

AD _____

Award Number: W81XWH-07-1-0424

TITLE: Annexin II-Dependent mechanism of breast cancer progression

PRINCIPAL INVESTIGATOR:

Mahesh C. Sharma, PhD

CONTRACTING ORGANIZATION:

Institute for Clinical Research Inc.
Washington, DC 20422-9745

REPORT DATE: November 2010

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.				
1. REPORT DATE (DD-MM-YYYY) 01-11-2010		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 JUN 2009 - 8 OCT 2010
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, PhD			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Institute for Clinical Research Inc. Washington, DC 20422-9745			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, MD 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 (neovascularization). Neovascularization is a significant independent prognostic indicator in early stage breast cancer (1). Delineating the molecular mechanism(s) of neovascularization 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 UU	18. NUMBER OF PAGES 26
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U		
				19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4-5
Body.....	5
Key Research Accomplishments.....	5-6
Reportable Outcomes.....	6-7
Conclusion.....	7
References.....	7-9
Appendices.....	10-26

INTRODUCTION

Sprouting of new blood vessels 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 sprouting of new blood vessels therefore plays a critical role in breast cancer (1). Analysis of the enzymes of the plasminogen/plasmin system clearly 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 conversion of zymogen plasminogen to active enzyme plasmin in breast cancer are poorly understood. In the physiological system two different mechanisms of plasmin generation have been established. The first mechanism is involved in binding of uPA to its receptor uPAR while the second mechanism converts plasminogen to plasmin by binding of tissue type plasminogen activator (tPA) to its receptor annexin II. This annexin II dependent plasmin exerts fibrinolytic activity that maintains continuous blood flow in vessels by dissolving intravascular fibrin clots.

Annexin II is reported to be an endothelial cell receptor which provides binding sites for plasminogen and tPA for efficient plasmin generation (4, 5). A number of reports have shown 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) and thus plays an important role in neoangiogenesis (10). There are speculations that annexin II derived neoangiogenesis may be involved in metastasis, and tumor progression. This might explain why we and other investigators have consistently found over expression of annexin II in various cancers including breast cancer (11-19). Overexpression of annexin II in cancer seems to correlate with advanced stage with poor prognosis and metastasis (11, 17, 18, 20).

Breast cancer growth and metastasis requires extensive neoangiogenesis. The fact is that neoangiogenesis is an independent and highly significant prognostic indicator for overall and relapse-free survival in patients with early-stage breast carcinoma (21). Angiostatin, a potent inhibitor of angiogenesis, was reported to inhibit human breast cancer growth and metastasis in xenograft model (22, 23) suggesting that anti-angiogenic therapy could be a potential therapeutic approach for breast cancer. Previously we have reported that angiostatin binds to endothelial cell surface annexin II (24). These findings, together with observations that angiostatin inhibits neoangiogenesis dependent growth of breast cancer may argue that angiostatin inhibits neoangiogenesis dependent breast cancer growth and metastasis by interfering with annexin II/tPA dependent plasmin generation in tumor microenvironment.

tPA is secreted by endothelial cells (EC) and is a ligand for cell surface annexin II. Emerging reports and clinical observations link tPA to neoplastic transformation and the invasive phenotype of highly aggressive tumors such as glioblastoma (25), melanoma (26) pancreatic cancer (27) and breast cancer (28, 29). The functional role of tPA and its receptor annexin II in breast tumor biology is not well-understood. Recent studies have provided evidence that showed association of tPA with the invasion of highly aggressive MDA-MB-435s breast cancer cells (30). Since tPA plays a major role in plasmin generation, consistently plasmin has been linked to invasive and metastatic breast cancer (1). Indeed, recent studies have clearly demonstrated over expression of annexin II in many cancer types and may argue that annexin II functions as a

regulator of neoangiogenesis *in vivo*. However the precise mechanism by which annexin II is involved in cancer progression and metastasis is still unclear.

In this report we investigated if tPA interaction on breast cell surface annexin II is a molecular switch to regulate localized plasmin generation *in vitro* and neoangiogenesis *in vivo*. Taken together, this research may provide a clue for the annexin II-dependent mechanism of neoangiogenesis and its dependent breast cancer progression and metastasis. Annexin II may be a potential target for the development of effective therapeutic strategies to inhibit angiogenesis and breast cancer.

BODY

We have successfully completed task 1 of this idea award as proposed. Part of the results of task 1 has been reported in 2008 and 2009 annual report. In 2009 our lab has moved from the Drexel University to the VA Medical center, Washington DC. This move has caused disruption of our research activities. However despite of this disruption, we were able to complete some experiments as we outlined in our task 2 of the SOW. Detailed staining and data analysis is still going on which will be reported after completion of the grant in November, 2011. One experiment as outlined in task 1 of SOW that is evaluation of therapeutic efficacy of anti-ANX II antibody in breast cancer metastasis is underway and will be completed in January 2011. The results obtained in these studies has been published in *Experimental and Molecular Pathology* 88 (2010) 278–286 and one paper was presented in annual meeting of *American Association for Cancer Research Proc. Am. Assoc. Can. Res. (2010) 15, 313* (Full manuscript is being submitted for publication). We published one review article in *Current Pharmaceutical Design*, 2009, 15, 1949-1955 (see attached reprints). The results are summarized below.

KEY ACHIEVEMENTS:

Task2: To determine whether the pattern of Annexin II expression in breast cancer correlates with neoangiogenesis and worsening stage of breast cancer and predictive of poor outcome.

One experiment planned in task 1d entitled effect of anti-annexin II antibody on mouse model of breast cancer metastasis is in underway and likely to be completed in January 2011. Results will be reported in final report

To investigate the role of annexin II in human breast cancer progression we analyzed human breast tissue samples by immunohistochemical staining. Immunohistochemical studies revealed expression of ANX II mainly on the surface of invasive cancerous cells (Fig. 1B). In contrast, epithelial cells in normal ducts were completely negative (Fig. 1G). Consistent with annexin II expression, immunostaining of

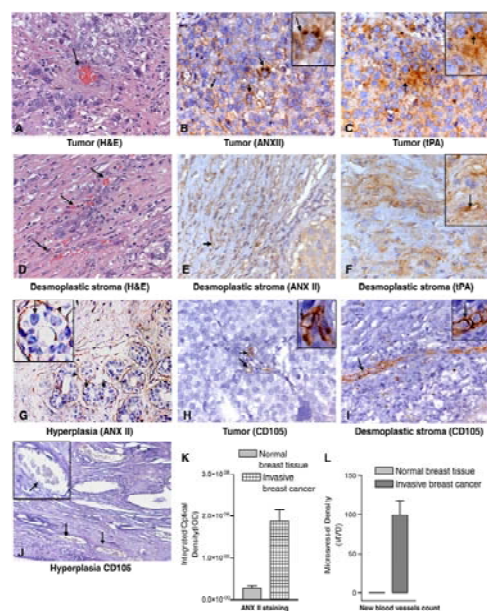


Fig. 1. Immunostaining for annexin II, tPA and anti-CD 105 in normal and human breast cancer tissues. Annexin II is undetectable in normal acinar and ductal epithelial cells (G, inset arrows). Anti-annexin II antibodies show a very strong reactivity on the surface of ductal epithelial cells (B, inset), tumor stroma (E) 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 (F inset), ductal epithelial cells (C, inset) and in tumor stroma. Anti-CD105 antibodies show a selective strong reactivity with newly formed blood vessels (H) but not in existing blood vessels of normal tissue (J). Panel J 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 K. CD 105 positive endothelial cells were counted and plotted by GraphPad Prism software Panel L.

thin serial sections also revealed high levels of tPA in tumor stroma (Fig. 1C). Careful investigation of immunostained sections revealed collagenous stroma composed of fibroblasts, macrophages, lymphocytes and ECM indicating an inflammatory response and annexin II in desmoplastic stroma (Fig. 1E) suggesting chronic inflammation in the tumor microenvironment and may be indicative of neoangiogenic activity. Staining pattern of CD 105 revealed numerous sprouting neovessels in tumor as well in desmoplastic stroma confirming neoangiogenic activity (Fig. 1H and I). Quantitative analysis of neoangiogenic activity as measured by microvascular density (MVD) appears to correlate with annexin II expression patterns (Fig. 1K and L).

1. Annexin II expression is appeared to be on the surface of aggressive human breast cancer patients. Very low or no expression was found on normal ducts.
2. Annexin II expression seems to correlate with neoangiogenic activity in human breast cancer patients.

REPORTABLE OUTCOMES: The results of this study have been published in following Journals/meetings.

1. *Experimental and Molecular Pathology* 88 (2010) 278–286. One paper was presented in annual meeting of American Association for Cancer Research

2. *Proc. Am. Assoc. Can. Res. (2010) 15, 313* (Full manuscript is being submitted for publication). One review article was published in
3. *Current Pharmaceutical Design, 2009, 15, 1949-1955*

CONCLUSIONS:

Our Immunohistochemical staining results indicate selective expression of annexin II in invasive breast cancer specimens. Annexin II expression appears to correlate with neoangiogenic activity in invasive breast cancer patients.

REFERENCES:

1. Stephens RW, Brunner N, Janicke F, Schmitt M. The urokinase plasminogen activator system as a target for prognostic studies in breast cancer. *Breast Cancer Res Treat* 1998; 52: 99-111.
2. Janicke F, Schmitt M, Ulm K, Gossner W, Graeff H. Urokinase-type plasminogen activator antigen and early relapse in breast cancer. *Lancet* 1989; 2: 1049.
3. Look MP, van Putten WL, Duffy MJ, et al. Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. *J Natl Cancer Inst* 2002; 94: 116-28.
4. Cesarman GM, Guevara CA, Hajjar KA. An endothelial cell receptor for plasminogen/tissue plasminogen activator (t-PA). II. Annexin II-mediated enhancement of t-PA-dependent plasminogen activation. *J Biol Chem* 1994; 269: 21198-203.
5. Hajjar KA, Jacovina AT, Chacko J. An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. *J Biol Chem* 1994; 269: 21191-7.
6. Diaz VM, Hurtado M, Thomson TM, Reventos J, Paciucci R. Specific interaction of tissue-type plasminogen activator (t-PA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion in vitro. *Gut* 2004; 53: 993-1000.
7. Brownstein C, Deora AB, Jacovina AT, et al. Annexin II mediates plasminogen-dependent matrix invasion by human monocytes: enhanced expression by macrophages. *Blood* 2004; 103: 317-24.
8. Mignatti P, Rifkin DB. Biology and biochemistry of proteinases in tumor invasion. *Physiol Rev* 1993; 73: 161-95.
9. Tarui T, Majumdar M, Miles LA, Ruf W, Takada Y. Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of angiostatin. *J Biol Chem* 2002; 277: 33564-70.
10. Ling Q, Jacovina AT, Deora A, et al. Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. *J Clin Invest* 2004; 113: 38-48.
11. Sharma M, Koltowski L, Rothman VL, Tuszyński GP, and RTO, Sharma MC. Angiogenesis Associated Protein Annexin II in Breast Cancer: Selective Expression in Invasive Breast Cancer and Contribution to Tumor Invasion and Progression. *Exp Mol Pathol* 2006; 81: 146-56.
12. Chuthapisith S, Bean BE, Cowley G, et al. Annexins in human breast cancer: Possible predictors of pathological response to neoadjuvant chemotherapy. *Eur J Cancer* 2009; 45: 1274-81.

13. Zimmermann U, Woenckhaus C, Pietschmann S, et al. Expression of annexin II in conventional renal cell carcinoma is correlated with Fuhrman grade and clinical outcome. *Virchows Arch* 2004; 445: 368-74.
14. Zhong LP, Wei KJ, Yang X, et al. Increased expression of Annexin A2 in oral squamous cell carcinoma. *Arch Oral Biol* 2009; 54: 17-25.
15. Pei H, Zhu H, Zeng S, et al. Proteome analysis and tissue microarray for profiling protein markers associated with lymph node metastasis in colorectal cancer. *J Proteome Res* 2007; 6: 2495-501.
16. Guedj N, Zhan Q, Perigny M, et al. Comparative protein expression profiles of hilar and peripheral hepatic cholangiocarcinomas. *J Hepatol* 2009; 51: 93-101.
17. Ohno Y, Izumi M, Kawamura T, Nishimura T, Mukai K, Tachibana M. Annexin II represents metastatic potential in clear-cell renal cell carcinoma. *Br J Cancer* 2009; 101: 287-94.
18. Shiozawa Y, Havens AM, Jung Y, et al. Annexin II/annexin II receptor axis regulates adhesion, migration, homing, and growth of prostate cancer. *J Cell Biochem* 2008; 105: 370-80.
19. Syed SP, Martin AM, Haupt HM, Arenas-Elliot CP, Brooks JJ. Angiostatin receptor annexin II in vascular tumors including angiosarcoma. *Hum Pathol* 2007; 38: 508-13.
20. Wu W, Tang X, Hu W, Lotan R, Hong WK, Mao L. Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. *Clin Exp Metastasis* 2002; 19: 319-26.
21. Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992; 84: 1875-87.
22. O'Reilly MS, Holmgren L, Chen C, Folkman J. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat Med* 1996; 2: 689-92.
23. Peyruchaud O, Serre CM, NicAmhlaoibh R, Fournier P, Clezardin P. Angiostatin inhibits bone metastasis formation in nude mice through a direct anti-osteoclastic activity. *J Biol Chem* 2003; 278: 45826-32.
24. Tuszynski GP, Sharma M, Rothman VL, Sharma MC. Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. *Microvasc Res* 2002; 64: 448-62.
25. Goh KY, Poon WS, Chan DT, Ip CP. Tissue plasminogen activator expression in meningiomas and glioblastomas. *Clin Neurol Neurosurg* 2005; 107: 296-300.
26. Stack MS, Gately S, Bafetti LM, Enghild JJ, Soff GA. Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation. *Biochem J* 1999; 340: 77-84.
27. Diaz VM, Planaguma J, Thomson TM, Reventos J, Paciucci R. Tissue plasminogen activator is required for the growth, invasion, and angiogenesis of pancreatic tumor cells. *Gastroenterology* 2002; 122: 806-19.
28. Rella C, Coviello M, Quaranta M, Paradiso A. Tissue-type plasminogen activator as marker of functional steroid receptors in human breast cancer. *Thromb Res* 1993; 69: 209-20.
29. Grondahl-Hansen J, Bach F, Munkholm-Larsen P. Tissue-type plasminogen activator in plasma from breast cancer patients determined by enzyme-linked immunosorbent assay. *Br J Cancer* 1990; 61: 412-4.

30. Chernicky CL, Yi L, Tan H, Ilan J. Tissue-type plasminogen activator is upregulated in metastatic breast cancer cells exposed to insulin-like growth factor-I. Clin Breast Cancer 2005; 6: 340-8.



Breast cancer cell surface annexin II induces cell migration and neoangiogenesis via tPA dependent plasmin generation

Meena Sharma^a, Robert T. Ownbey^b, Mahesh C. Sharma^{b,c,d,e,*}

^a University of Pennsylvania, Philadelphia, PA 19102, USA

^b Department of Surgery, Drexel University College of Medicine, Philadelphia, PA 19102, USA

^c Department of Pathology, Drexel University College of Medicine, Philadelphia, PA 19102, USA

^d Department of Biochemistry, George Washington University, Washington, DC 20037, USA

^e Veterans Affairs Medical Center, Washington, DC 20422, USA

ARTICLE INFO

Article history:

Received 6 November 2009

Available online 15 January 2010

Keywords:

Plasmin

Annexin II

Breast cancer

Neoangiogenesis

tPA

ABSTRACT

Annexin II, an abundant phospholipids binding cell surface protein, binds tPA and functions as a regulator of fibrinolysis. Annexin II also mediates angiogenesis and enhances tumor growth and metastasis. However, the mechanism supporting this role is not known. Using human breast cancer model we show that invasive human breast cancer cells (MDA-MB231) synthesize annexin II and tissue plasminogen activator (tPA). *In vitro* both annexin II and tPA interacts which in turn convert zymogen plasminogen to reactive enzyme plasmin. Cell surface produced plasmin inhibited the migration of MDA-MB231 cells. Silencing of annexin II gene in MDA-MB231 cells abolished tPA binding therefore inhibited tPA dependent plasmin generation. These annexin II suppressed MDA-MB231 cells showed reduced motility. Immunohistochemical analysis of prediagnosed clinical specimens showed abundant secretion of tPA and expression of annexin II on the surface of invasive human breast cancer cells which correlates with neovascularization of the tumor. Taken together, these data indicate that annexin II may regulate localized plasmin generation in breast cancer. This may be an early event switching breast cancer from the prevascular phase to the vascular phase and thus contributing to aggressive cancer with the possibility of metastasis. The data provide a mechanism explaining the role of annexin II in breast cancer progression and suggest that annexin II may be an attractive target for therapeutic strategies aimed to inhibit angiogenesis and breast cancer.

Published by Elsevier Inc.

Introduction

Sprouting of new blood vessels 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 sprouting of new blood vessels therefore plays a critical role in breast cancer (Stephens et al., 1998). Analysis of the enzymes of the plasminogen/plasmin system clearly suggests that high urokinase-type plasminogen activator (uPA) in human breast tumors predicts poor survival (Janicke et al., 1989; Look et al., 2002) and is significantly associated with a high rate of relapse for patients with breast cancer (Janicke et al., 1989). Despite the established role of the plasminogen/plasmin system in breast cancer, the receptor(s) which regulates conversion of zymogen plasminogen to active enzyme plasmin in breast cancer are poorly understood. In the physiological system two different mechanisms of plasmin generation have been established. The first mechanism is involved in binding of

uPA to its receptor uPAR while the second mechanism converts plasminogen to plasmin by binding of tissue type plasminogen activator (tPA) to its receptor annexin II. This annexin II dependent plasmin exerts fibrinolytic activity that maintains continuous blood flow in vessels by dissolving intravascular fibrin clots.

Annexin II is reported to be an endothelial cell receptor which provides binding sites for plasminogen and tPA for efficient plasmin generation (Cesarman et al., 1994; Hajjar et al., 1994). A number of reports have shown that cell surface annexin II regulates plasmin generation (Diaz et al., 2004; Hajjar et al., 1994) which in turn facilitates extracellular matrix (ECM) degradation, cell invasion (Brownstein et al., 2004; Diaz et al., 2004; Mignatti and Rifkin, 1993), and migration (Tarui et al., 2002) and thus plays an important role in neoangiogenesis (Ling et al., 2004). There are speculations that annexin II derived neoangiogenesis may be involved in metastasis, and tumor progression. This might explain why we and other investigators have consistently found over expression of annexin II in various cancers including breast cancer (Chuthapisith et al., 2009; Guedj et al., 2009; Ohno et al., 2009; Pei et al., 2007; Sharma et al., 2006a; Shiozawa et al., 2008; Syed et al., 2007; Zhong et al., 2009; Zimmermann et al., 2004). Overexpression of annexin II in cancer seems to correlate with advanced stage with poor prognosis and

* Corresponding author. Research Services (151A), Veterans Affairs Medical Center, 50 Irving street, NW, Washington, DC 20422, USA.

E-mail address: Mahesh.sharma@va.gov (M.C. Sharma).

metastasis (Ohno et al., 2009; Sharma et al., 2006a; Shiozawa et al., 2008; Wu et al., 2002).

Breast cancer growth and metastasis requires extensive neoangiogenesis. The fact is that neoangiogenesis is an independent and highly significant prognostic indicator for overall and relapse-free survival in patients with early-stage breast carcinoma (Weidner et al., 1992). Angiostatin, a potent inhibitor of angiogenesis, was reported to inhibit human breast cancer growth and metastasis in xenograft model (O'Reilly et al., 1996; Peyruchaud et al., 2003) suggesting that antiangiogenic therapy could be a potential therapeutic approach for breast cancer. Previously we have reported that angiostatin binds to endothelial cell surface annexin II (Tuszynski et al., 2002). These findings, together with observations that angiostatin inhibits neoangiogenesis dependent growth of breast cancer may argue that angiostatin inhibits neoangiogenesis dependent breast cancer growth and metastasis by interfering with annexin II/tPA dependent plasmin generation in tumor microenvironment.

tPA is secreted by endothelial cells (EC) and is a ligand for cell surface annexin II. Emerging reports and clinical observations link tPA to neoplastic transformation and the invasive phenotype of highly aggressive tumors such as glioblastoma (Goh et al., 2005), melanoma (Stack et al., 1999) pancreatic cancer (Diaz et al., 2002) and breast cancer (Grondahl-Hansen et al., 1990; Rella et al., 1993). The functional role of tPA and its receptor annexin II in breast tumor biology is not well-understood. Recent studies have provided evidence that showed association of tPA with the invasion of highly aggressive MDA-MB-435s breast cancer cells (Chernicky et al., 2005). Since tPA plays a major role in plasmin generation, consistently plasmin has been linked to invasive and metastatic breast cancer (Stephens et al., 1998). Indeed, recent studies have clearly demonstrated over expression of annexin II in many cancer types and may argue that annexin II functions as a regulator of neoangiogenesis *in vivo*. However the precise mechanism by which annexin II is involved in cancer progression and metastasis is still unclear.

In this report we investigated if tPA interaction on breast cell surface annexin II is a molecular switch to regulate localized plasmin generation *in vitro* and neoangiogenesis *in vivo*. Taken together, this research may provide a clue for the annexin II-dependent mechanism of neoangiogenesis and its dependent breast cancer progression and metastasis. Annexin II may be a potential target for the development of effective therapeutic strategies to inhibit angiogenesis and 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 (Sharma et al., 2006b). 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) (Sharma

et al., 2006a). Cell cultures were maintained in plastic flasks and incubated at 37 °C in a humidified chamber containing 5% CO₂.

Immunohistochemistry

Previously 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 μ m sections were prepared on albumin-coated slides. Sections were deparaffinized and incubated in 3% H₂O₂ 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 (Sharma et al., 2006a). 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.

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 (0.5 μ g/ml) 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 °C for 4 min. Immune complex was resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane, incubated with anti-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 the membrane was developed by ECL as we have reported previously (Sharma et al., 2006a,b; Tuszynski et al., 2002).

tPA binding and plasmin generation

Binding of tPA with breast cancer cell (MDA-MB 231) surface was studied by analyzing the capacity of cell bound tPA to activate plasminogen to plasmin. Human breast cancer cells were cultured, detached and counted. About 5000–7000 cells were incubated with 300 units of recombinant human tPA ligand for 30 min on ice in PBS to allow tPA to bind cell surface annexin II. Cells were washed three times with chilled PBS to remove unbound tPA. Breast cancer cells with bound tPA were incubated with plasminogen and specific chromogenic substrate chromozyme PL in a total volume of 100 μ l to determine plasminogen activation activity. tPA dependent plasmin generation was recorded at 405 nm for 40 min in 96-well-plate reader according to our published protocol (Sharma et al., 2006a,b). 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 was considered as negative control indicative of auto degradation of plasminogen to

plasmin during the reaction process. In a parallel experiment an equal amount of tPA unbound to cells were used as control. Absorbance of control was subtracted from experimental readings. Data were analyzed by non-linear regression algorithms using GraphPad Prism software.

Annexin II gene silencing

Antisense phosphorothionate oligonucleotides (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). Three potential sites were identified in annexin II gene and antisense oligonucleotides were synthesized against these regions (5′CAA CAT GTC CAC TTC ACT - 3′ location: 923–940 bases of total sequence) (5′ - CAT TTT CCA GGT CTC CTT - 3′ location: 882–899 bases of total sequence) (5′ - TCA TCC ACA CCT TTG GTC - 3′ location: 277–294 bases of total sequence). The antisense oligonucleotides were synthesized as 18-mer targeted annexin II gene sequences. The corresponding controls sequence was also synthesized as 18 mer (5′GGA TTT ACC TAT TGC TGG 3′) (5′ ACT ACG ACC TAC GTG AC 3′) (5′GGA TTT ACC TAT TGC TGG 3′). Control antisenses were used in all parallel experiments. Hereafter, the antisense and sense annexin II oligonucleotides will be referred to 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 h. In contrast, the half-life of antisense ODNs in serum containing culture media is >48 h. 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.

Transfection

To optimize transfection efficiencies MDA-MB231 cells were incubated with 3 different concentrations of RNAi ANX II and control ANX II (0.5, 2.0 and 4.0 $\mu\text{mol/L}$) for 12, 24, 48, and 72 h to determine the rate of transfection. Our optimizing experiments showed that the uptake of RNAi ANX II was maximal with 0.5 $\mu\text{mol/L}$ and after incubation for 48 h. At this time point, annexin II protein was suppressed >90% (see results). Therefore we used these optimum conditions to suppress annexin II gene to determine tPA bindings, its dependent plasminogen activation and role in cell migration.

Scratch wound healing assay for cell migration

Cell migration was assayed as described (Sharma et al., 2004). Briefly, about 8000 cells were seeded and grown to confluency in 96 well

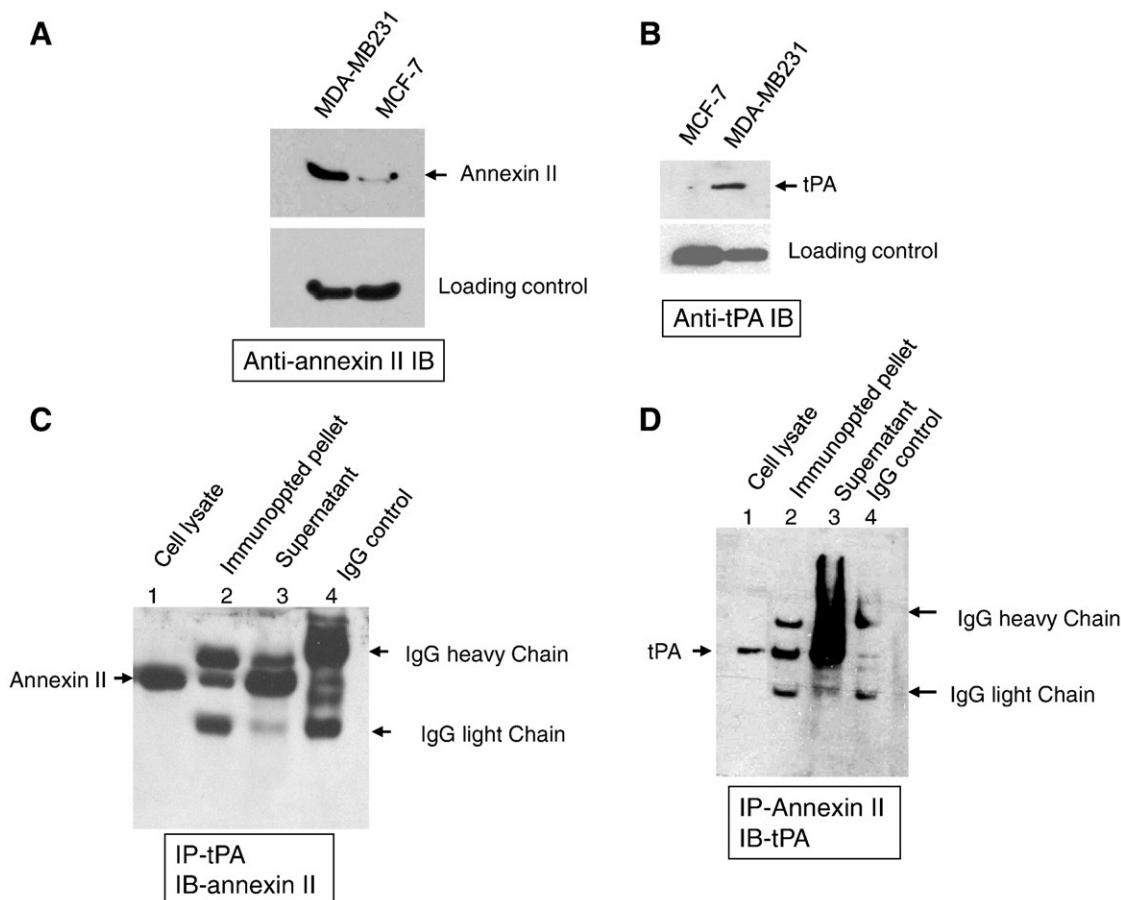


Fig. 1. Expressions and interactions of annexin II and tPA in human breast cancer cells. MDA-MB231 cell lysate (20 μg) was separated by SDS-PAGE and protein expression was analyzed by immunoblot analysis. Fig. 2 displays selective expression of annexin II and tPA in invasive breast cancer cell line MDA-MB231 (Fig. 2A and B). Cell lysate was immunoprecipitated (IP) with anti-tPA antibodies and the 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 the 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.

plates. A wound was created in monolayer of cells by scratching with a sterile pipette tip. The remaining adherent cells were washed twice with PBS. Media was replaced with 2% serum-supplemented medium. Cell motility was measured by counting the number of cells repopulated in the cleared area. Ten different fields (20X) were viewed to count migrating cells. Data were plotted using GraphPad Prism software.

Boyden chamber migration assay

The measurement of cell migration was performed as described by (Terranova et al., 1986). Chemotaxis cell migration assay was performed using a 96 well plate fitted with 8 μ m pore Boyden migration chambers (CHEMICON, Inc. cat # ECM510). Briefly, cells starved in serum free medium were harvested and plated at a density of 2.5×10^4 cells in 100 μ l without chemo-attractant in a Boyden chamber (upper well) in a 96 well plate. This assay is based on the Boyden chamber principle. The bottom well was filled (100 μ l) with medium containing 10% fetal bovine serum (FBS). Test reagents (plasminogen, plasmin, angiostatin, ϵ -aminocaproic acid, or antibodies) were added to the upper chamber and incubated for 24 h at 37 °C in a humidified chamber (5%CO₂). After 24 h, the plate was

flipped to remove the cells and medium. Migrated cells adhered on the other side of membrane were detached using detachment buffer and lysed in medium containing a fluorescent dye according to manufacturer instructions. The plate was read in a fluorescence plate reader (Bio-Tek, Inc., VT) using a 480/520 nm filter set. Serum-free medium alone was used as a negative control. Purified human plasmin was used as positive control. Separate controls were used against each reagent added. For example, mouse IgG was used as control for anti-annexin II antibody, reduced angiostatin was used as control for angiostatin and buffer in which ϵ -aminocaproic acid was dissolved was used as a control for ϵ -aminocaproic acid. Statistical analysis and graphic presentation of the data were performed with GraphPad Prism software.

Results

Cell surface annexin II interacts with recombinant human tPA in human breast cancer MDA-MB231 cells

Previous clinical studies including from our laboratory have shown over expression of annexin II in cancer patients. Over expression has

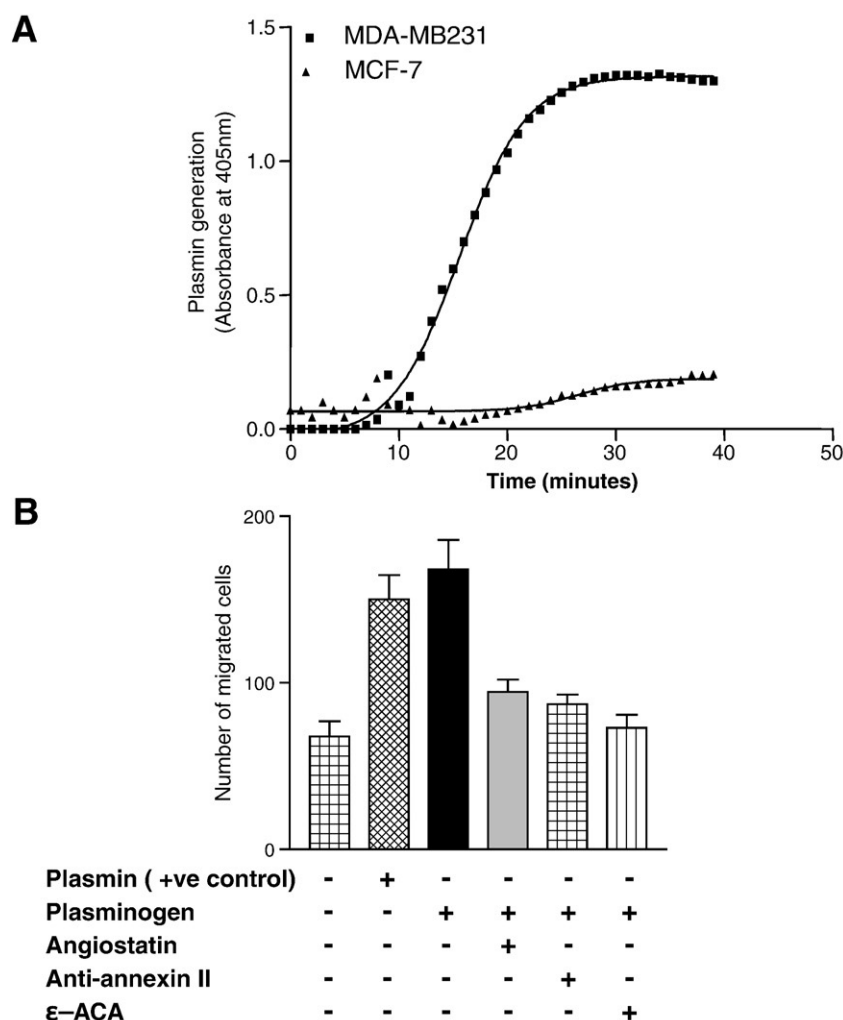


Fig. 2. Interaction of tPA with cell surface annexin II accelerate plasmin generation which in turn facilitates cell migration. MDA-MB231 cells were cultured in 12 well-plates. Cells were detached, washed and incubated and counted. About 20,000 cells were incubated with recombinant human tPA and allowed to bind on the cell surface annexin II for 30 min on ice. Cells were thoroughly washed to remove unbound tPA. tPA bound cells were incubated with plasminogen and chromozyme PL. Kinetics of plasminogen activation were monitored at 405 nm. Linearity of plasminogen conversion to plasmin was observed in about 20 min. Non invasive human breast cancer cells MCF-7 lacking annexin II failed to bind tPA and were unable to convert plasminogen to plasmin (A). tPA dependent plasmin generation induced cell migration. This enhanced cell migration could 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).

been correlated well with poor clinical outcome with advanced stages of cancers (Brichory et al., 2001; Roseman et al., 1994; Sharma et al., 2006a; Sharma and Sharma, 2007; Singh, 2007; Syed et al., 2007; Wu et al., 2002; Zimmermann et al., 2004). Because tPA is a ligand for annexin II and known to activate cell surface bound plasminogen to plasmin, we first tested whether annexin II and tPA proteins are synthesized by human breast cancer cells. Immunoblot analysis indicated selective expression of annexin II and tPA in highly invasive MDA-MB231 cells but not in non invasive MCF-7 cells (Fig. 1A and B). Next we investigated whether tPA synthesized by MDA-MB231 cells interacts with annexin II. To determine interaction, we immunoprecipitated an MDA-MB231 cell lysate with anti-tPA monoclonal antibody, and the 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 analysis identified tPA bound protein as annexin II indicating that tPA synthesized by MDA-MB231 cells binds to annexin II (Fig. 1C). These results were confirmed by a converse experiment where lysate was immunoprecipitated by anti-annexin II monoclonal antibody and bound protein was recognized by anti-tPA antibody confirming that tPA binds to annexin II (Fig. 1D).

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

The assembly of tPA and plasminogen on the cell surface annexin II facilitates conversion of inactive plasminogen to reactive enzyme plasmin. To test whether interaction of tPA and annexin II in MDA-MB231 cells are physiologically active, we assayed plasminogen activation in tPA bound live cells. MDA-MB231 cells were incubated with recombinant tPA and analyzed for cell surface binding. Unbound tPA was removed by washing. In parallel experiments non-invasive MCF-7 cells that lack annexin II expression were used as control. Specific tPA binding was determined by its ability to convert plasminogen to plasmin. Indeed, tPA bound to the MDA-MB231 cell surface and was able to activate plasminogen into plasmin in a time-dependent manner. As expected, MCF-7 cells lacking annexin II expression therefore tPA were unable to bind and consequently failed to activate plasminogen (Fig. 2A). To further confirm if cell surface interaction of annexin II and tPA is critical to activate plasminogen, we silenced annexin II gene in MDA-MB231 cells (Fig. 3A). These annexin II silenced cells were analyzed for their ability to bind tPA and their capacity to activate plasminogen. Results shown in Fig. 3C indicate that annexin II silenced MDA-MB231 cells were unable to bind tPA. Therefore activation of plasminogen was significantly inhibited. In contrast, wild type cells bound with recombinant tPA that activated plasminogen to plasmin efficiently (Fig. 3C). Collectively, these results strongly suggest that MDA-MB231 cell surface annexin II is required for tPA binding and is required for efficient plasmin generation.

Annexin II mediated plasmin generation is crucial for cell migration

Cell motility is critically involved in metastasis, neoangiogenesis and tumor invasion (Stack et al., 1999). It has been reported that plasmin is a strong serine protease and known to hydrolyze ECM and basement membrane (BM) proteins and thereby facilitates cellular migration, invasion and angiogenesis (Bajou et al., 2001; Diaz et al., 2002; Pepper, 2001; Tarui et al., 2002). Several studies were performed to determine the ability of annexin II to influence breast cancer cell migration. First we used a Boyden chamber assay model and tested if annexin II dependent plasmin generation is required for MDA-MB231 cell migration. The results of this experiment revealed that when cells were incubated with plasminogen they activated plasminogen to plasmin which in turn degraded ECM proteins and invaded across the membrane (Fig. 2B). This cell migration was significantly blocked by anti-annexin II antibody (Fig. 2B) suggesting that annexin II is involved in plasmin generation and its dependent

cell migration. Additional experiments were performed to confirm that annexin II-dependent plasmin generation plays a critical role in breast cancer cell migration. Previously we have shown that

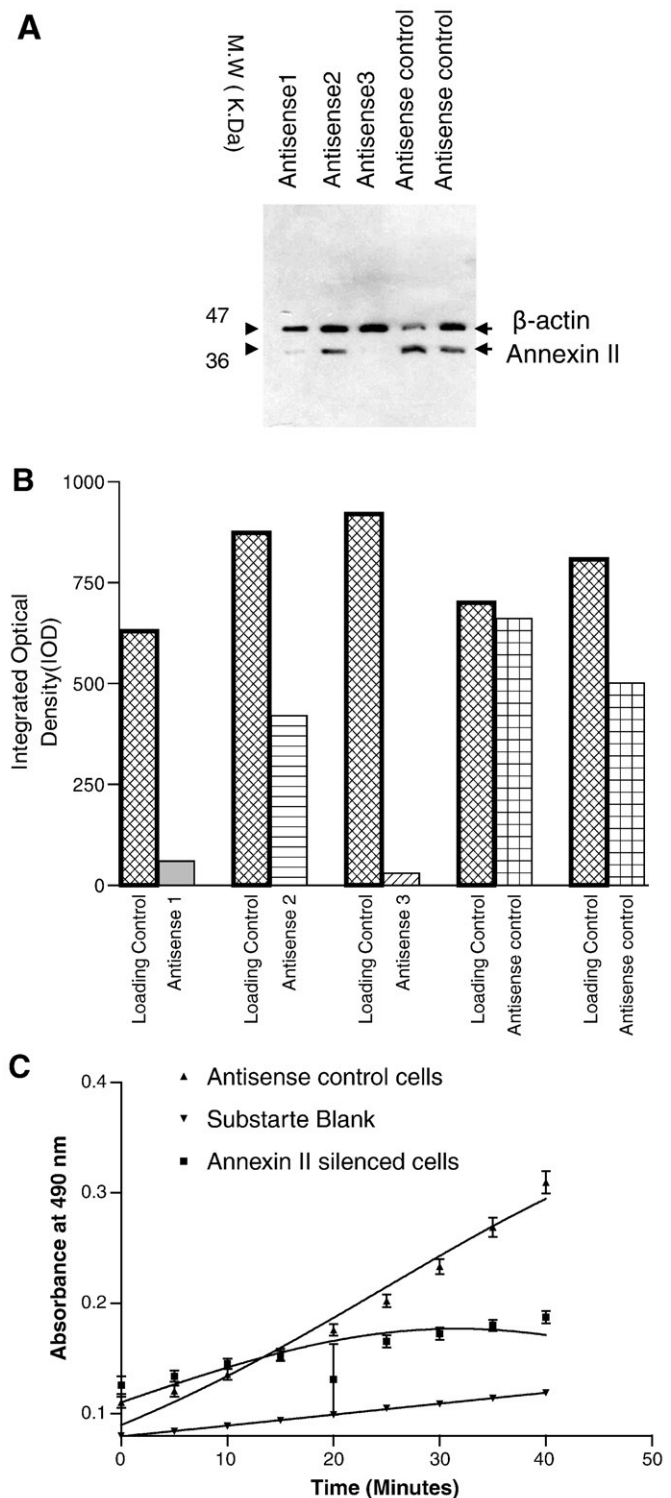


Fig. 3. Annexin II gene silencing inhibits tPA dependent plasmin generation. Three different antisense oligos were designed and synthesized as 16-mer dithionated oligonucleotide to silence annexin II gene. Immunoblot analysis suggests that two out of three antisense oligos were highly potent and almost completely silenced annexin II expression (A). β -actin was used as a loading control to confirm equal protein loading. Quantitative analysis of band densities showed more than 90% inhibition of annexin II expression as compared to β -actin loading control (B). Annexin II-silenced cells failed to bind tPA and significantly inhibited plasminogen activation (C).

angiostatin competes with plasminogen binding on endothelial cell surface annexin II because angiostatin and plasminogen share common binding sites and angiostatin competes for plasminogen binding (Tuszynski et al., 2002). We speculate that angiostatin may inhibit cancer invasion and migration (Stack et al., 1999; Tarui et al., 2002) by inhibiting plasmin generation. To test whether angiostatin blocks cell migration, we incubated angiostatin with the MB231 cells prior to plasminogen and examined whether this peptide inhibits annexin II-mediated breast cancer cell migration. As shown in Fig. 2B angiostatin indeed inhibited MDA-MB231 cell migration significantly suggesting that angiostatin blocks annexin II-dependent plasmin accumulation which in turn inhibits cell migration. Since lysine binding domains in plasminogen is critical for binding to cell surface receptors and activation to plasmin (Felez et al., 1996; Tuszynski et al., 2002), we next investigated the role of these residues in breast cancer cell migration using a lysine analogue ϵ -aminocaproic acid (EACA). Interestingly addition of EACA inhibited breast cancer cell migration significantly. These results support the conclusion that effective blocking of annexin II dependent plasmin generation may be critical for MDA-MB231 cell migration. In a second approach we silenced annexin II gene in MDA-MB231 cells (Fig. 3A) and assayed their abilities to activate plasminogen as well as migratory potential in a scratch wound healing model of cell migration. Results presented in Fig. 3C shows that silencing of annexin II expression significantly inhibited tPA binding and its dependent plasminogen activation. Annexin II silenced cells showed significant lower migratory activity as compared to wild type cells confirming that annexin II dependent plasmin generation is indeed required for MDA-MB231 cell migration (Fig. 4A and B).

Discussion

Tumor progression and metastasis involves degradation of ECM and BM proteins. Once this anatomic barrier is destroyed tumor cells start invading into adjacent host tissue and normal host cells start migrating into the tumor. Such migratory, invasive and tissue remodeling events are regulated by different proteolytic enzymes (Pepper, 2001). Among the proteases that play an active role in these processes is the serine protease plasmin. Plasmin is an end enzymatic product of plasminogen/plasmin pathway. Physiological roles of plasmin were believed to be limited to fibrinolysis, extracellular proteolysis, migration of macrophages to a site of injury, tissue repair/remodeling, invasion and angiogenesis (Lijnen, 2001; Pepper, 2001). Many advanced human tumors including breast cancer overproduce plasmin that is known to promote neoangiogenesis, cancer invasion and metastasis (Paciucci et al., 1998; Sharma et al., 2006a). Analysis of

the enzymes of plasminogen/plasmin system suggests that high urokinase-type plasminogen activator (Li et al., 1998) in breast tumors predicts poor survival and is associated with a high rate of relapse for patients with breast cancer (Janicke et al., 2001; Janicke et al., 1989; Look et al., 2002). These clinical observations suggest that the components of plasminogen/plasmin system can contribute to tumorigenesis in a variety of tissue types (Ossowski and Aguirre-Ghiso, 2000) including breast cancer. Consistent with this notion, inhibition of uPA activity seems to inhibit breast cancer in a mouse model (Rabbani and Gladu, 2002). Existing literature clearly indicates that under pathological conditions uncontrolled production of plasmin in the tumor microenvironment can accelerate the localized degradation of ECM and BM. Destruction of this anatomical structure may lead to release of matrix bound latent growth factors such as bFGF and VEGF which are known angiogenic mitogens (George et al., 2001; McColl et al., 2003). Earlier studies showed that proteolytic activity of plasmin is required to activate these growth factors which reprogram migrating endothelial cells for proliferation to establish new blood vessels (McColl et al., 2003).

Considering this fact our *in vitro* data may be of immense importance which shows that annexin II on the surface of breast cancer cells could be a molecular switch that specifically binds with tPA and thus regulates plasmin generation (Figs. 1 and 2A). Since noninvasive breast cancer cells MCF-7 that lacks annexin II expression failed to activate plasminogen may suggest specificity of invasive MDA-MB231 cells in plasmin generation (Figs. 1 and 2A). Because our data show interaction of tPA with annexin II in MDA-MB231 cells (Fig. 1.) that is capable to produce plasmin (Fig. 2A), we sought to determine if plasmin is involved in breast cancer cell migration. The data presented in Fig. 2B show that the cell migration was indeed plasmin dependent because ϵ -aminocaproic acid, Lys analogue, which is known to disrupt kringle-lysine interaction between plasminogen and their receptors, inhibits plasmin generation and migration in Boyden chamber model (Felez et al., 1996; Tuszynski et al., 2002).

Previously we have shown that angiostatin competes for plasminogen binding on EC surface annexin II and blocks plasmin generation (Sharma et al., 2006b; Tuszynski et al., 2002). Moreover angiostatin was reported to inhibit neoangiogenesis dependent breast cancer growth and metastasis (Griscelli et al., 1998; O'Reilly et al., 1996; Peyruchaud et al., 2003) in mouse model. Our data demonstrates that blocking of annexin II either by angiostatin or monoclonal antibody to annexin II effectively blocks MDA-MB231 cell migration indicating specificity of annexin II in cell migration (Fig. 2B). Based on our experimental evidence it can be argued that angiostatin mediated inhibition of cell migration may be due its ability to inhibit plasmin

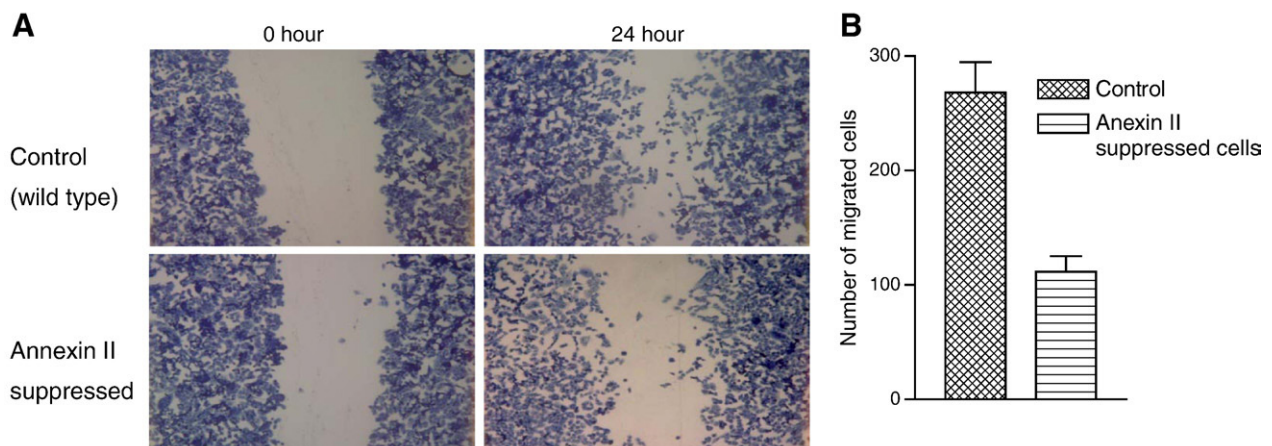


Fig. 4. Annexin II gene suppressed MDA-MB231 cells reduces cell motility. Annexin II gene silenced cells were grown in 96-well-plates. Cells were denuded by scraping and incubated for 24 h. After 24 h cells migrated across the wounded edge were counted and results are expressed as the mean $\pm$ SD of three experiments.

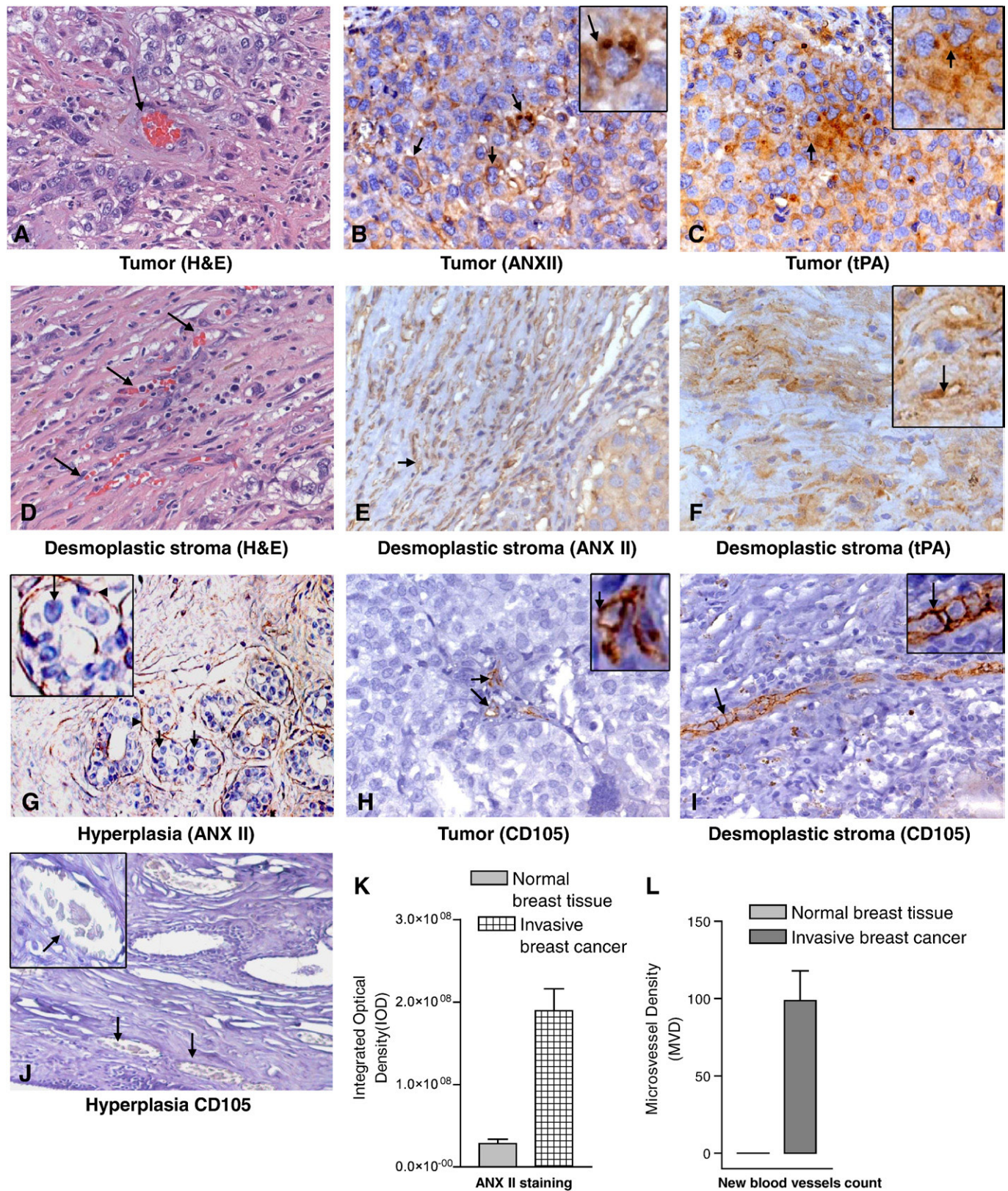


Fig. 5. Immunostaining for annexin II, tPA and anti-CD 105 in normal and human breast cancer tissues. Annexin II is undetectable in normal acinar and ductal epithelial cells (G, inset arrows). Anti-annexin II antibodies show a very strong reactivity on the surface of ductal epithelial cells (B, inset), tumor stroma (E) 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 (F inset), ductal epithelial cells (C, inset) and in tumor stroma. Anti-CD105 antibodies show a selective strong reactivity with newly formed blood vessels (H) but not in existing blood vessels of normal tissue (J). Panel J 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 K. CD 105 positive endothelial cells were counted and plotted by GraphPad Prism software Panel L.

generation by blocking plasminogen/tPA binding on cell surface annexin II (Brownstein et al., 2004; Stack et al., 1999; Tuszyński et al., 2002). Silencing of annexin II gene in MDA-MB231 cells inhibits their ability to bind tPA and plasminogen activation (Fig. 3). Furthermore these cells were less migratory as compared to wild type in a scratch wound healing model of migration assay (Fig. 4). Based on our data it is not surprising that Shiozawa et al. have found involvement of annexin II in adhesion, migration and homing of prostate cancer cells (Shiozawa et al., 2008). Together, these *in vitro* and *in vivo* experimental evidence links a potential role of annexin II in breast cancer.

It is possible that plasmin mediated destruction of the anatomic barrier of ECM and BM could support EC /cancer cell migration (Tarui et al., 2002) that may be the key for new blood vessel formation to support tumor expansion and metastasis (Ling et al., 2004; McColl et al., 2003; Semov et al., 2005). Our observations are further supported by the facts that tumors failed to grow on plasminogen (PLG-/-) gene knockout mice and unable to metastasize because in absence of plasmin tumor's ability to form new blood vessels was significantly inhibited (Bajou et al., 2001; Perides et al., 2006). Consistently a number of prior articles have described that direct inhibition of plasmin suppresses experimental metastasis, neoangiogenesis and tumor progression in Lewis Lung Carcinoma (LLC) model (Bugge et al., 1997; Tanaka et al., 1982).

Taken together, our data provides strong evidence that annexin II-dependent plasmin generation is required for breast cancer cell migration. It seems that annexin II is a regulatory switch for continuous plasmin generation in the tumor microenvironment and might explain the presence of annexin II on the cell surface of many different types of human cancers.

These data raised the possibility that annexin II dependent plasmin generation may be an endogenous regulator of neoangiogenesis (Ling et al., 2004). To investigate this we analyzed human breast tissue samples by immunohistochemical staining. Immunohistochemical studies revealed expression of ANX II mainly on the surface of invasive cancerous cells (Fig. 5B). In contrast, epithelial cells in normal ducts were completely negative (Fig. 5G). Consistent with annexin II expression, immunostaining of thin serial sections also revealed high levels of tPA in tumor stroma (Fig. 5C). In agreement with our findings earlier clinical studies have also noticed elevated levels of tPA in the plasma of human breast cancer (Grondahl-Hansen et al., 1990; Rella et al., 1993), glioblastoma and pancreatic cancers (Aguilar et al., 2004; Goh et al., 2005). Careful investigation of immunostained sections revealed collagenous stroma composed of fibroblasts, macrophages, lymphocytes and ECM indicating an inflammatory response and annexin II in desmoplastic stroma (Fig. 5E) suggesting chronic inflammation in the tumor microenvironment and may be indicative of neoangiogenic activity. Next, we investigated neoangiogenic activity in the serial sections of same tissue using anti-CD105, selective marker for sprouting neovessels (Gerber et al., 2006; Kumar et al., 1999). Staining pattern of CD105 revealed numerous sprouting neovessels in tumor as well in desmoplastic stroma confirming neoangiogenic activity (Fig. 5H and I). Quantitative analysis of neoangiogenic activity as measured by micro vascular density (MVD) appears to correlate with annexin II expression patterns (Fig. 5K and L).

Based on our *in vitro* and *in vivo* data, it is quite likely that over-expression of annexin II in human breast cancer regulates local plasmin generation which in turn degrades ECM and BM. Hydrolysis of matrix components by plasmin may release and activate matrix bound inactive angiogenic cytokine VEGF and may induce neoangiogenic activity (McColl et al., 2003). This could be an early event in the tumor microenvironment switching breast cancer from prevascular phase to the vascular phase (Ling et al., 2004) which is characterized by aggressive phase with the possibility of metastasis. Consistent with our observations neoangiogenic activity reported to be a significant prognostic indicator in early stage of breast cancer (Weidner et al., 1992).

Collectively, the data reported here and previously published reports strongly support the concept that annexin II on tumor/EC may be an angiogenic switch. This may be a potential reason why annexin II is being repeatedly identified as widely overexpressed protein in number of cancers and strongly associated with prognostic significance in metastasis (Brichory et al., 2001; Chuthapisith et al., 2009; Guedj et al., 2009; Pei et al., 2007; Roseman et al., 1994; Sharma et al., 2006a; Sharma and Sharma, 2007; Shiozawa et al., 2008; Singh, 2007; Syed et al., 2007; Wu et al., 2002; Zhong et al., 2009; Zimmermann et al., 2004). Whether annexin II could be a valuable target in inhibiting neoangiogenic activity and breast cancer *in vivo* is an interesting but unanswered question that is currently under investigation in our laboratory.

Acknowledgments

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 Chalon Saunders and Miriam Smith for technical assistance.

References

- Aguilar, S., et al., 2004. Tissue plasminogen activator in murine exocrine pancreas cancer: selective expression in ductal tumors and contribution to cancer progression. *Am. J. Pathol.* 165, 1129–1139.
- Bajou, K., et al., 2001. 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.
- Brichory, F.M., et al., 2001. An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. *Proc. Natl. Acad. Sci. U. S. A.* 98, 9824–9829.
- Brownstein, C., et al., 2004. Annexin II mediates plasminogen-dependent matrix invasion by human monocytes: enhanced expression by macrophages. *Blood* 103, 317–324.
- Bugge, T.H., et al., 1997. Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice. *Blood* 90, 4522–4531.
- Cesarman, G.M., et al., 1994. An endothelial cell receptor for plasminogen/tissue plasminogen activator (t-PA). II. Annexin II-mediated enhancement of t-PA-dependent plasminogen activation. *J. Biol. Chem.* 269, 21198–21203.
- Chernicky, C.L., et al., 2005. Tissue-type plasminogen activator is upregulated in metastatic breast cancer cells exposed to insulin-like growth factor-I. *Clin. Breast Cancer* 6, 340–348.
- Chuthapisith, S., et al., 2009. Annexins in human breast cancer: possible predictors of pathological response to neoadjuvant chemotherapy. *Eur. J. Cancer* 45, 1274–1281.
- Diaz, V.M., et al., 2004. Specific interaction of tissue-type plasminogen activator (t-PA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion *in vitro*. *Gut* 53, 993–1000.
- Diaz, V.M., et al., 2002. Tissue plasminogen activator is required for the growth, invasion, and angiogenesis of pancreatic tumor cells. *Gastroenterology* 122, 806–819.
- Felez, J., et al., 1996. Characterization of cellular binding sites and interactive regions within reactants required for enhancement of plasminogen activation by tPA on the surface of leukocytic cells. *Thromb. Haemost.* 76, 577–584.
- George, S.J., et al., 2001. Plasmin-mediated fibroblast growth factor-2 mobilisation supports smooth muscle cell proliferation in human saphenous vein. *J. Vasc. Res.* 38, 492–501.
- Gerber, S.A., et al., 2006. 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.
- Goh, K.Y., et al., 2005. Tissue plasminogen activator expression in meningiomas and glioblastomas. *Clin. Neurol. Neurosurg.* 107, 296–300.
- Griscelli, F., et al., 1998. 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.
- Grondahl-Hansen, J., et al., 1990. Tissue-type plasminogen activator in plasma from breast cancer patients determined by enzyme-linked immunosorbent assay. *Br. J. Cancer.* 61, 412–414.
- Guedj, N., et al., 2009. Comparative protein expression profiles of hilar and peripheral hepatic cholangiocarcinomas. *J. Hepatol.* 51, 93–101.
- Hajjar, K.A., et al., 1994. An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. *J. Biol. Chem.* 269, 21191–21197.
- Janicke, F., et al., 2001. Randomized adjuvant chemotherapy trial in high-risk, lymph node-negative breast cancer patients identified by urokinase-type plasminogen activator and plasminogen activator inhibitor type 1. *J. Natl. Cancer Inst.* 93, 913–920.
- Janicke, F., et al., 1989. Urokinase-type plasminogen activator antigen and early relapse in breast cancer. *Lancet* 2, 1049.
- Kumar, S., et al., 1999. Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. *Cancer Res.* 59, 856–861.

- Li, H., et al., 1998. Adenovirus-mediated delivery of a uPA/uPAR antagonist suppresses angiogenesis-dependent tumor growth and dissemination in mice. *Gene Ther.* 5, 1105–1113.
- Lijnen, H.R., 2001. Plasmin and matrix metalloproteinases in vascular remodeling. *Thromb. Haemost.* 86, 324–333.
- Ling, Q., et al., 2004. Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. *J. Clin. Invest.* 113, 38–48.
- Look, M.P., et al., 2002. 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.
- McColl, B.K., et al., 2003. Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. *J. Exp. Med.* 198, 863–868.
- Mignatti, P., Rifkin, D.B., 1993. Biology and biochemistry of proteinases in tumor invasion. *Physiol. Rev.* 73, 161–195.
- O'Reilly, M.S., et al., 1996. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.* 2, 689–692.
- Ohno, Y., et al., 2009. Annexin II represents metastatic potential in clear-cell renal cell carcinoma. *Br. J. Cancer* 101, 287–294.
- Ossowski, L., Aguirre-Ghiso, J.A., 2000. Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. *Curr. Opin. Cell Biol.* 12, 613–620.
- Paciucci, R., et al., 1998. The plasminogen activator system in pancreas cancer: role of t-PA in the invasive potential in vitro. *Oncogene* 16, 625–633.
- Pei, H., et al., 2007. Proteome analysis and tissue microarray for profiling protein markers associated with lymph node metastasis in colorectal cancer. *J. Proteome Res.* 6, 2495–2501.
- Pepper, M.S., 2001. Extracellular proteolysis and angiogenesis. *Thromb. Haemost.* 86, 346–355.
- Perides, G., et al., 2006. The fibrinolytic system facilitates tumor cell migration across the blood-brain barrier in experimental melanoma brain metastasis. *BMC Cancer* 6, 56.
- Peyruchaud, O., et al., 2003. Angiostatin inhibits bone metastasis formation in nude mice through a direct anti-osteoclastic activity. *J. Biol. Chem.* 278, 45826–45832.
- Rabbani, S.A., Gladu, J., 2002. Urokinase receptor antibody can reduce tumor volume and detect the presence of occult tumor metastases in vivo. *Cancer Res.* 62, 2390–2397.
- Rella, C., et al., 1993. Tissue-type plasminogen activator as marker of functional steroid receptors in human breast cancer. *Thromb. Res.* 69, 209–220.
- Roseman, B.J., et al., 1994. Annexin II marks astrocytic brain tumors of high histologic grade. *Oncol. Res.* 6, 561–567.
- Semov, A., et al., 2005. Metastasis-associated protein S100A4 induces angiogenesis through interaction with Annexin II and accelerated plasmin formation. *J. Biol. Chem.* 280, 20833–20841.
- Sharma, M., et al., 2006a. 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.
- Sharma, M., et al., 2006b. 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.
- Sharma, M.C., Sharma, M., 2007. The role of annexin II in angiogenesis and tumor progression: a potential therapeutic target. *Curr. Pharm. Des.* 13, 3568–3575.
- Sharma, M.R., et al., 2004. 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.
- Shiozawa, Y., et al., 2008. Annexin II/annexin II receptor axis regulates adhesion, migration, homing, and growth of prostate cancer. *J. Cell. Biochem.* 105, 370–380.
- Singh, P., 2007. Role of Annexin-II in GI cancers: interaction with gastrins/progastrins. *Cancer Lett.* 252, 19–35.
- Stack, M.S., et al., 1999. Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation. *Biochem. J.* 340, 77–84.
- Stephens, R.W., et al., 1998. The urokinase plasminogen activator system as a target for prognostic studies in breast cancer. *Breast Cancer Res. Treat.* 52, 99–111.
- Syed, S.P., et al., 2007. Angiostatin receptor annexin II in vascular tumors including angiosarcoma. *Hum. Pathol.* 38, 508–513.
- Tanaka, N., et al., 1982. Ultrastructural study of the effects of tranexamic acid and urokinase on metastasis of Lewis lung carcinoma. *Br. J. Cancer* 46, 428–435.
- Tarui, T., et al., 2002. Plasmin-induced migration of endothelial cells. A potential target for the anti-angiogenic action of angiostatin. *J. Biol. Chem.* 277, 33564–33570.
- Terranova, V.P., et al., 1986. Basement membrane and the invasive activity of metastatic tumor cells. *J. Natl. Cancer Inst.* 77, 311–316.
- Tuszynski, G.P., et al., 2002. Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. *Microvasc. Res.* 64, 448–462.
- Weidner, N., et al., 1992. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J. Natl. Cancer Inst.* 84, 1875–1887.
- Wu, W., et al., 2002. Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. *Clin. Exp. Metastasis* 19, 319–326.
- Zhong, L.P., et al., 2009. Increased expression of Annexin A2 in oral squamous cell carcinoma. *Arch. Oral Biol.* 54, 17–25.
- Zimmermann, U., et al., 2004. Expression of annexin II in conventional renal cell carcinoma is correlated with Fuhrman grade and clinical outcome. *Virchows Arch.* 445, 368–374.

Inflammation, Microenvironment, and the Immune System in Cancer Progression

Andrew Keibel¹, Vinod Singh² and Mahesh C. Sharma^{1,*}

¹Departments of Surgery and Pathology, Drexel University College of Medicine, Philadelphia, PA 19102, USA and

²Departments of Microbiology Barakatullah University, Bhopal, MP, India

Abstract: Since Virchow first proposed in 1863 that tumors could originate from sites of chronic inflammation, it has been well established that chronic inflammation both contributes to cancer progression and predisposes tissue to various types of cancer. Experimental, clinical, and epidemiological studies have all demonstrated the strong association between chronic inflammation and cancer, and many studies have correlated the prolonged presence of the inflammatory milieu with an increased risk for developing cancer. Proinflammatory cytokines, chemokines and adhesion molecules, which regulate the sequential recruitment of leukocytes, are frequently observed in tumor microenvironment. These early desmoplastic changes could stimulate fibroblasts and endothelial cell division and produce components for tissue remodeling and neovascularization, ultimately promoting neoplastic processes. In this review article we overview the current understanding of the role of chronic inflammation in neoangiogenesis, tumor initiation, promotion, and progression.

Key Words: Inflammation, microenvironment, neoangiogenesis, cancer.

INTRODUCTION

Neoplasia ("new growth") is the uncontrolled proliferation of transformed cells. The term *tumor*, which was originally used to describe the swelling caused by inflammation, is now used interchangeably with *neoplasm*. Transformation is the multi-step process in which normal cells acquire malignant characteristics. Each step in the neoplastic process reflects a genetic alteration that confers a growth advantage over normal cells. There are a number of essential alterations in cell physiology that collectively enable malignant growth: self-sufficiency in growth signals, evasion of programmed cell death (apoptosis), a avoidance of immune detection and destruction, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These characteristics are shared by most, if not all, human tumors. Despite tremendous progress in detection as well as treatment of cancer, rising cancer deaths requires a reevaluation of our basic understanding about the etiology of cancer and how to control it.

For the past four decades investigators have been trying to halt tumor growth by directly attacking cancer cells. This approach has provided limited clinical success coupled with a high degree of toxicity. Continuous failure has indicated that it is time to change our strategies and look beyond the cancer cells. Studies that have done such, through the implementation of tissue, gene and protein microanalysis, have shown significant alteration in the tumor microenvironment known as tumor stroma. These new investigations argue against our old understanding that carcinogenesis occurs in individual cells, and support a new idea that carcinogenesis

occurs in tissue. The heterogeneous nature of the tumor is a result of continuous crosstalk between different cell types within tumor and its surrounding stroma.

Inflammation is the body's response to tissue insult. When tissue is damaged, either by physical trauma or by pathogen, a complex cascade of events is triggered. Numerous inflammatory cells and complexes collaborate to attack the invading pathogen, clear debris, reconstitute the extracellular matrix (ECM) and assist in the proliferation and transfer of healthy cells to the target site. The inflammatory response is often initiated by mast cells. When damaged, mast cells release histamine, an amino acid that increases the permeability of adjacent capillaries. Increased blood flow and vessel permeability enable the recruitment of inflammatory cells, including interleukins, neutrophils, cytokines and monocytes to the damaged tissue.

Ideally, inflammation is a self-limiting process, subsiding once the wounded tissue has healed. If inflammation does not subside within the normal course, the very mechanisms that the body uses to heal can promote unintended results, including neoplastic processes, such as cancer.

This heterogeneous nature of tumor stroma is very similar to granulation tissue formed during wound healing. Interestingly, Hal Dvorak in 1986 proposed that tumor build up their stroma by triggering host wound-healing response, which ultimately leads to fibrin deposition at the tumor site. This fibrin provides temporary ECM that forms a provisional scaffold for cancer cells, inflammatory cells and newly formed blood vasculature. Thus he proposed the tumor to be "wounds that do not heal" [1].

Indeed, this relationship between chronic inflammation and cancer has been well established both *in vitro* and *in vivo*. For example, skin inflammation is associated with

*Address correspondence to this author at the Department of Surgery and Pathology, Drexel University College of Medicine, MS 435, 245 N 15th Street, Philadelphia, PA 19102, USA;
Email: Mahesh.sharma@drexelmed.edu

melanoma, bladder inflammation with bladder carcinoma, chronic pancreatitis with pancreatic carcinoma, chronic viral hepatitis with liver cancer, chronic HPV infection with cervical cancer, inflammatory bowel disease with colorectal carcinoma, smoking (which inflames the lungs) with lung cancer, and hepatitis C viral infection with hepatocellular carcinoma [2]. Components of inflammation have been reported to frequently be overexpressed and active within the tumor environment. The cancer-associated inflammation milieu is defined by the expression of: leukocyte infiltration, cytokines such as tumor necrosis factor α (TNF- α) and various interleukins, chemokines such as cyclooxygenase-2 (COX-2), monocytes derivatives called tumor-associated macrophages (TAM), matrix metalloproteinases (MMP), reactive oxygen and nitrogen species (ROS and RNS), and an activated nuclear factor κ B (NF- κ B) [3]. These components of cancer-associated inflammation work in a concerted effort to promote tumorigenesis in four major ways: by promotion of angiogenesis, by facilitation of cancer cell and tumor proliferation, by suppression of anti-tumor immunity, and by inhibition of apoptotic events.

In this article, we review the current literature supporting the relationship between chronic inflammation and cancer, as well as the current body of research that explains the possible mechanisms that define this relationship.

TUMOR ASSOCIATED MACROPHAGES (TAMs)

Derived from monocytic precursors circulating in the blood [4], tumor associated macrophages (TAM) are a significant component of inflammatory infiltrates in the tumor microenvironment [2]. In rare cases of breast cancer, TAM were found to represent more than 50% of tumor mass [5]. TAM concentration within the tumor environment appears to play a significant role in patient prognosis: a significant difference exists between the survival rates of patients with tumors defined as having low TAM density compared to patients with tumors defined as having high TAM density [6]. It was previously believed that optimally stimulated TAM were capable of killing *in vitro* tumor cells. This theory has been replaced with a general acceptance that TAM mostly, although not always, serve pro-tumoral functions [4]. For example, when activated by interleukin 12 (IL-12) TAM regulates T-cell dependent eradication of tumors [7]. However, it is more common for TAM to undertake the following pro-tumoral roles: assuaging tumor survival in hypoxic conditions due to angiogenesis, suppression of anti-tumor immunity, and aiding in neoplastic cell proliferation [8].

Development of a high density vessel network which connects the tumor to host circulation, termed the 'angiogenic switch' is crucial for the tumor's progression to malignancy [9]. Peri-tumoral accumulation of macrophages has been associated with blood vessel density in tumors [10]. Additionally, it has been demonstrated in mice that macrophages infiltrated the tumor site during the nonmalignant adenoma stage [10], the stage associated with the angiogenic switch. Together, these findings indicate that macrophages have a more significant role on tumor progression to malignancy rather than tumor growth. One suggested mechanism behind TAM infiltration and the subsequent triggering of angiogenesis states that tumors induce hypoxic stress, which in

turn recruits TAM [7]. Once arriving at the site of hypoxia, TAM become angiogenically active; producing multiple proangiogenic growth factors and proteinases such as vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9) and uPA [9].

TAM have been shown to suppress immune response through both poor antigen-presenting activity [11] as well as inhibition T-cell proliferation. TAM inhibits T-cell proliferation through the release of prostaglandins, IL-10 and TGF- β [11]. Prostaglandins have been established to suppress T-cell proliferation in various malignant disorders including Hodgkin's disease [12]. T-cells that are recruited to the site of tissue injury also have diminished anti-tumoral capabilities as the result of TAM, as TAM have been shown to release chemokines that recruit T-cells that lack any cytotoxic function. As the cellular source of the chemokine CCL18, TAM was found to have significantly higher concentrations in the ascitic fluids from patients with ovarian carcinoma versus nonovarian carcinoma patients [13].

The proliferative effects of TAM were demonstrated *in vivo* in mice with mammary cancer. The macrophage colony stimulating factor (CSF-1) has been observed within human breast carcinomas, and correlated with poor patient prognosis [14]. Using a mouse model, tumor progression was compared between mice containing an inactivating mutation in the CSF-1 gene and wild-type mice. Expression of CSF-1 in the wild-type mice accelerated tumor progression and increased pulmonary metastasis [7]. Based upon these findings, CSF-1 is implicated as promoting metastatic potential by regulating TAM infiltration at the tumor site. TAM has also been shown to promote neoplastic proliferation by producing and secreting neoplastic factors including IL-1, IL-10 and platelet-derived growth factor into the tumor stroma [15]. Finally, TAM also produce MMP, which degrades all structural components of the extracellular matrix (ECM) [16], allowing for easier tumor infiltration and cell proliferation.

MATRIX METALLOPROTEINASES (MMPs)

Expression and activation of MMP, a proteolytic enzyme, is increased in almost all human cancers compared to non-cancer tissue [17]. Furthermore, expression of MT1-MMP and MMP2 are associated with poor prognosis in patients with various carcinomas [18]. In animal studies, up-regulation of MMP expression increases the aggressiveness of benign cancer cells, while down-regulation has the opposite effect. MMP appear to play a paradoxical role in cancer progression while acting in both pro and anti angiogenic roles. As a tumor promoter, MMP aids in neoplastic cell proliferation and immunosuppression.

MMP-1, MMP-2, MMP-9 and MT-MMPs are all known to promote angiogenesis through various mechanisms [19]. These mechanisms include control of endothelial cell tube morphogenesis in three dimension ECM assay, regulation of vessel stabilization by controlling pericyte recruitment [19], and mediating fibrinolytic and collagenolytic activity [20]. Recent studies provide evidence that the pro-angiogenic effect of MT-MMP is mediated by an up-regulation of VEGF. VEGF expression, in turn, has been reported to be controlled

by MMP-9. MMP-9 controls VEGF bioavailability by cleaving it from the ECM [21]. Once released from the ECM, MMP-1, MMP-3, MMP-7 and MMP-13 selectively degrade connective tissue growth factor bound to VEGF, thereby increasing the reactive angiogenic activity of VEGF [21].

Conversely, MMP-7, MMP-12, MMP-13 and MMP-20 are believed to be negative regulators of tumor angiogenesis as a result of their capacity to generate angiogenic inhibitors. These angiogenic inhibitors are produced through the cleavage of plasminogen type IV or type XVIII collagens [22].

Similar to TAM, MMP have diverse effects on T-cell efficacy as an anti-tumor agent. Interleukin-2 receptor α (IL-2R α) has a direct role in the production of functional T-cells. It has been demonstrated in cervical cancer, where tissues over express MMP-9, that MMP-9 mediates cleavage of IL-2R α and reduces the functionality of cancer-encountered T-cells, ultimately decreasing the function of tumor-reactive cytotoxic lymphocytes [23]. This finding was supported by the restoration of T-cell proliferation when MMP inhibitors blocked MMP induced IL-2R α cleavage.

MMP also affects cell invasion. E-cadherin is a cell-cell adhesion molecule responsible for epithelial cell aggregation along with the ability to suppress tumor invasion. Disturbance of E-cadherin resulting in a loss of cell-cell adhesion during tumor progression is positively correlated with tumor invasiveness [24]. An *in vitro* study conducted with a human breast cell line demonstrated that the MMP matrilysin and stromelysin 1 both cleave E-cadherin at the cell surface. It was concluded that this direct cleavage is capable of inhibiting epithelial cell aggregation, as well as facilitating invasion of epithelial cells in a paracrine manner [25]. MMP also act as a broad spectrum protease, indiscriminately degrading ECM and thus priming tissue for pathogen invasiveness [25].

MMP's role as a tumor promoter has been demonstrated both in humans and in mice. *In vivo*, mice lacking MMP-9 displayed reduced proliferation at all neoplastic stages and a decreased incidence of invasive tumors [20]. MMP-9 from bone marrow-derived inflammatory cells has also been proven to contribute to the HPV16 squamous carcinogenesis pathway [20].

TUMOR NECROSIS FACTOR α (TNF- α)

Once the inflammatory response has been initiated, tumors actively recruit inflammation-associated infiltrate cells, which upon arrival produce their own set of proteins: cytokines and chemokines [26]. One of the most prominent cytokines in the inflammatory infiltrate is TNF- α , a pro inflammatory cytokine that regulates growth development, differentiation, wound healing and immune response. TNF- α plays a dual role in tumor progression, switching between pro and anti-tumoral roles in a dose dependent manner. At high concentrations, TNF- α destroys tumor vasculature and induces tumor necrosis. When chronically produced in relatively low concentrations, as it frequently is in chronic inflammation, TNF- α acts as a tumor promoter [26].

TNF- α has been shown to play an especially important role in early stage tumor promotion both *in vitro* and in mouse models. TNF- α deficient mice developed 5-10% the

number of tumors developed by wild-type mice during the initiation and promotion phase of tumor progression [27]. After the initiation and promotion phase, TNF- α deficient mice developed 25% the number of tumors as wild-type mice, indicating that TNF- α is integral in the initiation of the tumor as opposed to tumor growth [27].

TNF- α also affects tumor stroma through the induction of chemokines and MMP, and by stimulating fibroblast growth and function; all being angiogenic factors. TNF- α also expresses receptors for angiogenic cytokines. Working in concert with IL-1, TNF- α produces the angiogenic factor VEGF, while simultaneously destroying ECM. ECM is also degraded when TNF- α is chronically expressed as a result of TNF- α stimulated production of u-P A. Activated u-P A induces proteolytic cleavage of ECM, causing the release of additional pro angiogenic factors.

As mentioned herein, high concentrations of TNF- α can induce tumor necrosis. To combat this, tumors are capable of producing soluble TNF- α receptors. Produced by TAM, these receptors function as a defense mechanism, protecting the tumor from the tumoricidal and immunoactivating effects of TNF- α .

TNF- α also induces production of DNA damaging reactive oxygen species, while simultaneously inhibiting the ability of repair enzymes to repair damaged DNA. Further damage occurs to DNA when TNF- α is initially recruited to the damaged tissue site. TNF- α recruitment increases uptake of oxygen at the damaged tissue site, which leads to the release of free radicals that interfere with healthy epithelial and stromal cells. Ultimately, the processes that result from an environment characterized by persistently low TNF- α concentration lead to carcinogenesis by disrupting targets and pathways that the body requires to maintain homeostasis.

INTERLEUKIN FAMILY

Another pro-inflammatory cytokine that contributes to cancer is interleukin 6 (IL-6). Along with IL-4 and IL-10, IL-6 is highly expressed in hormone refractory prostate cancer. Additionally, prostatic carcinoma usually metastasizes IL-6 rich tissues such as the liver, lymph nodes and bone [28]. Hodgkin lymphoma shows high levels of IL-6, evidence of which is associated with poor patient outcome. It has been hypothesized that a heritable abnormality in IL-6 regulation may be a predisposition for young adult Hodgkin lymphoma [29]. *In vitro*, IL-6 has been shown to stimulate colony formation of Isoco 2, a cell line derived from liver metastasis, indicating that IL-6 may be a stimulator of metastatic colon carcinoma cell growth [30]. Inhibition of IL-6 production and subsequent IL-6 signal transduction as a result of TGF- β production in tumor infiltrating lymphocytes suppresses tumor growth in the colon [31]. It was demonstrated that IL-6 signal transduction was regulated by soluble IL-6R, and therefore, tumor growth is controlled by IL-6-trans-signaling via the soluble form of IL-6R α . IL-6R α is one of the two subunits that constitute the IL-6 heterodimer. The other subunit, glycoprotein 130 (gp130), triggers phosphorylation of STAT3 protein, which has a well documented role in malignant cell proliferation and survival rates [31].

IL-6 is also indirectly induced by IL-1 α and IL-1 β , which are secreted by macrophages alongside TNF- α , and help directly initiate inflammation. IL-1 α and IL-1 β are also responsible for inducing other pro-inflammatory factors such as inducible nitric oxide synthase (iNOS) and cyclooxygenase type 2 (COX-2). Similar to TNF- α , IL-1 can both assist and inhibit tumor progression based on concentration within the tumor environment. When expressed in small quantities, IL-1 produces a stimulatory inflammatory response that can be suppressed by normal anti-tumor immunity. However, as a result of its paracrine effects on stromal cells in the tumor's microenvironment, large amounts of IL-1 can induce major metastasis that is not susceptible to anti-tumor immunity [32]. Indeed, high IL-1 β concentration is correlated with other accepted parameters of aggressive tumors in advanced breast carcinomas [33].

CYCLOOXYGENASE 2 (COX 2)

As mentioned above, COX-2 is induced by pro-inflammatory cytokines. It has been demonstrated to promote tumor progression, and is up-regulated in cancers of the pancreas, [34] prostate, [35] colon, [36] breast, [37] lung, [38] as well as in multiple squamous cell carcinoma of the head, [39] neck, [39] and esophagus [40]. COX-2 aids tumor progression by induction of angiogenesis, facilitation of cell proliferation, and prevention of apoptosis.

Two cytokines which induce COX-2 expression are the pro-angiogenic factors TNF- α and IL-1 β . Using a mouse cornea model, it was demonstrated that COX-2 expression regulates IL-1 β induced angiogenesis, as the corneas of COX-2 null mice induced less angiogenesis than the corneas of wild-type [41]. The mechanism behind this can be attributed to the biological function of COX-2. COX-2 is one of two isoforms of cyclooxygenase, an enzyme that catalyzes prostaglandin from arachidonic acid (AA). It is this COX-2 production of prostaglandins, specifically E₂ (PGE₂) and thromboxane A₂ (TXA₂) that contributes to promotion of angiogenesis [42]. Metabolism of AA in order to form prostaglandins has been shown to be significantly higher in malignant prostatic tissue versus benign tissue [43]. In the above mouse experiment, PGE₂ receptor (EP₂ and EP₄) agonists and a TXA₂ receptor agonist induced angiogenesis both *in vitro* and also in mouse cornea. IL-1 β -induced angiogenesis is inhibited by a PGE₂ receptor, EP₄, a antagonist and a TXA₂ antagonist. Together these findings implicate COX-2 activated prostanoids as inflammatory angiogenesis regulators [42]. It is believed that both nonspecific and specific COX-2 inhibitors also provide an effective tool for reduction of angiogenic processes by inactivating MAPK and by blocking COX-2 production of VEGF [44].

During chronic inflammation, excessive COX-2 production of prostaglandins E₂, PGE₂ and PGI₂ not only effects angiogenesis, but also enhances cell proliferation and invasiveness in both tumor and normal cells. When treated with COX-2 inhibitors, human colonic adenocarcinoma cell line (HCA-7) growth was reduced in colorectal carcinoma. It is interesting to note that COX-2 inhibition more effectively reduced the cancer growth when combined with HER-2/*neu* inhibitors [45]. COX has also been shown to disturb the bal-

ance between MMPs and tissue inhibitors of metalloproteinases (TIMP) [46]. A disruption of this balance can lead to overexpression of MMP, which as discussed earlier has multiple pro-tumoral roles. COX role on cell invasiveness was also clearly demonstrated in prostate cancer progression. The hypothesis that COX metabolites including AA and PGE₂ affect prostate cancer progression by increasing invasion of basement-membrane (BM) was supported by the subsequent inhibition of PGE₂. Inhibition of this isoform reduced *in vitro* invasiveness by 85%, an effect attributed to PGE₂ mediation of BM degradation [46].

It was also revealed that COX-2 specific as well as non-specific nonsteroidal anti-inflammatory drugs (NSAIDs) caused apoptosis in a variety of cancer cells. A study using colon cancer cell lines showed that NSAID induced apoptosis was more severe in colon cancers expressing COX-2 [47]. Likewise, rat intestinal epithelial cells (RIE) expressed elevated COX-2 protein levels and demonstrated increased resistance to induced apoptosis. Effects were reversed by administering sulindac sulfide, a COX inhibitor [18]. These findings demonstrate the significance of the COX-2 pathway in apoptosis regulation in many cell types, and the potential of NSAIDs to reverse the effects of this pathway [44]. The preventative role of COX-2 in apoptosis is attributed to two possible mechanisms: apoptosis could be mediated by generation of prostaglandin products or by the removal of the substrate AA [44]. By inhibiting COX-2, AA metabolism is mitigated in order to stimulate the production of ceramide, a mediator of apoptosis [3].

INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) AND REACTIVE OXYGEN SPECIES (ROS)

COX-2, a stimulator of proangiogenic factors and vascular permeability, is activated by nitric oxide (NO). NO is a major component produced by iNOS, and is induced by a combination of cytokines including TNF- α and IL-1 β [48]. Increased expression of iNOS has been observed in multiple inflammatory conditions associated with cancer, such as Crohn's disease, H-Pylori gastritis, ulcerative colitis and viral hepatitis [49]. NOS activity correlates with lymph node metastases, indicating that increased metastatic behavior is associated with high NOS activity [50]. iNOS production of NO has been documented to directly damage DNA by causing DNA lesions [51]. NO becomes more reactive with DNA when exposed to reactive oxygen species (ROS), transforming into a reactive nitrogen species (RNS). RNS can also inactivate enzymes that are critical to DNA repair. In high concentrations, as is often the case with chronic inflammation, RNS inhibits p53 function, a protein that induces cell death in damaged DNA [49]. The combination of these events creates an accumulation of damaged DNA, enhancing the invasiveness of cancer.

NO are also centrally involved in angiogenesis. It has been demonstrated that tumor produced VEGF requires a NO pathway within the endothelial compartment to promote neovascular growth [49, 51]. Specifically, NO activates COX-2, which as discussed can stimulate the production of proangiogenic factors and prostaglandins resulting in increased vascular permeability [52]. Additionally, NO medi-

ates angiogenesis induced by platelet activating factor (PAF), as well as angiogenesis induced by TNF, a factor mediated by production of PAF [53]. Inhibitors of NO synthase have also been shown to suppress angiogenesis [54].

NUCLEAR FACTOR κ B (NF- κ B)

Chronic inflammation is associated with persistent activation of gene-transcription factor NF- κ B, which has been implicated in cancer progression [55]. Specifically, NF- κ B activation has been associated with several aspects of tumorigenesis including promoting cancer cell proliferation, preventing apoptosis, inducing RNS, and increasing tumor angiogenic and metastatic potential [44]. NF- κ B is believed to promote cancer during the tumor's promotion phase as opposed to the tumor initiation [56]. During the tumor initiation phase, degrees of proliferation, hyperploidy and dysplasia were found to be comparable in mice with active NF- κ B when compared to mice with inactivated NF- κ B. As the mice matured, a discernable difference was noticed between mice with active NF- κ B versus inactive NF- κ B. Thus, inactivation of NF- κ B activity in the hepatocytes led to a dramatic decrease in tumor promotion [56].

During tumor promotion, a colitis-associated cancer model displayed increased epithelial cells after deletion of I κ B in intestinal epithelial cells. I κ B kinase is the pathway responsible for cytokine activation of NF- κ B. While inflammation remained constant, there was a dramatic decrease in tumor incidence [57]. Further supporting the argument that NF- κ B activation is integral for malignant conversion, as opposed to early stages or initiation, is the link that NF- κ B assists in the survival of hepatocytes and their progression to malignancy [55]. Selective deletion of NF- κ B in hepatocytes induced programmed cell death of hepatocytes that had undergone malignant transformation, and reduced the incidence of liver tumors [55].

The RelA (p65) subunit of NF- κ B is required for induction of TNF- α dependent genes [58]. Treatment of RelA-deficient mouse fibroblasts and macrophages with TNF- α resulted in significant reduction in viability, whereas RelA positive cells were unaffected. Cytotoxicity to both cell types was mediated by TNFR1. Reintroduction of RelA into the RelA null fibroblasts resulted in enhanced survival, demonstrating that the presence of RelA is required for protection from TNF- α [58].

NF- κ B's effect on angiogenesis has been displayed in human ovarian cancer cells, where NF- κ B blocking has shown to suppress angiogenesis in human ovarian cancer. NF- κ B signaling blockade also inhibited both *in vitro* and *in vivo* expression of VEGF and IL-8, two angiogenic factors [59].

CONCLUSIONS

The evidence reviewed in this article demonstrates that chronic inflammation results in the production of multiple factors that can either stimulate or inhibit tumor growth and progression by modulating tumor microenvironment. For this reason, an effective therapeutic strategy may involve normalizing the function of tumor stroma including macrophages, granulocytes, lymphocytes, endothelial cells and

their associated pericytes, as well as fibroblasts in the microenvironment. Most proinflammatory cytokines produced by either host immune cells or tumor cells themselves, have been demonstrated to promote tumor development. By contrast, proapoptotic (TRAIL) and anti-inflammatory cytokines (IL-10, TGF- β) interfere with tumor development itself. This illuminates the complicated and paradoxical role of inflammation in tumor development. Each major player in the inflammatory process has the ability to both promote malignant tumor progression, as well as aid in the body's fight against cancer. As described above, during chronic inflammation many pathways are either activated or deactivated, leading to modulation of the tumor microenvironment milieu. Because signaling pathways in this milieu are either regulated by host immune system or by tumor cells, it has been difficult to identify a single component of the inflammatory pathways in the microenvironment. However, recent attempts to target tumor microenvironment have yielded exciting results. For example, statins reduce cancer incidence, and it has been suggested that possible mechanisms for this effect involve the suppression of inflammation and angiogenesis in the microenvironment [60]. Studies have further shown that the chronic use of NSAIDs, which is already known to reduce colon cancer, [61] is also associated with a striking reduction in the risk of breast cancer [62]. This study found an approximately 50% reduction in breast cancer risk with aspirin use, and relative risk dropped to 0.29 with specific COX2 inhibitors. These results unequivocally support the notion that targeting tumor microenvironment may be an effective strategy for the treatment of cancer. This strategy may prove more effective by targeting multiple molecules using a combinatorial approach in the tumor microenvironment.

Pathogenic infection causes acute inflammation which initiates sequence of events such as activation of mast cells, macrophages, and dendritic cells which in turn facilitates entry of granulocytes. This event is followed by further recruitment of macrophages. These sequential events set the stage for infiltration of immune cells (lymphocytes) to initiate immune response against tissue injury. Finally, the tissue remodeling process begins at the injury site by recruiting mesenchymal cells such as endothelial cells and fibroblasts. These cells synthesize collagen and extracellular matrix (ECM) also form new blood vessels (neovascularization). Ultimately, the pathogen is destroyed, and the damaged tissue is repaired. Therefore, all sequences of events recede to their normal state and homeostasis maintained. Contrast to orderly and organized sequence of events, carcinogenesis is the chaotic disorganization of inflammation and repair mechanism as described in Fig. (1). Since carcinogenic process begins with cell mutation and hyperproliferation, inflammation never ends (chronic inflammation). Unresolved inflammation leads to chronic inflammation and homeostasis is not achieved. This disruption of tissue homeostasis mechanism can in turn lead to another wave of inflammatory response, which is trying to repair injured tissue by further promoting neovascularization. This continuous neovascularization leads to invasive and metastatic state of cancer. This is consistent with the idea proposed by Hal Dvorkin that "tumors are wounds that do not heal".

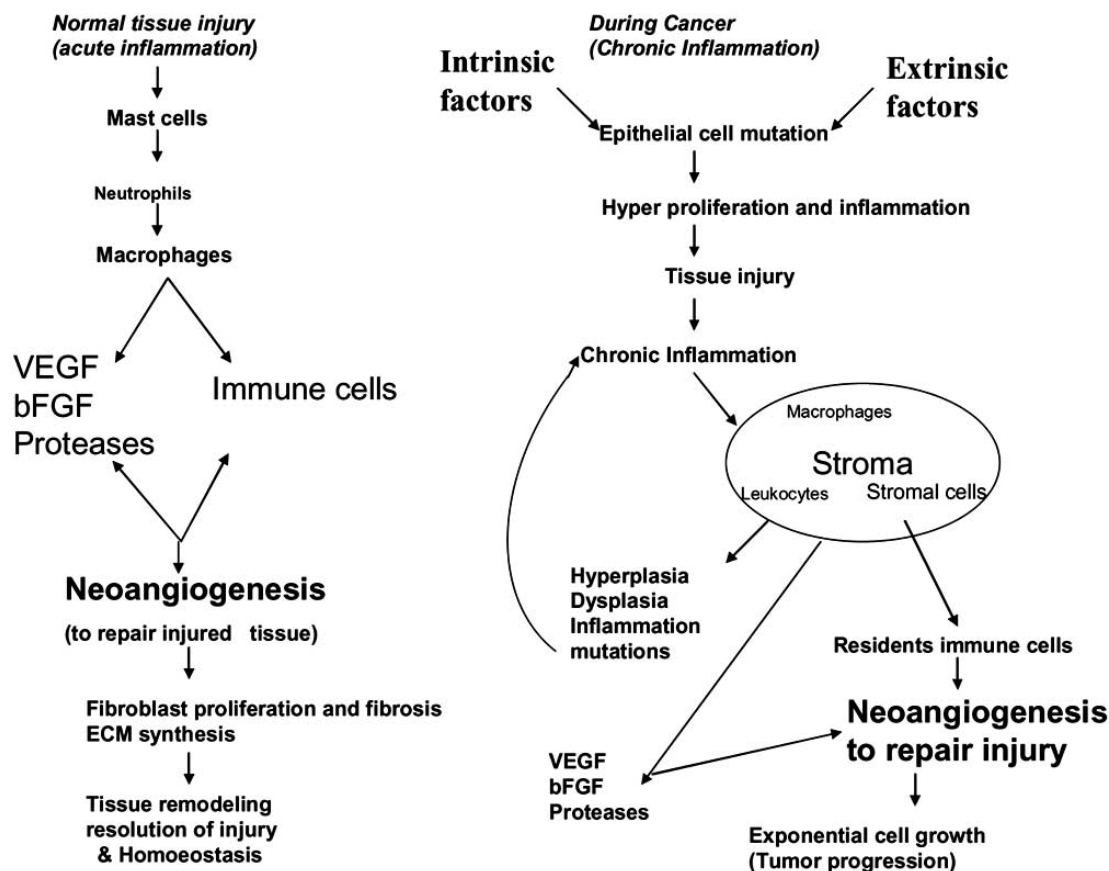


Fig. (1). Inflammation and tissue repair mechanism during normal tissue injury (acute inflammation) and cancer induced tissue injury (chronic inflammation).

REFERENCES

- [1] Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986; 315 (26): 1650-9.
- [2] Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420 (6917): 860-7.
- [3] Lucia MS, Torkko KC. Inflammation as a target for prostate cancer chemoprevention: pathobiological and laboratory rationale. *J Urol* 2004; 171 (2 Pt 2): S30-34; discussion S35.
- [4] Allavena P, Sica A, Solinas G, Porta C, Mantovani A. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol/Hematol* 2008; 66 (1): 1-9.
- [5] Pollard JW. Macrophages define the invasive microenvironment in breast cancer. *J Leukocyte Biol* 2008; 84 (3): 623-30.
- [6] Takanami I, Takeuchi K, Kodaira S. Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. *Oncology* 1999; 57 (2): 138-42.
- [7] Tsung K, Dolan JP, Tsung YL, Norton JA. Macrophages as effector cells in interleukin 12-induced T cell-dependent tumor rejection. *Cancer Res* 2002; 62 (17): 5069-75.
- [8] Sica A, Allavena P, Mantovani A. Cancer related inflammation: the macrophage connection. *Cancer Lett* 2008; 267 (2): 204-15.
- [9] Lin EY, Pollard JW. Tumor-associated macrophages repress the angiogenic switch in breast cancer. *Cancer Res* 2007; 67 (11): 5064-6.
- [10] Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res* 1996; 56 (20): 4625-9.
- [11] Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 2002; 23 (11): 549-55.
- [12] Fisher RI, Ostick-Bruton F. Depressed T cell proliferative responses in Hodgkin's disease: role of monocyte-mediated suppression via prostaglandins and hydrogen peroxide. *J Immunol* 1982; 129 (4): 1770-4.
- [13] Schutysse E, Struyf S, Proost P, Opdenakker G, Laureys G, Verhasselt B, et al. Identification of biologically active chemokine isoforms from ascitic fluid and elevated levels of CCL18/pulmonary and activation-regulated chemokine in ovarian carcinoma. *J Biol Chem* 2002; 277 (27): 24584-93.
- [14] Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* 2001; 193 (6): 727-40.
- [15] Wahl LM, Kleinman HK. Tumor-associated macrophages as targets for cancer therapy. *J Nat Cancer Inst* 1998; 90 (21): 1583-4.
- [16] Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* (New York, NY) 2002; 295 (5564): 2387-92.
- [17] Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev* 2002; 2 (3): 161-74.
- [18] Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* 1995; 83 (3): 493-501.
- [19] Chabottaux V, Noel A. Breast cancer progression: insights into multifaceted matrix metalloproteinases. *Clin Exp Metastasis* 2007; 24 (8): 647-56.
- [20] Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000; 103 (3): 481-90.
- [21] Sounni NE, Noel A. Membrane type-1 matrix metalloproteinases and tumor progression. *Biochimie* 2005; 87 (3-4): 329-42.
- [22] Noel A, Jost M, Maquoi E. Matrix metalloproteinases at cancer tumor-host interface. *Seminars in cell & developmental Biol* 2008; 19 (1): 52-60.

- [23] Sheu BC, Hsu SM, Ho HN, Lien HC, Huang SC, Lin RH, *et al.* A novel role of metalloproteinase in cancer -mediated immunosuppression. *Cancer Res* 2001; 61 (1): 237-42.
- [24] Herzig M, Savarese F, Novatchkova M, Semb H, Christofori G. Tumor progression induced by the loss of E-cadherin independent of beta-catenin/Tcf-mediated Wnt signaling. *Oncogene* 2007; 26 (16): 2290-8.
- [25] Noe V, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, *et al.* Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 2001; 114 (Pt 1): 111-8.
- [26] Brigati C, Noonan DM, Albin A, Benelli R. Tumors and inflammation in filtrates: friends or foes? *Clin Exp Metastasis* 2002; 19 (3): 247-58.
- [27] Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, *et al.* Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat Med* 1999; 5 (7): 828-31.
- [28] Siegall CB, Schwab G, Nordan RP, Fitzgerald DJ, Pastan I. Expression of the interleukin 6 receptor and interleukin 6 in prostate carcinoma cells. *Cancer Res* 1990; 50 (24): 7786-8.
- [29] Cozen W, Gill PS, Ingles SA, Masood R, Martinez-Maza O, Cockburn MG, *et al.* IL-6 levels and genotype are associated with risk of young adult Hodgkin lymphoma. *Blood* 2004; 103 (8): 3216-21.
- [30] Schneider MR, Hoefflich A, Fischer JR, Wolf E, Sordatt B, Lahm H, *et al.* Interleukin-6 stimulates clonogenic growth of primary and metastatic human colon carcinoma cells. *Cancer Lett* 2000; 151 (1): 31-8.
- [31] Becker C, Fantini MC, Schramm C, Lehr HA, Wirtz S, Nikolaev A, *et al.* TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 2004; 21 (4): 491-501.
- [32] Apte RN, Voronov E. Interleukin-1--a major pleiotropic cytokine in tumor-host interactions. *Semin Cancer Biol* 2002; 12 (4): 277-90.
- [33] Jin L, Yuan RQ, Fuchs A, Yao Y, Joseph A, Schwall R, *et al.* Expression of interleukin-1beta in human breast carcinoma. *Cancer* 1997; 80 (3): 421-34.
- [34] Yoshida S, Ujiki M, Ding XZ, Pelham C, Talamonti MS, Bell RH, *et al.* Pancreatic stellate cells (PSCs) express cyclooxygenase-2 (COX-2) and pancreatic cancer stimulates COX-2 in PSCs. *Mol Cancer* 2005; 4: 27.
- [35] Kirschenbaum A, Liu X, Yao S, Levine AC. The role of cyclooxygenase-2 in prostate cancer. *Urology* 2001; 58 (2 Suppl 1): 127-31.
- [36] Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, *et al.* Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J Clin Invest* 1997; 99 (9): 2254-9.
- [37] Pan MR, Hou MF, Chang HC, Hung WC. Cyclooxygenase-2 up-regulates CCR7 via EP2/EP4 receptor signaling pathways to enhance lymphatic invasion of breast cancer cells. *J Biol Chem* 2008; 283 (17): 11155-63.
- [38] Cui X, Zhang L, Luo J, Rajasekaran A, Hazra S, Cacalano N, *et al.* Unphosphorylated STAT6 contributes to constitutive cyclooxygenase-2 expression in human non-small cell lung cancer. *Oncogene* 2007; 26 (29): 4253-60.
- [39] Jaekel EC, Raja S, Tan J, Das SK, Girod DA, *et al.* Correlation of expression of cyclooxygenase-2, vascular endothelial growth factor, and peroxisome proliferator-activated receptor delta with head and neck squamous cell carcinoma. *Arch Otolaryngol--Head Neck Surg* 2001; 127 (10): 1253-9.
- [40] Kuo KT, Chow KC, Wu YC, Lin CS, Wang HW, Li WY, *et al.* Clinicopathologic significance of cyclooxygenase-2 overexpression in esophageal squamous cell carcinoma. *Annals Thorac Surg* 2003; 76 (3): 909-14.
- [41] Kuwano T, Nakao S, Yamamoto H, Tsuneyoshi M, Yamamoto T, Kuwano M, *et al.* Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *FASEB J* 2004; 18 (2): 300-10.
- [42] Ono M. Molecular links between tumor angiogenesis and inflammation: in inflammatory stimuli of macrophages and cancer cells as targets for therapeutic strategy. *Cancer Sci* 2008; 99 (8): 1501-6.
- [43] Chaudry A, McClinton S, Moffat LE, Wahle KW. Essential fatty acid distribution in the plasma and tissue phospholipids of patients with benign and malignant prostatic disease. *Br J Cancer* 1991; 64 (6): 1157-60.
- [44] Cao Y, Prescott SM. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. *J Cellular Physiol* 2002; 190 (3): 279-86.
- [45] Mann M, Sheng H, Shao J, Williams CS, Pisacane PI, Sliwkowski M, *et al.* Targeting cyclooxygenase 2 and HER-2/neu pathways inhibits colorectal carcinoma growth. *Gastroenterology* 2001; 120 (7): 1713-9.
- [46] Attiga FA, Fernandez PM, Weeraratna AT, Manyak MJ, Patierno SR. Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases. *Cancer Res* 2000; 60 (16): 4629-37.
- [47] Li M, Wu X, Xu XC. Induction of apoptosis by cyclo-oxygenase-2 inhibitor NS398 through a cytochrome C-dependent pathway in esophageal cancer cells. *Int J Cancer* 2001; 93 (2): 218-23.
- [48] Ganster RW, Taylor BS, Shao L, Geller DA. Complex regulation of human inducible nitric oxide synthase gene transcription by Stat1 and NF-kappa B. *Proc Natl Acad Sci USA* 2001; 98 (15): 8638-43.
- [49] Sawa T, Ohshima H. Nitritative DNA damage in inflammation and its possible role in carcinogenesis. *Nitric Oxide* 2006; 14 (2): 91-100.
- [50] Gallo O, Masini E, Morbidelli L, Franchi A, Fini-Storchi I, Vergari W, *et al.* Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. *J Nat Cancer Inst* 1998; 90 (8): 587-96.
- [51] Streuli C. Extracellular matrix remodelling and cellular differentiation. *Curr Opin Cell Biol* 1999; 11 (5): 634-40.
- [52] Jaiswal M, LaRusso NF, Gores GJ. Nitric oxide in gastrointestinal epithelial cell carcinogenesis: linking inflammation to oncogenesis. *Am J Physiol* 2001; 281 (3): G626-34.
- [53] Montrucchio G, Lupia E, de Martino A, Battaglia E, Arrese M, Tizzani A, *et al.* Nitric oxide mediates angiogenesis induced *in vivo* by platelet-activating factor and tumor necrosis factor-alpha. *Am J Pathol* 1997; 151 (2): 557-63.
- [54] Morbidelli L, Chang CH, Douglas JG, Granger HJ, Ledda F, Ziche M, *et al.* Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. *Am J Physiol* 1996; 270 (1 Pt 2): H411-5.
- [55] Balkwill F, Coussens LM. Cancer: an inflammatory link. *Nat* 2004; 431 (7007): 405-406.
- [56] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, *et al.* NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004; 431 (7007): 461-6.
- [57] Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, *et al.* IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 2004; 118 (3): 285-96.
- [58] Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science (New York, NY)* 1996; 274 (5288): 782-4.
- [59] Huang S, Robinson JB, Deguzman A, Bucana CD, Fidler IJ. Blockade of nuclear factor-kappaB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. *Cancer Res* 2000; 60 (19): 5334-9.
- [60] Demierre MF, Higgins PD, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. *Nat Rev* 2005; 5 (12): 930-42.
- [61] Mann JR, Backlund MG, DuBois RN. Mechanisms of disease: Inflammatory mediators and cancer prevention. *Nat Clin Pract* 2005; 2 (4): 202-10.
- [62] Harris RE, Beebe-Donk J, Alshafie GA. Reduction in the risk of human breast cancer by selective cyclooxygenase-2 (COX-2) inhibitors. *BMC Cancer* 2006; 6: 27.

Inhibition of human breast cancer cell (MDA-MB231) migration, neoangiogenesis and tumor growth by selective disruption of annexin II function: in vitro and in vivo studies

¹Meena Sharma, ²Robert T. Ownbey, ^{4,*}Marc R. Blackman, ^{4, 2*}Mahesh C. Sharma

⁴V.A Medical Center Washington, DC 20422, *George Washington University School of Medicine, Washington, DC 20052., ¹University of Pennsylvania, Philadelphia, PA 19104.
²Drexel University College of Medicine, Philadelphia, PA 19102.

Annexin II, an abundant phospholipid binding cell surface protein, binds tissue plasminogen activator (tPA) and functions as a regulator of fibrinolysis. Annexin II also mediates angiogenesis and enhances tumor growth and metastasis. However, the mechanism supporting this role is not known. Using a human breast cancer model we found that invasive human breast cancer cells (MDA-MB231) synthesize annexin II and tPA. Annexin II interacted with tPA *in vitro* leading to conversion of zymogen plasminogen to the reactive enzyme plasmin. Cell surface generated plasmin inhibited the migration of MDA-MB 231 cells. Silencing of the annexin II gene in MDA-MB231 cells abolished tPA binding thereby inhibiting tPA dependent plasmin generation. Moreover these annexin II suppressed MDA-MB231 cells exhibited reduced motility. Immunohistochemical analysis of pre-diagnosed clinical specimens showed abundant secretion of tPA and expression of annexin II on the surface of invasive cancer cells which correlates with neovascularization of the tumor. Intravenous administration of the anti-annexin II antibody to mice bearing MDA-MB231 tumors significantly inhibited tumor growth possibly due to blocked neoangiogenic activity. Taken together, these data suggest that annexin II dependent plasmin generation may be an early event switching breast cancer from the prevascular phase to the vascular phase with the possibility of metastasis. Annexin II may be an attractive target for therapeutic strategies aimed to inhibit neoangiogenesis and breast cancer metastasis.