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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

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Abstract

Angiostatin, the N-terminal four kringles (K1–4) of parent molecule plasminogen, is reported to block Lewis Lung Carcinoma (LLC) tumor growth and metastasis. However, angiostatin's mechanism of action is unclear. We earlier reported that angiostatin binds to cell surface annexin II through the lysine-binding domain (kringles 1–4) [Tuszynski, G.P., Sharma, M., Rothman, V.L., Sharma, M.C., 2002. Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. Microvasc. Res. 64:448–462.]). We now show that annexin II on the cell surface of LLC cells regulates conversion of plasminogen to plasmin. Activation of plasminogen to plasmin is time-dependent, with the linear activation lasting up to 120 min. Monoclonal antibodies to annexin II reduced plasminogen activation by 92.6%, suggesting a specific role of annexin II in plasmin generation. Angiostatin also reduced plasmin generation by 81.6%, suggesting that angiostatin may be competing with plasminogen through lysine-binding domain. ε-Aminocaproic acid, a lysine analogue, effectively blocked plasminogen activation indicating that, indeed, the lysine-binding site of the kringles domain is required for activation. These data suggest that annexin II may be a receptor target for angiostatin's action. Therefore, we tested the effect of high affinity monoclonal antibody to annexin II in mouse model of LLC. A single dose of antibody treatment inhibited LLC tumor growth almost 70% with concomitant inhibition of circulating plasmin generation and its proteolytic activity. Taken together, it is possible that inhibition of LLC tumor growth and metastasis reported by angiostatin therapy may be due to blocking of annexin-II-dependent plasmin generation. Plasmin is known to influence angiogenic, invasive and metastatic capability of tumors.

Keywords: Plasmin; Angiogenesis; Annexin II; Invasion; Lewis Lung Carcinoma; Angiostatin; Metastasis; Tumor growth; ECM

Introduction

Angiogenesis and metastasis are two processes that are central to the progression of cancer. As such, they have become important targets for the development of anti-cancer agents. Angiostatin, the N-terminal four kringles (K1–4) of plasminogen, blocks tumor-mediated angiogenesis (O'Reilly et al., 1994). Angiostatin suppresses LLC tumor growth and metastasis (O'Reilly et al., 1994). Several independent groups of investigators verified the powerful anti-angiogenic, anti-cancer and anti-metastatic activity of angiostatin in mouse as well as in human cancer models (Griscelli et al., 1998; O'Reilly et al., 1996; Peyruchaud et al., 2003; Sim et al., 1997; Wu et al., 1997). In an effort to uncover angiostatin's mechanism, at least four receptor/binding proteins have been identified (Moser et al., 1999; Tarui et al., 2001; Troyanovsky et al., 2001) including from our laboratory (Tuszynski et al., 2002).

Both angiogenesis and metastasis require a proteolytic cascade that involves serine, cysteine and metalloproteases. This proteolytic cascade degrades the extracellular matrix (ECM) and basement membrane which surrounds blood vessels (Pepper et al., 1996). During angiogenesis, the resulting lesion in the basement membrane allows endothelial/epithelial cells to extend into the neighboring tissues and form new blood vessels. During metastasis, cancer cells penetrate through the degraded basement membrane and ECM, become implanted in the underlying tissues and subsequently form secondary tumors

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(Liotta, 1992). Thus, cell migration, invasion and cell proliferation rely on a proteolytic/anti-proteolytic balance based on cell-surface-restricted reactions. Receptors which regulate the proteolytic cascade may be attractive targets for blocking these processes. Cell surface receptors for plasminogen activators (Blasi, 1993) or cellular binding sites for plasminogen (Cesarman et al., 1994) are positioned for localized generation of plasmin. Plasmin is a serine protease that plays an important role in the proteolytic cascade (Pepper, 2001). This protease acts directly by hydrolyzing components of the basement membrane such as fibrin, type IV collagen, fibronectin and laminin and also acts indirectly by activating other enzymes in the cascade such as matrix metalloproteases, collagenases and VEGF (McColl et al., 2003). Degradation of the basement membrane by plasmin is a multi-step process. For example, during the first step in fibrin hydrolysis, plasminogen, which is the inactive precursor to plasmin, binds to receptor via a lysine-binding site which is also known as K1–4 (Brownstein et al., 2004). Next, plasminogen is converted to active plasmin in a reaction that is catalyzed by plasminogen activators (Diaz et al., 2004). It has been reported that the proteolytic activity of plasmin contributes to angiogenesis (Ling et al., 2004; Pepper, 2001), invasion/metastasis (Bajou et al., 2001; Kobayashi et al., 1995; Tanaka et al., 1982) and morbidity and mortality of LLC (Bugge et al., 1997).

Annexin II is a Ca^{++}- and phospholipids-binding protein and an in vivo substrate for tyrosine kinase and PKC. It is generally believed that annexin II at the cell surface initiates proteolytic activation of plasminogen to plasmin (Diaz et al., 2004; Hajjar et al., 1994), which in turn leads to breakdown of the ECM and thereby promotes cellular invasion/migration (Blasi and Stoppelli, 1998; Tarui et al., 2002). This conclusion is supported by the fact that annexin II and t-PA are highly expressed on the surface of human tumor cells or by surrounding stromal cells and that they are both independent prognostic indicators in human cancers (Aguilar et al., 2004; Banerjee et al., 2003; Brichory et al., 2001a; Diaz et al., 2004; Zimmermann et al., 2004). In addition, annexin II expression was associated with metastasis of head and neck cancer (Wu et al., 2002). Because of the extensive structural similarity within kringle domains, it has always been speculated that somehow angiostatin regulates plasminogen activation. We previously reported that angiostatin binds to endothelial cell surface annexin II and competes for plasminogen binding (Tuszynski et al., 2002). In this study, we examined whether LLC cells regulate plasminogen activation through binding to cell surface annexin II. We also investigated whether angiostatin blocks annexin-II-dependent plasminogen activation. We found that LLC cell surface annexin II regulates plasminogen activation to plasmin. Angiostatin or anti-annexin II antibody blocks annexin-II-dependent plasmin generation. We provide evidence that antibody-mediated targeted blocking of annexin II inhibits LLC tumor growth in mouse model. Inhibition of tumor growth correlates with the inhibition of plasmin generation. These results suggest that anti-angiogenic and anti-metastatic action of angiostatin may be due to direct blocking of plasmin generation possibly by competitive inhibition of plasminogen binding to annexin II.

Materials and methods

Human plasminogen and ε-aminocaproic acid were obtained from Sigma Chemical Company, St. Louis, MO. Human plasmin was purchased from Calbiochem, San Diego, CA. Electrophoretic reagents were procured from Bio-Rad Richmond, CA. Anti-plasminogen (K1–3) monoclonal antibody was purchased from Enzyme Research Inc., Chicago, IL. The specific synthetic substrate for plasmin, chromozyme PL was purchased from Roche Molecular Biochemicals, IN. Anti-annexin II monoclonal antibody and angiostatin were generated in our laboratory as described earlier (Sharma and Tuszynski, 2002; Tuszynski et al., 2002).

Angiostatin generation and purification

Angiostatin was generated by limited proteolytic digestion of human plasminogen using pancreatic elastase. The angiostatin fragment was purified from the reaction mixture using lysine-affinity chromatography as we reported previously (Tuszynski et al., 2002).

Anti-annexin II antibody

Annexin II was purified from the membranes of Bovine Aortic Endothelial (BAE) cells as reported previously (Tuszynski et al., 2002). Hybridoma clones were generated by Washington Biotechnology, (Baltimore, MD). Five positive clones were identified using an annexin-II-specific ELISA. One of the clones was selected for antibody production. Monoclonal antibodies were purified using Protein A–Sepharose affinity chromatography and characterized by Western blotting.

Cell line maintenance and treatments

Lewis Lung Carcinoma (LLC) cells were maintained in DMEM media containing 10% fetal calf serum (FCS) supplemented with glucose, L-glutamine and antibiotics according to our published protocol (Zhou et al., 2004). In all experiments, angiostatin, antibody to annexin II and ε-aminocaproic acid were incubated with cell membranes for 30 min prior to adding plasminogen.

Immunofluorescence staining

The surface immunofluorescence staining was done using as described previously (Dudani and Ganz, 1996). For surface proteins on nonpermeabilized cells, LLC 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. Cover slips were washed in PBS and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Cells were viewed under fluorescence microscope using FITC filter.

Preparation of LLC cell membranes

Previously, we identified and purified annexin II from endothelial cell membranes (Tuszynski et al., 2002). Interaction of angiostatin with annexin II was characterized in a purified system. Similar approach was used to isolate LLC membranes. Briefly, confluent LLC cells were scraped from the plates, washed three times with chilled PBS, homogenized in ice cold Tris–sucrose buffer, pH 7.4, containing protease inhibitors, leupeptin (4.2 μM), antipain (3.3 μM) and phenyl methylsulfonylflouride (PMSF; 0.57 mM). Homogenate containing plasma membranes was fractionated by centrifugation as we described before (Sharma and Shapiro, 1995). The purified plasma membrane fraction was solubilized in homogenizing buffer and stored at −20°C until further use.

Western blot analysis

To determine annexin II expression in LLC cell membranes, 10 μg of membrane proteins was fractionated on 12% sodium dodecyl sulfate-