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Research Article

The inhibitory effects of endostatin on endothelial cells are modulated by extracellular matrix

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ABSTRACT

We investigated the ability of extracellular matrix (ECM) proteins to modulate the response of endothelial cells to both promoters and inhibitors of angiogenesis. Using human dermal microvascular endothelial cells (HDMEC), we found that cells demonstrated different adhesive properties and proliferative responses to the growth factor VEGF depending upon which ECM protein with which they were in contact, with fibronectin having the most impact on VEGF-induced HDMEC proliferation and survival. More importantly, we observed that ECM could modulate the ability of the angiogenic inhibitor endostatin to prevent endothelial cell proliferation, survival and migration. We observed that growth on vitronectin or fibronectin impaired the ability of endostatin to inhibit VEGF-induced HDMEC proliferation to the greatest extent as determined by BrdU incorporation. We found that, following growth on collagen I or collagen IV, endostatin only inhibited VEGF-induced HDMEC proliferation at the highest dose tested (2500 ng/ml). In a similar manner, we observed that growth on ECM proteins modulated the ability of endostatin to induce endothelial cell apoptosis, with growth on collagen I, fibronectin and collagen IV impairing endostatin-induced apoptosis. Interestingly, endostatin inhibited VEGF-induced HDMEC migration following culture on collagen I, collagen IV and laminin, while migration was not inhibited by endostatin following HDMEC culture on other matrices including vitronectin, fibronectin and tenascin-C. These results suggest that different matrix proteins may affect different mechanisms of endostatin inhibition of angiogenesis. Taken together, our results suggest that the ECM may have a profound impact on the ability of angiostatic molecules such as endostatin to inhibit angiogenesis and thus may have impact on the clinical efficacy of such inhibitors.

Introduction

Following their initial discovery, the use of anti-angiogenic agents as a therapeutic approach to cancer treatment was heralded as the new cure for cancer. Subsequent clinical trials investigating the use of endostatin as a novel anti-cancer agent demonstrated that the treatment was well tolerated and safe to administer, however, anti-tumor responses were not readily observed [1,2]. The apparent lack of efficacy of endostatin in the clinic has led to further investigations of the mode of action of this inhibitor.

Endostatin is a cleavage product containing the NC1 domain of the α1 chain of collagen XVIII and has been shown to be an inhibitor of tumor angiogenesis in mouse

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models of tumor growth [3–8]. In spite of extensive studies delivering endostatin by a variety of different routes or vector systems, the mechanism of inhibition of tumor angiogenesis by endostatin remains unclear. Endostatin inhibits angiogenesis mainly via its ability to inhibit endothelial cell proliferation [4,9] and migration, as has been previously demonstrated [10–13]. However, the exact mechanism by which endostatin mediates this inhibition has not yet been elucidated. Endostatin has been shown to bind a number of cell surface molecules including heparin sulfate proteoglycans [14,15], glypicans [16], vascular endothelial growth factor receptor 2 (VEGFR2) [17] and integrins [11,13,18]. Furthermore, endostatin has been shown to modulate a number of intracellular pathways including Wnt/β-catenin signaling [15,19], VEGFR2 signaling [17], Ca^{2+} signaling [20] and kinase signaling via association with the Shb protein [21]. Despite these observations, a defined signaling mechanism for tumor vessel inhibition by endostatin has not yet been determined.

Extracellular matrix (ECM) is a component of the tumor microenvironment that can be vastly different than that found in the normal tissue counterparts. Elevated levels of the ECM proteins collagen I, fibronectin, vitronectin and tenascin-C have been found in a variety of tumors [22–27]. Additionally, loss of normal basement membrane ECM proteins, such as collagen IV and laminin, has also been observed during tumor progression [28,29]. ECM matrices signal to cells via binding of integrins, heterodimeric surface molecules composed of an α and a β subunit that can associate with one another in different combinations to form 24 known integrin molecules with different affinities for various ECM proteins [30–32]. Signaling in cells following binding of integrins appears to be primarily via activation of two main kinases, focal adhesion kinase (FAK) [33–35] and integrin-linked kinase (ILK) [36–38].

Elevated levels of ECM proteins may also have profound effects on the induction and inhibition of tumor-associated angiogenesis as some of the same matrices found elevated in tumors have also been found to modulate endothelial cell proliferation, migration and survival [39–44]. Recently, our group has determined that the ECM protein collagen I can impair the ability of the angiostatic molecules thrombospondin-1 (TSP-1) and interferon-inducible protein 10 (IP-10/CXCL10) to inhibit endothelial cell proliferation, survival and migration [45]. However, in contrast, we observed that endostatin retained the ability to inhibit endothelial cell migration and tube formation on collagen I [45]. These observations led us to investigate the effects of other tumor-associated matrices on the ability of endostatin to inhibit endothelial cell processes such as adhesion, proliferation, survival and migration. In addition, we have begun to examine the role of integrin signaling via FAK in this process. We found that preincubation of endothelial cells with endostatin reduced the adhesion of the cells to all the matrices tested, however, to a lesser extent than following incubation with an adhesion neutralizing antibody to β_1 integrin. We observed that, with respect to inhibition of endothelial cell proliferation, survival and migration, endostatin most potently inhibited proliferation when cells were cultured on collagen I, collagen IV or laminin. Furthermore, endostatin induced apoptosis of endothelial cells cultured on vitronectin, tenascin-C, laminin and collagen IV, while it did not induce apoptosis following culture on collagen I or fibronectin. Endostatin was also found to inhibit VEGF-induced endothelial cell migration on collagen I, collagen IV and laminin, while it had little effect on cell migration in the presence of the other ECM proteins. Additionally, in contrast to previously reported observations [17], we observed no correlation of FAK activity with the inhibition of angiogenic activities by endostatin, as measured by detection of phospho-specific FAK isoforms.

Materials and methods

Endothelial cells, recombinant proteins and antibody reagents

Human dermal microvascular endothelial cells (HDMEC) derived from neonatal tissue were obtained from Cambrex Corporation (Walkersville, MD) and were propagated in EGM-2MV supplemented media (Cambrex Corp., Walkersville, MD). All experiments were performed using cells between passages 4 and 8. Recombinant $VEGF_{165}$ protein was obtained from R&D Systems (Minneapolis, MN), and recombinant human endostatin was purchased from Calbiochem (San Diego, CA) or Sigma (Oakville, ON). Mouse anti-FAK and mouse anti-phosphoFAKY397 antibodies were purchased from BD Biosciences Pharmingen (Mississauga, ON). Rabbit anti-phosphoFAKY861 antibody was obtained from Sigma (Oakville, ON), and phosphoFAKY925 antibody was from Cell Signaling Technology (Danvers, MA). The adhesion-blocking antibody to β_1 integrin was purchased from Chemicon International (Temecula, CA).

Preparation of matrix-coated tissue culture dishes

Tissue culture dishes were coated as follows:

Fibrillar Collagen I
Vitrogen 100 bovine dermal collagen (Cohesion Technologies, Palo Alto, CA) was used to coat tissue culture vessels. The acidified collagen solution was kept on ice and diluted to a concentration of 1.5 mg/ml and neutralized by addition of 10× PBS and 0.1 N NaOH to a pH of approximately 7.4. Plates were then incubated overnight at 37°C to allow gelation to occur. The collagen surfaces were then washed with Hanks Buffered Salt Solution (HBSS, Invitrogen, Carlsbad, CA) and were incubated in EGM-2MV for a minimum of 2 h to equilibrate the collagen prior to the addition of endothelial cells.

Monomeric Collagen I
Dishes were coated with Vitrogen 100 (Cohesion Technologies, Palo Alto, CA), diluted to 5 $\mu g/cm^2$ in 0.01 N HCl. The collagen was allowed to dry overnight onto the dishes or wells in a laminar-flow hood. The next day, the surface was washed twice with PBS before the seeding of cells.

Collagen IV
Dishes were coated with human collagen IV (Rockland Immunochemicals, Inc., Gilbertsville, PA), diluted in 0.1 M sodium acetate, to a concentration that resulted in 3.5 $\mu g/cm^2$