Mahesh C. Sharma[1,2]*

[1,2] From the Departments of Surgery and Pathology, Drexel University College of Medicine, [3]University of Pennsylvania, Philadelphia, PA 19102,

Footnotes:

This study was supported in part by grants from Pennsylvania Department of Public Health and Department of Defense Idea award # W81XWH-07-1-0424 and Concept award # DAMD1703-1-0761 to MCS. We thank Andrew Keibel for helping in preparation and organizing this manuscript.

Address correspondence to:

Mahesh C. Sharma, PhD

Associate Professor

Department of Surgery and Pathology

Drexel University College of Medicine

MS 435

245 N 15th Street, Philadelphia, PA 19102,

Email: Mahesh.sharma@drexelmed.edu

Conflict of interest:

No conflict of interest to disclose

Running Title: Role of annexin II in breast cancer progression

Key Words: Plasmin, annexin II, Breast Cancer, neoangiogenesis, tPA

ABSTRACT

Annexin II, a receptor for tissue plasminogen activator (tPA) and plasminogen, is absent from normal human breast tissue, and is over expressed in of human breast cancers. We have analyzed the expression of annexin II and its components in human breast tissue and in vitro using human breast cancer cell lines. We investigated annexin II-dependent potential mechanism of breast cancer progression. Annexin II was undetectable in normal breast ductal epithelial cells, ductal hyperplasia and in ductal complexes. Annexin II was found to stain ECM around the duct and blood vessels in normal breast tissues. By contrast, annexin II expression was consistently detected on the surface of DCIS, invasive and metastatic breast cancer cells. Large numbers of blood vessels were also found positive for annexin II. Annexin II staining correlated with CD105, a selective marker for proliferating endothelial cells, and tPA, indicating neoangiogenesis. Consistent with in vivo expression, annexin II and its ligand tPA expression was detected selectively in invasive human breast cancer cell line MDA-MB231. Both annexin II and tPA co localized in MDA-MB231 cells. Direct binding analysis showed that annexin and tPA interact, and this cell surface interaction accelerated plasmin generation efficiently. Annexin II induced plasmin generation facilitated cell migration, a key biological function required for neoangiogenesis and metastasis. These results suggest that annexin II-dependent plasmin generation in the tumor microenvironment could contribute to invasion and neoangiogenesis possibly by ECM degradation that makes annexin II an attractive target for anti-angiogenic and anti-cancer therapies.

Introduction

Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anti-cancer agents. The plasminogen/plasmin system, a serine protease pathway, is known to trigger angiogenesis and therefore plays a critical role in breast cancer (1). Analysis of the enzymes of the plasminogen/plasmin system suggests that high urokinase-type plasminogen activator (uPA) in human breast tumors predicts poor survival (2, 3) and is significantly associated with a high rate of relapse for patients with breast cancer(2). Despite the established role of the plasminogen/plasmin system in breast cancer, the receptor(s) which regulates plasminogen conversion to plasmin in breast cancer are poorly understood.

Annexin II is one of the most studied receptors for plasminogen and tissue plasminogen activator (tPA) (4, 5). It has been demonstrated that cell surface annexin II regulates plasmin generation (5, 6) which in turn facilitates extracellular matrix (ECM) degradation, cell invasion (6-8), and migration (9), leading to the formation of new blood vessels (neoangiogenesis) (10). Through this process annexin II regulates neoangiogenesis, metastasis, and tumor progression and is an independent prognostic indicator in cancer (6). The exact mechanism of the role of annexin II, however, is unclear. It is interesting to note that breast cancer growth and metastasis requires extensive neoangiogenesis (11). The fact is that microvessel density (MVD) in the area of the most intense neovascularization in invasive breast carcinoma is an independent and highly significant prognostic indicator for overall and relapse-free survival in patients with early-stage breast carcinoma (11). tPA is a ligand for annexin II secreted by endothelial cells, and plays a major physiological role in maintaining blood fluidity in vessels (5, 12). However, emerging reports and clinical observations link tPA to neoplastic transformation and the invasive phenotype of highly aggressive tumors such as glioblastoma (13), melanoma and pancreatic cancer(14). The function of tPA and its receptor annexin II in breast tumor biology is not well-understood. For the first time, we have shown that annexin II is selectively expressed in the invasive phenotype of human breast cancer. Interestingly, tPA, a ligand which binds to cell surface annexin II has also been associated with the invasion of highly aggressive MDA-MB-435s breast cancer cells (15).

These independent investigations provide strong links between tPA and annexin II in breast cancer progression/metastasis.

Since excessive plasmin generation has been linked to invasive and metastatic breast cancer, molecular switches which specifically regulate plasmin generation in the tumor microenvironment may be of immense importance. Receptor(s) which specifically regulate plasmin generation in the tumor microenvironment may provide a clue for the mechanism of neoangiogenesis, tumor progression and metastasis. Neoangiogenesis is an essential component of tumor progression during which new blood vessels nourish growing tumors and facilitate rapid tumor expansion. The prospect of inhibiting human cancers with angiogenesis inhibitors is becoming more realistic as knowledge behind angiogenesis and angiogenesis inhibitors has progressed.

We hypothesize that interaction of annexin II with tPA on the surface of breast cancer cells surface stimulates plasmin production. Excessive plasmin generation may trigger an angiogenic switch by ECM degradation which facilitate migration and invasion. In this report we have provided evidence that tPA synthesized by breast cancer cells interacts with cell surface annexin II, and induces plasmin production which facilitates cell migration and plays a pivotal role in cell migration. Annexin II may be a potential target for the development of effective therapeutic strategies for the treatment of breast cancer.

Materials and Methods

Human Lys-plasminogen, plasmin and recombinant tPA was purchased from Calbiochem, (La Jolla, CA). Lysine–Sepharose and ε-aminocaproic acid (ε-ACA) were procured from Sigma (St. Louis, MO). Chromatography and electrophoretic reagents were procured from BioRad, (Richmond, CA). Anti-plasminogen monoclonal antibody was purchased from Enzyme Research Inc. (Chicago, IL). Anti-tPA and Anti-CD105 monoclonal antibodies were purchased from American Diagnostica (Stamford, CT) and Neomarkers (Fremont, CA) respectively. Antibodies to annexin II were generated in our lab as reported earlier(16). Chromozyme PL was purchased from Roche Molecular Biochemicals (IN).

Immunohistochemistry reagents were procured from DAKO Corporation (CA). All other chemicals used in this study were of analytical grade.

Cell lines maintenance

Invasive and metastatic human breast cancer cell line MDA-MB231 which is known to cause tumors in athymic mice and the ER positive noninvasive breast cancer cell line MCF-7 were obtained from ATCC (Rockville, MD). These cell lines were maintained in RPMI 1640 media containing either 10% fetal calf serum (FCS) or serum free medium (0.1% BSA supplemented with L-glutamine and antibiotics) (17). Cell cultures were maintained in plastic flasks and incubated at 37°C in a humidified chamber containing 5% CO2.

Immunohistochemistry

Preeviously diagnosed paraffin-embedded human breast cancer samples were obtained from the tumor bank of the Department of Pathology, Drexel University College of Medicine. Serial 4 μm sections were prepared on albumin-coated slides. Sections were deparaffinized and incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Nonspecific protein binding was blocked with 3% bovine serum albumin (BSA)/PBS for 1 h. Sections were incubated with monoclonal antibodies overnight at room temperature. Staining was visualized using a DAKO kit with diaminobenzidine (DAB) as a chromogen followed by nuclear counterstaining with hematoxylin according to our published protocol (17). For each immunohistochemical staining, we performed additional staining without primary antibody in parallel and considered it as a control. The immunoreactive pattern was carefully recorded with the help of our pathologist and photographed.

Double Immunofluorescence labeling and Microscopy

MDA-MB 231 cells were grown on cover slips, fixed with 3% glutaraldehyde in PBS for 10 minute at room temperature. The fixed cells were washed with $NaBH_4$ to reduce aldehyde groups. Cells were incubated with 1% goat serum for 30 minutes to block non-specific binding. Fixed cells were incubated with primary anti-annexin II antibody for one hour and washed three times for 10 minutes each. After washing, cells were incubated with secondary antibody TRITC for 30 minutes in dark. The cells were

further blocked by 1% mouse serum for 30 minutes and reincubated with anti-tPA for one hour, washed and incubated with secondary antibody labeled with FITC. In parallel cover slips with cells incubated with secondary antibody labeled with FITC/ TRITC with out primary antibodies considered as controls. Cells were stained with DAPI to visualize nucleus. Fluorescence labeled cells were visualized using FITC, rodamine and DAPI filters under a Zeiss Immunofluorescence microscope (Axiovert 40) and photographed.

Immunoprecipitation and Western blot analysis

Cells grown in 12 well-plates, rinsed in ice-cold phosphate-buffered saline (PBS), pH 7.2, and lysed in 67 mmol/L Tris, pH 6.8, and 2% sodium dodecyl sulfate on ice for total extracts or in buffer A [50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4, 0.1% Triton X-100 plus protease and phosphatase inhibitors (200 mmol/L Pefabloc, 1 mmol/L aprotinin, 20 mmol/L leupeptin, 0.1 mmol/L sodium vanadate, 1 mmol/L sodium fluoride, and 10 mmol/L sodium pyrophosphate)] for immunoprecipitation. Protein concentration was measured using Bradford assay (Bio-Rad Laboratories, Hercules, CA). Immunoprecipitation were performed using 0.4 to 1 mg of cellular proteins incubated with anti- annexin II monoclonal antibody (0.5μg/ml) for overnight at 4°C. The immune complex was precipitated with protein A Sepharose beads. The immunocatcher system was used to purify immunoglobulin complex by catching the resins containing immobilized Protein A. Resins were washed three times and directly incubated with SDS sample dilution buffer and heated at 90^{o}C for 4 minutes. Immune complex was resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane, incubated with ant-tPA antibodies and analyzed by Western blot analysis. After incubation with the appropriate primary antibody, species-specific secondary antibodies conjugated to horseradish peroxidase were used and membrane was developed by ECL as we have reported previously (16-18).

tPA binding and plasmin generation

Interaction of tPA with the surface of MDA-MB 231 cells was studied by analyzing the plasminogen activating capacity of bound recombinant tPA. Cells were grown in 96-well plates, where they were then trypsnized and counted. About 5000-7000 cells were incubated with 300 units of recombinant human

tPA for 30 minutes on ice in PBS. Cells were washed three times with PBS to remove any unbound tPA. Bound tPA was determined by incubating cells directly with plasminogen and plasmin specific chromogenic substrate chromozyme PL in a total volume of 100µl. Kinetics of tPA dependent plasmin generation were recorded at 405nm for 40 minutes in 96-well-plate reader according to our published protocol (16, 17).

The plasmin generated in the reaction mixture hydrolyzes the chromozyme PL forming a yellow color of p-nitroaniline that absorbs light at 405 nm. The color absorbance was measured by an automated 96-well plate reader (Bio-TeK Inc., VT). The colorimetric change is a direct measurement of plasmin generation. Plasminogen incubated with chromozyme PL used to measure the auto degradation of plasminogen to plasmin during the reaction process, and was considered blank. Absorbance of control was subtracted from experimental. Equilibrium and saturation binding data were analyzed by non-linear regression algorithms using GraphPad Prizmsoftware. For saturation binding analysis, data were fit to a one or two binding site model.

In vitro wound healing assay for cell migration:

Cell migration was assayed as described (19). Briefly, about 8,000 cells were seeded and grown to confluency in 96 well plates. A wound was created in monolayer of cells by scratching with a sterile pipette tip. After 24 hours of culture in 2% serum-supplemented medium, cells were treated and in the each treatment group migration was evaluated by counting cells that migrated from the wound edge. Ten different fields (20X) were viewed to count migrating cells. Data were plotted using Prizm software.

RNAi mediated suppression of annexin II

Antisense Phosphorothionate oligonucleotide (ODNs) and sense ODNs (control) directed to 5'-3'coding sequence of the human annexin II mRNA were designed and manufactured by Biognostic, Germany (www.biognostik.com). The antisense oligonucleotides were synthesized as 16-mer targeted 5' – 3' sequences of annexin II. The corresponding control was a random16 mer hereafter, the antisense and sense annexin II oligonucleotides will be referred as RNAi ANX II and Control ANX II respectively.

MDA-MB231 cells were grown in 96-well plates in triplicates and transfected with RNAi ANX II and control ANX II by adding into the culture medium.

Antisense ODNs are actively taken up by cells, partially through fluid-phase endocytosis and possibly also through the putative receptor protein p80 that facilitates the cellular uptake of negatively charged molecules like ODNs or heparin. Fluorescein (FITC)-labeled phosphorothioate ODNs were used to monitor cellular uptake and distribution (data not shown). Labeled antisense ODNs met the same standards of purity and stability as antisense products. Whereas the cellular uptake of antisense ODNs may be enhanced through various cationic lipids, most of the cationic lipids are cytotoxic, and the treatment must be limited to 6 to 8 hours. In contrast, the half-life of antisense ODNs in serum containing culture media is >48 hours. Therefore, adding the antisense ODNs to the culture medium for the full duration of the experiment is more effective for experiments with a longer time frame. Details of the transfection process are provided below.

Trasnfection:

MDA-MB231 cells were incubated with 3 different concentrations of RNAi ANX II and control ANX II (0.5, 2.0 and 4.0 μmol/L) for 12, 24, 48, and 72 hours to determine the rate of transfection. Preliminary experiments showed that the uptake of RNAi ANX II was maximal with 0.5 μmol/L and after incubation for 48 hours. At this time point, annexin II protein was suppressed > 90% (see results). Therefore, to study the role of annexin II, we used these optimum conditions to investigate tPA bindings, plasminogen activation and cell migration.

RESULTS

Selective expression of annexin II and tPA in invasive human breast cancer:

Numbers of previous reports have shown that invasive breast cancers overproduce plasmin, a fibrinolytic enzyme, which may trigger an angiogenic switch and is correlated with a more invasive and metastatic phenotype of cancer (10, 17). Plasmin is a serine protease which can be converted directly from inactive enzyme plasminogen either by uPA or tPA after binding to their respective receptors. The first report on the role of the uPA in breast cancer prognosis was published by Look et al. (3) who

demonstrated that breast cancer patients with high levels of uPA activity in their primary tumors had a worse disease free survival (DFS) than patients with low levels. tPA binds to annexin II and facilitates plasminogen conversion to plasmin, however, this mechanism of annexin II/tPA induced plasmin generation in breast cancer has been overlooked for a long time. A recent clinical study of 33 patients with renal cell carcinoma (RCC) analyzed annexin II expression, and concluded that annexin II expression in RCC directly correlates with Fuhrman grade and clinical outcome (20). To understand the role of tPA and annexin II in breast cancer we investigated expression of tPA, annexin II and associated angiogenic markers (CD105) by immunohistochemistry in previously diagnosed human breast cancers. Normal human breast tissues were used to demonstrate the specificity of staining.

Immunostaining analysis showed that annexin II expression was not present in normal ducts(Fig.1A). In normal breast tissue annexin II staining was found in basement membrane around the normal ducts and in blood vessels (Fig.1A). By contrast, the cytoplasmic membrane of malignant ductal epithelial cells showed strong positive staining (Fig.1B, inset). Tumor stroma and fibrous tissue was also found to be strongly positive (Fig.1B, inset). Peritumor stroma and blood vessels also showed positive staining. Occasionally annexin II staining was also observed in the cytoplasm. Stromal cells such as fibroblasts and myoepithelial cells in the tumor microenvironment also showed strong annexin II staining (Fig.1B, inset). Consistent with annexin II strong staining of tPA was observed in invasive breast tumors suggesting excessive presence of both ligand (tPA) and receptor (annexin II) in these invasive tumors (Fig.1C, bottom inset). Since annexin II (10) and tPA (21, 22) have both been reported to play important role in neoangiogenesis, we analyzed CD 105 expression, a selective marker of neoangiogenesis in invasive breast cancer (23). Consistent with tPA and annexin II expression we found that CD 105 highlights many newly formed blood vessels in invasive breast cancer suggesting excessive neoangiogenesis activity (Fig.1D). Interestingly, CD105 failed to stain existing blood vessels in normal breast tissue (Fig.1E) indicating remarkable degree of specificity of CD105 for proliferating endothelial cells.

With in vivo data we found selective expression of annexin II and tPA in the highly invasive and metastatic cell line MDA-MB 231 but not in the nonmetastatic/less aggressive cell line MCF-7 (Fig.2.A)

Cell surface annexin II interacts with tPA in MDA-MB231cells.

Our immunostaining data, as well as in vitro results, clearly suggest expression of annexin II on the surface of invasive breast cancer cells. Next we investigated whether tPA, which is synthesized by invasive breast cancer cells (Fig.2.A), interacts with cell surface annexin II. To demonstrate interaction we immunoprecipitated cell lysate with anti-tPA monoclonal antibody, immune complex was resolved on SDS-PAGE and transferred onto nitrocellulose membranes. Bound proteins were identified by anti-annexin II monoclonal antibody by immunoblotting. Immunoblot data unequivocally identified tPA bound protein as annexin II (Fig2C). These results were further confirmed by a converse experiment where lysate was immunoprecipitated by anti-annexin II monoclonal antibody and bound protein was identified as tPA by immunoblotting using anti-tPA monoclonal antibody (Fig.2D). We further investigated whether this in vitro interaction is physiological. To demonstrate this we cultured MDA-MB 231 cells on coverslips, then fixed and incubated the cells with anti-tPA monoclonal antibody followed by FITC labeled secondary antibody. Cells were thoroughly washed and reincubated with anti-annexin II monoclonal antibody followed by TRITC labeled secondary antibody. Finally cells were washed and labeled with DAPI and visualized using fluorescence microscope and photographed. Cells stained with DAPI are presented in (Fig.3A) stained with anti-tPA-FITC (Fig.3B), stained with anti-annexin II-TRITC (Fig.3C). Figure 3D is the merged photograph of panels A, B and C. The merged photograph shows an orange color indicating co-localization of tPA and annexin II in the MDA-MB231 cells. These results suggest that tPA and annexin II indeed interact physiologically.

tPA binding to cell surface annexin II is required for plasminogen activation to plasmin in breast cancer:

Annexin II has been reported to be a receptor for tPA, plasminogen and angiostatin (5, 18) in endothelial cells. The assembly of tPA and plasminogen on the cell surface annexin II facilitates conversion of

inactive plasminogen to the highly reactive enzyme plasmin. The main physiological role of plasmin is to remove blood clots and maintain uninterrupted blood flow in blood vessels. Available clinical data indicates high serum levels of tPA in the early stage breast cancer (24, Rella, 1993 #1528, 25). Consistently, excessive plasmin generation has also been linked to the more aggressive phenotype of breast cancer (26). These data provide strong evidence supporting the liklihood that tPA and annexin II interaction may be involved in plasmin generation. To test this, we used non-invasive MCF-7 cells that lack annexin II expression and MDA-MB 231 cells which over expressed annexin II, and analyzed tPA binding kinetics. Both cell lines were grown to 70% confluency, trypsinized and washed. About 5000 cells were incubated with recombinant human tPA for 30 min. Cells were washed three times to remove unbound tPA. tPA binding was determined by measuring plasminogen conversion to plasmin. Consistent with our ex-vivo data we found that tPA bound saturably to the surface of MDA-MB231 cells in a time-dependent manner. As expected, tPA failed to bind to MCF-7 cells because they lack annexin II (Fig.4A). Kinetics of binding suggest linearity up to 20 minutes and after that it reaches plateau (Fig.4A). Next, we tested if these cells were capable of activating plasminogen to plasmin. Indeed MCF-7 failed to activate plasminogen to plasmin while in MDA-MB231 cells bound tPA presumably to cell surface annexin II, activated plasminogen to plasmin efficiently. To confirm these results we silenced annexin II expression in MDA-MB231 cells and determined tPA binding in wild type and annexin II suppressed MDA-MB231cells. Annexin II suppressed cells completely failed to bind tPA, and consequently there was no tPA dependent plasminogen activation. By contrast, wild type cells were bound to tPA in a time-dependent manner and were capable of activating plasminogen to plasmin. Taken together, these results suggest that tPA binding to cell surface annexin II is required for efficient plasmin generation which may be a prerequisite mechanism for cellular migration and neoangiogenesis. These results might explain the poor invasive capacity of MCF-7 cells because of inefficient plasmin generation.

Annexin II/ tPA dependent plasmin generation may be crucial for cell migration and neoangiogenesis.

Cancer cell migration plays a pivotal role in neoangiogenesis (new blood vessel formation), tumor invasion, and metastasis (27). Numbers of published articles including from our lab have shown that plasmin degrades ECM which in turn facilitates cellular migration and plays key biological functions for tumors to become angiogenic and invasive phenotype(16, 28). Previous reports have shown that plasmin generation is required for angiogenesis and cell invasion (14, 29)

We investigated whether tPA/annexin II mediated plasmin generation is involved in breast cancer cell migration. Cell migration was assayed by scratch wound healing assay as described (30). Cells were grown to confluency in 96 well-plates and a wound was created by using a pipette tip. Cells were treated with various reagents (see legends). After 24-36 hours of treatment cell migration was recorded by counting cells. Cell migration was assayed without adding any reagents as a negative control, and since plasmin has been reported to induce cell migration (9), we used plasmin as a positive control. Results (Fig. 4B) showed a significant increase in numbers of migrating cells after plasmin treatment. As evident from our previous experiment, tPA and plasminogen binding to cell surface annexin II facilitates plasmin generation. We treated cells with plasminogen + tPA and determined cell migration. The number of migrating cells was almost the same as observed in plasmin treated cells, indicating breast cancer cells were able to convert plasminogen to plasmin which induced cellular migration (Fig. 4B). EACA is a Lys analogue, which is known to block interaction of plasminogen with their receptors (17, 31). When cells were treated with EACA, migration was completely inhibited (Fig. 4B), suggesting that binding of plasmin to their receptors (annexin II) is critical for activation to plasmin. Next we tested whether assembly of plasminogen and tPA on annexin II is required for plasmin generation leading to cellular migration. We used angiostatin, which is known to bind to annexin II and compete for plasminogen binding (16, 17). Results suggest (Fig. 4B) that either angiostatin or anti-annexin II monoclonal antibody can inhibit MDA-MB 231 cell migration, indicating that annexin II dependent plasmin generation is crucial for breast cancer cell migration. To further provide evidence we used annexin II silenced MDA-MB231 cells. Not to our surprise, MDA-MB231 cells lacking annexin II

expression significantly reduced cell migration (61%) indicating cell surface annexin II is crucial for plasmin generation which facilitates cell migration.

Decades of extensive research have provided evidence that tumors are undetectable in the non-vascular phase (11, 32). Consistently neoangiogenesis is reported to be a significant independent prognostic indicator for poor prognosis in early stage breast cancer (11). We propose that annexin II expression in endothelial cells (EC) tumor cells is crucial for plasmin generation leading to ECM and basement membrane degradation which surrounds blood vessels and may be crucial for neoangiogenesis. We evaluated neoangiogenesis in breast tumor tissues by staining with monoclonal antibody to CD105, a specific marker of neoangiogenesis (23, 33). Consistent with previous reports, we have found that CD 105 selectively stains new blood vessels (Fig.1D) but not existing blood vessels (Fig.1E). With analysis of about 40 patients we concluded that newly formed blood vessels in invasive carcinoma correlate with annexin II staining. Our results indicate that breast cancer cells generate excessive plasmin in the tumor microenvironment leading to ECM degradation and tissue remodeling and consequently neoangiogenesis.

DISCUSSION

In this present investigation we have extended our prior observations that annexin II plays an important role in human breast cancer progression. Here we describe a potential mechanism by which annexin II facilitates breast cancer progression. Annexin II is a fibrinolytic receptor which binds to plasminogen and tPA (34). This trimolecular assembly is known to accelerate tPA dependent plasminogen activation by 60 fold.

The physiological role of plasmin was believed to be limited to fibrinolysis such as maintenance of blood fluidity by removing fibrin plugs from the vascular bed (35). However emerging clinical observations document activation of fibrinolytic pathways during cancer progression, specifically in breast cancer (36). Laboratory investigation provides strong support to these clinical observations and suggests that molecules of fibrinolytic pathways facilitate uncontrolled plasmin generation in the tumor microenvironment (17). Plasmin triggers ECM degradation which in turn allows EC/tumor cell

migration across the anatomic barrier and paves the way for invasion and neoangiogenesis(10). This in turn feeds the metabolic demand of the tumor to support rapid growth (32).

Because our data demonstrated selective over expression of annexin II in human breast cancer (17), we sought to investigate annexin II-dependent molecular mechanisms in breast cancer. Fig.2B shows that tPA is (ligand for annexin II) selectively synthesized by invasive human breast cancer cells MDA-MB231 whereas the less invasive human breast cancer cells MCF-7 failed to express both annexin II and tPA (Fig.2A&B). These observations explain why MCF-7 cells are poorly capable of plasmin generation (17, 37). Consistent with our in vitro observations, clinical studies have also reported elevated plasma tPA levels during breast cancer (24, 25). Elevated tPA levels were detected even in early stages of breast cancer (25). These clinical data and our own observations indicate activation of fibrinolytic pathways occurs during the early stage of breast cancer. Next, we investigated the molecular mechanism of activation of fibrinolytic pathways. Our data demonstrate that tPA and annexin II are co-localized in MDA-MB231 cells suggesting potential physiological interaction (Fig.3D). The fact is that tPA bound to the cell surface of MDA-MB231 cells in a time dependent manner, and activated plasminogen to plasmin efficiently. The linearity of plasmin generation was observed up to 20 minutes (Fig.4A). MCF-7 cells lacking annexin II expression failed to bind tPA and also failed to generate plasmin indicating specificity (Fig.4A) of annexin II and tPA interaction. Selective interaction was further confirmed by RNAi mediated suppression of annexin II in MDA-MB231 cells. Annexin II suppressed cells failed to bind tPA on the cell surface annexin II and were therefore unable to generate plasmin (Fig.5C).

Tumor angiogenesis is a significant poor prognostic indicator even in early stage breast cancer. It is possible that early stage detection of tPA (25) and annexin II (17) in clinical samples may establish a plasmin generation pathway. Excess localized plasmin may turn on an angiogenic switch by destruction of ECM and BM. This is supported by the fact that tPA, annexin II and plasmin all have been reported to play role in neoangiogenesis (10, 21, 28).

The receptors which regulate plasmin generation may predict the subsequent clinical course of breast cancer. Because cell surface annexin II can serve as a receptor for tPA which activates plasminogen to plasmin, localized plasmin generation in the tumor microenvironment may trigger neoangiogenic activity.

Our results show excessive neoangiogenesis activity (Fig.1D) as determined by monoclonal antibody to CD105, a specific marker for proliferating endothelial cells, which identifies only newly formed blood vessels (33). These data show that annexin II expression correlates with tPA and CD105 positive endothelial cells, indicating neoangiogenic activity (Fig.1D). These data further suggest that cell surface annexin II may provide binding sites for tPA and plasminogen, therefore increasing the rate of local plasmin generation. This localized plasmin allows pericellular ECM and BM degradation, therefore switching tumors from the prevascular to vascular phase with metastatic possibility.

Recently published reports examined angiogenic competence of vascular explants culture obtained from the mice lacking tPA-/- and plasminogen (PLG-/-). Results suggest that tPA deficient vessels showed complete failure of angiogenesis (21). A similar angiogenic failure was seen in plsminogen deficient vessels (28). Other investigators reported that neutralizing antibody to tPA blocks angiogenesis, confirming the importance of tPA in angiogenesis (38). Furthermore, mice lacking annexin II gene (ANX-/-) have also reported to lack angiogenesis (10). The prognostic significance of annexin II has been reported in patients with renal cell carcinoma (RCC). Clinical analysis of 36 patients confirmed a correlation with annexin II and clinical outcome (20). Taken together, for the first time, we have provided strong evidence that cell surface annexin II interacts with tPA in breast cancer. This annexin II and tPA interaction may be a molecular signature for efficient plasmin generation in the breast tumor microenvironment leading to an angiogenic switch. Annexin II could be of prognostic significance and potential therapeutic target for the treatment of breast cancer.

REFERENCES:

1. Stephens, R. W., Brunner, N., Janicke, F., and Schmitt, M. The urokinase plasminogen activator system as a target for prognostic studies in breast cancer. Breast Cancer Res Treat, *52:* 99-111, 1998.

2. Janicke, F., Schmitt, M., Ulm, K., Gossner, W., and Graeff, H. Urokinase-type plasminogen activator antigen and early relapse in breast cancer. Lancet, *2:* 1049, 1989.

3. Look, M. P., van Putten, W. L., Duffy, M. J., Harbeck, N., Christensen, I. J., Thomssen, C., Kates, R., Spyratos, F., Ferno, M., Eppenberger-Castori, S., Sweep, C. G., Ulm, K., Peyrat, J. P., Martin, P. M., Magdelenat, H., Brunner, N., Duggan, C., Lisboa, B. W., Bendahl, P. O., Quillien, V., Daver, A., Ricolleau, G., Meijer-van Gelder, M. E., Manders, P., Fiets, W. E., Blankenstein, M. A., Broet, P., Romain, S., Daxenbichler, G., Windbichler, G., Cufer, T., Borstnar, S., Kueng, W., Beex, L. V., Klijn, J. G., O'Higgins, N., Eppenberger, U., Janicke, F., Schmitt, M., and Foekens, J. A. Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. J Natl Cancer Inst, *94:* 116-128, 2002.

4. Cesarman, G. M., Guevara, C. A., and Hajjar, K. A. An endothelial cell receptor for plasminogen/tissue plasminogen activator (tPA). II. Annexin II-mediated enhancement of tPA-dependent plasminogen activation. J Biol Chem, *269:* 21198-21203, 1994.

5. Hajjar, K. A., Jacovina, A. T., and Chacko, J. An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. J Biol Chem, *269:* 21191-21197, 1994.

6. Diaz, V. M., Hurtado, M., Thomson, T. M., Reventos, J., and Paciucci, R. Specific interaction of tissue-type plasminogen activator (tPA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion in vitro. Gut, *53:* 993-1000, 2004.

7. Brownstein, C., Deora, A. B., Jacovina, A. T., Weintraub, R., Gertler, M., Khan, K. M., Falcone, D. J., and Hajjar, K. A. Annexin II mediates plasminogen-dependent matrix invasion by human monocytes: enhanced expression by macrophages. Blood, *103:* 317-324, 2004.

8. Mignatti, P. and Rifkin, D. B. Biology and biochemistry of proteinases in tumor invasion. Physiol Rev, *73:* 161-195, 1993.

9. Tarui, T., Majumdar, M., Miles, L. A., Ruf, W., and Takada, Y. Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of angiostatin. J Biol Chem, *277:* 33564-33570, 2002.

10. Ling, Q., Jacovina, A. T., Deora, A., Febbraio, M., Simantov, R., Silverstein, R. L., Hempstead, B., Mark, W. H., and Hajjar, K. A. Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. J Clin Invest, *113:* 38-48, 2004.

11. Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. N., Moore, D. H., Meli, S., and Gasparini, G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst, *84:* 1875-1887, 1992.

12. Hajjar, K. A. Cellular receptors in the regulation of plasmin generation. Thromb Haemost, *74:* 294-301, 1995.

13. Goh, K. Y., Poon, W. S., Chan, D. T., and Ip, C. P. Tissue plasminogen activator expression in meningiomas and glioblastomas. Clin Neurol Neurosurg, *107:* 296-300, 2005.

14. Diaz, V. M., Planaguma, J., Thomson, T. M., Reventos, J., and Paciucci, R. Tissue plasminogen activator is required for the growth, invasion, and angiogenesis of pancreatic tumor cells. Gastroenterology, *122:* 806-819, 2002.

15. Chernicky, C. L., Yi, L., Tan, H., and Ilan, J. Tissue-type plasminogen activator is upregulated in metastatic breast cancer cells exposed to insulin-like growth factor-I. Clin Breast Cancer, *6:* 340-348, 2005.

16. Sharma, M., Rothman, V. L., Tuszynski, G. P. a., and Sharma, M. C. Antibody-directed targeting of angiostatin's receptor annexin II inhibits Lewis Lung Carcinoma tumor growth via blocking of plasminogen activation: Possible biochemical mechanism of angiostatin's action. Exp Mol Pathol, *81:* 136-145, 2006.

17. Sharma, M., Koltowski, L., Rothman, V. L., Tuszynski, G. P., and, R. T. O., and Sharma, M. C. Angiogenesis Associated Protein Annexin II in Breast Cancer: Selective Expression in Invasive Breast Cancer and Contribution to Tumor Invasion and Progression. Exp Mol Pathol, *81:* 146-156, 2006.

18. Tuszynski, G. P., Sharma, M., Rothman, V. L., and Sharma, M. C. Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. Microvasc Res, *64:* 448-462, 2002.

19. Sharma, M. R., Tuszynski, G. P., and Sharma, M. C. Angiostatin-induced inhibition of endothelial cell proliferation/apoptosis is associated with the down-regulation of cell cycle regulatory protein cdk5. J Cell Biochem, *91:* 398-409, 2004.

20. Zimmermann, U., Woenckhaus, C., Pietschmann, S., Junker, H., Maile, S., Schultz, K., Protzel, C., and Giebel, J. Expression of annexin II in conventional renal cell carcinoma is correlated with Fuhrman grade and clinical outcome. Virchows Arch, *445:* 368-374, 2004.

21. Brodsky, S., Chen, J., Lee, A., Akassoglou, K., Norman, J., and Goligorsky, M. S. Plasmin-dependent and -independent effects of plasminogen activators and inhibitor-1 on ex vivo angiogenesis. Am J Physiol Heart Circ Physiol, *281:* H1784-1792, 2001.

22. Sharma, M. C. and Sharma, M. The role of annexin II in angiogenesis and tumor progression: a potential therapeutic target. Curr Pharm Des, *13:* 3568-3575, 2007.

23. Kumar, S., Ghellal, A., Li, C., Byrne, G., Haboubi, N., Wang, J. M., and Bundred, N. Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. Cancer Res, *59:* 856-861, 1999.

24. Grondahl-Hansen, J., Bach, F., and Munkholm-Larsen, P. Tissue-type plasminogen activator in plasma from breast cancer patients determined by enzyme-linked immunosorbent assay. Br J Cancer, *61:* 412-414, 1990.

25. Rella, C., Coviello, M., Quaranta, M., and Paradiso, A. Tissue-type plasminogen activator as marker of functional steroid receptors in human breast cancer. Thromb Res, *69:* 209-220, 1993.

26. Chappuis, P. O., Dieterich, B., Sciretta, V., Lohse, C., Bonnefoi, H., Remadi, S., and Sappino, A. P. Functional evaluation of plasmin formation in primary breast cancer. J Clin Oncol, *19:* 2731-2738, 2001.

27. Stack, M. S., Gately, S., Bafetti, L. M., Enghild, J. J., and Soff, G. A. Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation. Biochem J, *340:* 77-84, 1999.

28. Bajou, K., Masson, V., Gerard, R. D., Schmitt, P. M., Albert, V., Praus, M., Lund, L. R., Frandsen, T. L., Brunner, N., Dano, K., Fusenig, N. E., Weidle, U., Carmeliet, G., Loskutoff, D., Collen, D., Carmeliet, P., Foidart, J. M., and Noel, A. The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic strategies. J Cell Biol, *152:* 777-784, 2001.

29. Pepper, M. S. Extracellular proteolysis and angiogenesis. Thromb Haemost, *86:* 346-355, 2001.

30. Yarrow, C., Benoit, G., and Klein, M. C. Outcomes after vacuum-assisted deliveries. Births attended by community family practitioners. Can Fam Physician, *50:* 1109-1114, 2004.

31. Tarui, T., Miles, L. A., and Takada, Y. Specific interaction of angiostatin with integrin alpha(v)beta(3) in endothelial cells. J Biol Chem, *276:* 39562-39568, 2001.

32. Folkman, J. The role of angiogenesis in tumor growth. Semin Cancer Biol, *3:* 65-71, 1992.

33. Gerber, S. A., Rybalko, V. Y., Bigelow, C. E., Lugade, A. A., Foster, T. H., Frelinger, J. G., and Lord, E. M. Preferential attachment of peritoneal tumor metastases to omental immune aggregates and possible role of a unique vascular microenvironment in metastatic survival and growth. Am J Pathol, *169:* 1739-1752, 2006.

34. Hajjar, K. A., Mauri, L., Jacovina, A. T., Zhong, F., Mirza, U. A., Padovan, J. C., and Chait, B. T. Tissue plasminogen activator binding to the annexin II tail domain. Direct modulation by homocysteine. J Biol Chem, *273:* 9987-9993, 1998.

35. Hajjar, K. A. Changing concepts in fibrinolysis. Curr Opin Hematol, *2:* 345-350, 1995.

36. Korte, W. Changes of the coagulation and fibrinolysis system in malignancy: their possible impact on future diagnostic and therapeutic procedures. Clin Chem Lab Med, *38:* 679-692, 2000.

37. Westphal, J. R., Van't Hullenaar, R., Geurts-Moespot, A., Sweep, F. C., Verheijen, J. H., Bussemakers, M. M., Askaa, J., Clemmensen, I., Eggermont, A. A., Ruiter, D. J., and De Waal, R. M. Angiostatin generation by human tumor cell lines: involvement of plasminogen activators. Int J Cancer, *86:* 760-767, 2000.

38. Sato, Y., Okamura, K., Morimoto, A., Hamanaka, R., Hamaguchi, K., Shimada, T., Ono, M., Kohno, K., Sakata, T., and Kuwano, M. Indispensable role of tissue-type plasminogen activator in growth factor-dependent tube formation of human microvascular endothelial cells in vitro. Exp Cell Res, *204:* 223-229, 1993.

FIGURE LEGENDS

Fig.1.

Immunostaining for annexin II, tPA and anti-CD 105 in normal and human breast cancer tissues

Annexin II is undetectable in normal acinar and ductal epithelial cells (A, inset arrows). Anti-annexin II antibodies show a very strong reactivity on the surface of ductal epithelial cells (B, left inset) , tumor stroma (B, right inset) and vascular endothelial cells (B, arrows) in invasive breast cancer. Staining in ductal epithelial cells was found mainly on the cell surface with occasional cytoplasmic staining. Anti-tPA antibodies show intense staining in vascular endothelial cells(C, top inset), ductal epithelial cells (C, bottom inset) and in tumor stroma. Anti-CD105 antibodies show a selective strong reactivity with newly formed blood vessels (D) but not in existing blood vessels of normal tissue (E). Panel E arrows indicate existing blood vessels in normal breast tissue are not stained with anti-CD105 antibody suggesting specificity of CD105 to identify proliferating endothelial cells (Magnification 20X). Staining intensity of

annexin II was analyzed in 20 different fields by ImagePro Software Panel F. CD 105 positive endothelial cells were counted and plotted by GraphPad Prizm software Panel F.

Fig.2.

Expressions of annexin II and tPA in human breast cancer cells and their interaction

MDA-MB231cell lysate (20μg) was separated by SDS-PAGE and protein expression was analyzed by immunoblot analysis**.** Figure 2 displays selective expression of annexin II and tPA in invasive breast cancer cell line MDA-MB231 (Fig.2A and B). Cell lysate was immunoprecipitated (IP) with anti-tPA antibodies and immune complex was electrophoresed and immunoblotted with anti-annexin II (Panel 2C, Lane 1 positive control, lane 2 IP pellet, lane 3 supernatant and lane 4 is IgG negative control). Panel D: cell lysate was immunoprecipitated (IP) with anti-annexin II antibodies and immune complex was electrophoresed and immunoblotted with anti-tPA((Panel C, Lane 1 positive control, lane 2 IP pellet, lane 3 supernatant and lane 4 is IgG negative control). Co-immunoprecipitated pellet in lane 2 of both gels suggest interaction of tPA and annexin II.

Fig.3.

Co-localization of annexin II and tPA in MDA-MB231 cells

Human breast cancer cells (MDA-MB231) were cultured on cover slip, fixed and double immunofluorescence labeled with anti-annexin II (Fig.3B) and tPA ((Fig.3C). Panel A shows DAPI staining for nuclei. Merged photographed of panel A, B and C is shown in panel D. Orange color in panel D demonstrates co-localization of tPA and annexin II in the breast cancer cells.

Fig.4.

tPA binding to cell surface annexin II accelerate plasmin generation which in turn facilitates cell migration

MDA-MB231 cells were cultured in 12 wells plates. Cells were detached, washed and incubated and counted. About 20000 cells were incubated with recombinant human tPA and allowed to bind on the cell surface annexin II for 30 minutes on ice. Cells were thoroughly washed to remove unbound tPA. tPA bound cells were incubated with plasminogen and chromozyme PL. Kinetics of plasminogen

activation was monitored at 405nm. Linearity of plasminogen conversion to plasmin was observed about 20 minutes (A, inset). Non invasive Human breast cancer cells MCF-7 lacking annexin II failed to bind tPA and unable to convert plasminogen to plasmin (A). tPA dependent plasmin generation induced cell migration. This enhanced cell migration can be blocked either by direct blocking of annexin II using anti-annexin II monoclonal antibodies or angiostatin which binds to cell surface annexin II and blocks plasminogen binding . Migration was also inhibited by blocking of lysine residue of plasminogen which is required for interaction with annexin II (B)

Fig.5.

RNAi mediated suppression of annexin II gene inhibits cell migration

Three different antisenses were designed and synthesized as 16-mer dithionated oligonucleotide to silence annexin II gene. Immunoblot analysis suggests that two out of three antisenses were highly potent and almost completely silenced annexin II expression (A). β-actin was used as loading control to confirm equal protein loading. Quantitative analysis of band density shows more than 90% inhibition of annexin II expression as compared to β-actin loading control (B). Annexin II silenced cells failed to bind tPA(C) and significantly inhibited plasminogen activation (D). Annexin II silenced cells show significant inhibition of cell migration (E, F) as determined by scratch wound healing assay for cell migration.