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Antibody-directed neutralization of annexin II (ANX II) inhibits neoangiogenesis and human breast tumor growth in a xenograft model

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ABSTRACT

Activation of the fibrinolytic pathway has long been associated with human breast cancer. Plasmin is the major end product of the fibrinolytic pathway and is critical for normal physiological functions. The mechanism by which plasmin is generated in breast cancer is not yet fully described. We previously identified annexin II (ANX II), a fibrinolytic receptor, in human breast tumor tissue samples and observed a strong positive correlation with advanced stage cancer (Sharma et al., 2006a). We further demonstrated that tissue plasminogen activator (tPA) binds to ANX II in invasive breast cancer MDA-MB231cells, which leads to plasmin generation (Sharma et al., 2010). We hypothesize that ANX II-dependent plasmin generation in breast tumor is necessary to trigger the switch to neoangiogenesis, thereby stimulating a more aggressive cancer phenotype. Our immunohistochemical studies of human breast tumor tissues provide compelling evidence of a strong positive correlation between ANX II expression and neoangiogenesis, and suggest that ANX II is a potential target to slow or inhibit breast tumor growth by inhibiting neoangiogenesis. We now report that administration of anti-ANX II antibody potently inhibits the growth of human breast tumor in a xenograft model. Inhibition of tumor growth is at least partly due to attenuation of neoangiogenic activity within the tumor. *In vitro* studies demonstrate that anti-ANX II antibody inhibits angiogenesis on three dimensional matrigel cultures by eliciting endothelial cell (EC) death likely due to apoptosis. Taken together, these data suggest that selective disruption of the fibrinolytic activity of ANX II may provide a novel strategy for specific inhibition of neoangiogenesis in human breast cancer.

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Introduction

Sprouting of new blood vessels (neoangiogenesis) from existing vasculature is the hallmark of progression and metastasis of human breast cancer (Weidner et al., 1992). Clinical studies suggest that neoangiogenesis is an independent and highly significant prognostic indicator of overall and relapse-free survival in patients with early-stage breast cancer (Weidner et al., 1992). Not surprisingly, targeting neoangiogenesis has been a central focus of the development of single and multi-drug chemotherapy for breast cancer (O'Reilly et al., 1996). To date, however, the molecular mechanism(s) that triggers neoangiogenic activity in the breast tumor microenvironment remains poorly understood. Angiostatin (AS), an internal fragment of plasminogen, is a powerful inhibitor of angiogenesis. Experimental studies demonstrated that recombinant AS inhibits human breast cancer and related bone metastases in xenograft models (O'Reilly et al., 1996). In an attempt to understand AS's mechanism, we discovered its interaction with the endothelial cell surface receptor, ANX II (Tuszynski et al., 2002). We proposed that the ability of AS to inhibit neoangiogenesis is due to its interaction with cell surface ANX II. We recently demonstrated selective expression of ANX II in highly invasive human breast cancer MDA-MB231 cells, and suggested that ANX II may play an important role in breast cancer progression and metastasis by facilitating neoangiogenesis (Sharma et al., 2010). Our initial findings have been confirmed in various cancer model systems including breast cancer (Chuthapisith et al., 2009) prostate cancer (Braden et al., 2009) angiosarcoma, (Syed et al., 2007) hepatocelluar carcinoma(Yu et al., 2007), gastrointestinal cancer(Singh, 2007), oral carcinoma(Qi et al., 2007), clear-cell renal cell carcinoma (Ohno

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et al., 2009), renal carcinoma (Zimmermann et al., 2004), lung cancer,(Brichory et al., 2001), head and neck cancer(Wu et al., 2002), and pancreatic cancer (Diaz et al., 2004).

Because of the frequent overexpression of ANX II in diverse clinical and experimental models of cancer, investigators are trying to develop highly sensitive techniques such as immunosensors or ELISA to assess the utility ANX II in the early diagnosis (Ji et al., 2009; Kim et al., 2009).

Several groups have reported that cell surface ANX II regulates plasmin generation (Diaz et al., 2004) which facilitates extracellular matrix (ECM) degradation, and consequently cell invasion (Brownstein et al., 2004; Diaz et al., 2004) and migration (Tarui et al., 2002), biological activities which are required for neoangiogenesis.

Previously we and others demonstrated that ANX II is critical for neoangiogenesis and tumor growth (Semov et al., 2005). ANX II knockout mice exhibited defective neoangiogenesis (Ling et al., 2004). Many investigators have reported ANX II among the proteins differentially regulated in neoangiogenesis, cancer and metastasis (Aitkenhead et al., 2002; Pei et al., 2007; Zhang et al., 2009) suggesting that ANX II may serve as a molecular signature of neoangiogenesis in the tumor progression. ANX II may provide a potential therapeutic target to inhibit neoangiogenesis and its dependent tumor growth and metastasis in patients with breast cancer and other malignant diseases (Kesavan et al., 2009; Lima e Silva et al., 2010; Ling et al., 2004; Zhang et al., 2009). The plasminogen/plasmin system comprises of plasminogen activators (PA) [tissue type (tPA) or urokinase type (uPA)], their receptors such as uPAR and ANX II respectively and plasminogen activator inhibitor (PAI). Although both tPA and uPA bind to their respective cognate receptors and convert inactive plasminogen to the highly reactive enzyme plasmin. To date most of our knowledge about the plasmin generation in breast cancer is derived from studies uPA/uPAR-dependent mechanism. ANX II is a well established receptor for tPA and known to regulate plasmin generation which physiologically dissolves intravascular fibrin clots, a process known as fibrinolysis (Cesarman et al., 1994). Although hyperfibrinolysis is a well-known phenomenon in patients with advanced stage breast cancer however, the role of tPA/ANX II-mediated fibrinolytic pathway in breast cancer is not known.

Previously, we reported high levels of ANX II expression in advanced stage breast cancer patients concurrent with excessive tPA secretion in the tumor microenvironment (Sharma et al., 2010). These initial findings from our laboratory have now been confirmed in clinical studies, which identified tPA as a single major factor that contributes to the development of metastasis in breast cancer patients (Naina et al., 2010). Previous clinical studies have also observed an association of tPA with aggressive and metastatic breast cancer (Grondahl-Hansen et al., 1990; Rella et al., 1993) with neoplastic transformation and invasion of other cancers (Aguilar et al., 2004; Chernicky et al., 2005; Diaz et al., 2002; Goh et al., 2005; Stack et al., 1999).

In the present study, we investigated whether selective targeting of ANX II inhibits human breast cancer growth and neoangiogenesis in a xenograft mouse model. To test this concept, we used human breast cancer MDA-MB231 cells, which are known to express high levels of annexin II (Sharma et al., 2006a), to induce tumors in BALB/c *nu/nu* mice. Tumor bearing mice were then treated with anti-ANX II monoclonal antibody to target cell surface ANX II. We now report that anti-ANX II antibody arrests xenograft breast tumor growth by inhibiting neoangiogenesis.

Materials and methods

Human Lys-plasminogen, plasmin and recombinant tPA were purchased from Calbiochem, (La Jolla, CA). Electrophoresis reagents were procured from BioRad, (Richmond, CA). Anti-tPA monoclonal antibodies were purchased from American Diagnostica (Stamford, CT). Antibodies to ANX II were generated in our laboratory as reported earlier (Sharma et al., 2006b). Chromozyme PL was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Anti-CD31 monoclonal antibodies and immunohistochemical staining kit were procured from DAKO Corporation (Carpinteria, CA). Apoptosis kit was procured from Oncogene Research Products (San Diego, CA). Angiostatin was produced in our laboratory (Tuszynski et al., 2002) and all other chemicals used in this study were of analytical grade.

Cell culture and maintenance

The human invasive breast cancer MDA-MB231 cell line was provided by Dr. George Tuszynski, Temple University in Philadelphia, PA. MDA-MB231 cells were grown in RPMI 1640 media containing 10% fetal calf serum (FCS) supplemented with L-glutamine and antibiotics as we reported earlier (Sharma et al., 2006a). Bovine Aortic Endothelial (BAE) cells were grown in Ham's F12 K containing either 10% fetal calf serum (FCS) or serum free media (0.1% BSA) supplemented with L-glutamine and antibiotics (Tuszynski et al., 2002).

Immunoflorescence staining

The surface immunofluorescence staining of MDA-MB231 cells was performed as we described previously (Sharma et al., 2006b). For surface proteins on non-permeabilized MDA-MB231 cells, cells grown on gelatin-coated cover slips were washed three times in PBS and then incubated for 3 h at 4 °C with anti-annexin II monoclonal antibodies followed by FITC-conjugated secondary antibody. Cells were washed three times with PBS and fixed in 3.7% formaldehyde for 5 min and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Cells were viewed under fluorescence microscope using FITC filter.

Plasmin generation in tumor tissue

Pieces of tumor tissues were excised and snap frozen in liquid nitrogen for plasmin generation assay, as described previously, with minor modifications (Mulligan-Kehoe et al., 2001; Sharma et al., 2010). Tumor tissues were homogenized in chilled PBS and protein concentration was determined using the Micro BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL). Ten μg of total protein was incubated with plasminogen (2 μM final concentration) and plasmin-specific chromogenic substrate (2 mM Chromozyme PL) in a total volume of 100 μl to determine activation of plasminogen (Brownstein et al., 2004; Sharma et al., 2006b). The change in color at 405 nm is a direct measure of plasmin generation. Appropriate controls were included in this experiment to determine non-specific plasmin generation. Control well 1 was designed to assess self-degradation of plasminogen to plasmin by incubating plasminogen with Chromozyme PL. Control well 2 was included to assess any plasmin activity in tissues by incubating tumor tissues with Chromozyme PL. Non-specific plasmin generated in these wells was subtracted to calculate tumor specific plasmin generation.

Immunohistochemistry

Tumor tissues were excised and immediately submerged in phosphate buffered formalin, embedded in paraffin, and 4 μm sections were cut on albumin-coated slides. Staining was performed according to our established protocol (Sharma et al., 2006a, 2010). Briefly, sections were deparaffinized and incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Nonspecific protein binding was blocked with 3% BSA/PBS for 1 h. Sections were incubated with monoclonal antibodies (1:1000) overnight at room temperature followed by HRP labeled secondary antibody (1:2500) for 1 h. Staining was visualized by diaminobenzidine (DAB) followed by nuclear counterstaining with hematoxylin. In parallel experiments