Fibrinogen E-fragment Inhibits the Migration and Tubule Formation of Human Dermal Microvascular Endothelial Cells in Vitro

Carolyn A. Bootle-Wilbraham, S. Tazzyman, J. M. Marshall, and Claire E. Lewis

Tumor Targeting Group, Section of Pathology, Division of Genomic Medicine, University of Sheffield Medical School, Sheffield S10 2RX [C. A. B.-W., S. T., C. E. L.], and Oxford Bioresearch Laboratory, Magdalen Centre, Oxford OX4 4GA [J. M. M.], United Kingdom

Abstract

Angiogenesis, the development of new blood vessels from an existing vascular bed, is essential for the growth and spread of malignant tumors. Several endogenous angiogenesis inhibitors have been discovered and shown to suppress endothelial cell function in vitro and tumor growth in vivo. Several of these are proteolytic fragments of larger, endogenous proteins. Here we show that a M, 50,000 polypeptide derived from the plasmin cleavage of fibrinogen, fibrinogen E-fragment, inhibits endothelial cell migration and tubule formation induced by both proangiogenic growth factors, vascular endothelial growth factor and basic fibroblast growth factor, in vitro.

Introduction

Angiogenesis, the outgrowth of new capillaries from preexisting vessels, is a complex multistep process that involves the degradation of components of the extracellular matrix and then the migration, proliferation, and differentiation of endothelial cells to form sprouts and tubes and eventually new vessels (1). This is a critical step in the growth of tumors and is thought to result from a change in the local balance of proangiogenic and antiangiogenic factors. Proangiogenic growth factors such as VEGF2 and bFGF are produced by both malignant cells and such stromal cells as macrophages to maintain a high level of angiogenesis in tumors (2). Conversely, a number of endogenous inhibitors have also been discovered and are currently being evaluated in clinical trials for the treatment of cancer. Angiostatin and endostatin are formed by the proteolytic cleavage of plasminogen and collagen XVIII, respectively. Both factors suppress endothelial cell responses to VEGF and bFGF in vitro and markedly reduce the vascularization and growth of experimental tumors in animal models (3). In the present report, we have investigated the angiogenic properties of another endogenous protein found in tumors, fibrinogen, and some of its major breakdown products. Fibrinogen, the soluble circulating precursor of fibrin, is a dimeric molecule containing pairs of nonidentical chains, i.e., the α-, β-, and γ-chains. These are arranged as three discrete domains, the two outer D-domains and the central E-domain (4). Fibrinogen can be digested either by plasmin or thrombin. Plasmin cleavage of each fibrinogen molecule gives rise to two D fragments, a number of smaller fragments including a small peptide, β 1-42 (the amino terminal of the β chain), and one E fragment (called fibrinogen E-fragment) consisting of the NH2-terminal regions of the α-, β-, and γ-chains chains held together by disulfide bonds (5). By contrast, cleavage of fibrinogen by thrombin produces a fibrin monomer and two copies of fibrinopeptides A and B (Fig. 1; Ref. 4). Fibrinogen has been shown to accumulate mainly around leaky blood vessels in solid tumors (6) and to polymerize at the host-tumor interface to form fibrin networks that promote tumor angiogenesis by supporting the adhesion, migration, proliferation, and differentiation of endothelial cells (7). Fibrin E-fragment, produced by proteolytic cleavage of fibrin, stimulates angiogenesis in the chick chorioallantoic membrane assay (8). Here we have compared the effects of fibrinogen and its breakdown products on the proliferation, migration, and tubule formation of HuDMECs in response to VEGF and bFGF in vitro. We confirm the proangiogenic effects of fibrin E-fragment and demonstrate for the first time that fibrinogen E-fragment is a potent inhibitor of angiogenesis in vitro.

Materials and Methods

Cell Culture. Adult HuDMECs were obtained commercially (TCS Biologicals, Buckinghamshire, United Kingdom) and cultured in microvascular endothelial cell growth medium. This medium contains heparin (10 ng/ml), hydrocortisone, human epidermal growth factor (10 ng/ml), human fibroblast growth factor (10 ng/ml; such endothelial growth factors are necessary for routine passaging of HuDMECs in culture), and dibutyryl cyclic AMP. This was supplemented with 5% heat-inactivated FCS, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B (TCS Biologicals). Cells were grown at 37°C in a 100% humidified incubator with a gas phase of 5% CO2 and routinely screened for Mycoplasma.

Proteins and Peptides. Commercial human fibrinogen (plasminogen/plasmin and thrombin free) was obtained from Enzyme Research Laboratories (Swansea, United Kingdom). The fibrinogen did not clot at any point during the experiments, indicating that there was no enzyme activity within the preparation to change its conformation. Human fibrinogen E-fragment was purchased from Diagnostica Stago (Asnieres, France). This was produced by plasmin cleavage of fibrinogen and purified by electrophoresis, immunoelectrophoresis, ion exchange, and gel filtration. To generate human fibrin E-fragment, fibrinogen E-fragment was digested with human thrombin (Sigma-Aldrich Co, Dorset, United Kingdom), as described previously (9). To control for the possible effects of trace amounts of thrombin in the fibrin E-fragment preparation on our assays, the same amount of thrombin (0.5 unit/ml) was added to control media used in experiments using fibrin E-fragment. HPLC-purified fibrinopeptide was obtained commercially from Bachem Ltd. (Saffron Walden, United Kingdom). This peptide was included in the study because the NH2 termini of the two α fragments are retained in the fibrinogen E-fragment but are missing in the fibrin E-fragment (i.e., because the fibrinopeptide A portion of this is missing). We, therefore, compared the effects of equimolar amounts of fibrinopeptide A and fibrinogen E-fragment in the assays described below to ascertain whether effects induced by fibrinogen E-fragment were attributable to an active site located in the fibrinopeptide A part of the molecule. Human recombinant endostatin (originally obtained from Calbiochem, La Jolla, CA) was a kind gift from E. Bishop (BioCure Ltd., Aberdeen, United Kingdom).

Migration Assay. The Boyden chamber technique was adapted from Malinda et al. (10) and used to evaluate HuDMEC migration across a porous membrane toward a concentration gradient of 10 ng/ml of recombinant human
Fig. 1. Schematic illustration of the role of the enzymes, plasmin and thrombin, in the generation of the fibrinogen breakdown products. Fibrinogen consists of two of each of the polypeptide chains, α, β, and γ, joined by disulfide bonds to form a symmetric dimeric structure. The NH₂-terminal regions of all six chains form the central E-domain. This fibrinogen molecule, when cleaved by plasmin, releases two D-fragments (the COOH termini of the α- and β-chains), one E-fragment (the NH₂ termini of the α- and γ-chains), and several smaller fragments including a small peptide, β 1-42 (the NH₂ terminus of the β-chain). Cleavage by thrombin releases the two fibrinopeptides A and B (FpA and FpB) from the NH₂ termini of the fibrinogen molecule, when cleaved by plasmin, releases two D-fragments (the COOH termini of the α- and β-chains), one E-fragment (the NH₂ termini of the α- and γ-chains), and several smaller fragments including a small peptide, β 1-42 (the NH₂ terminus of the β-chain). Cleavage by thrombin releases the two fibrinopeptides A and B (FpA and FpB) from the NH₂ termini of the fibrinogen molecule, when cleaved by plasmin, releases two D-fragments (the COOH termini of the α- and β-chains), one E-fragment (the NH₂ termini of the α- and γ-chains), and several smaller fragments including a small peptide, β 1-42 (the NH₂ terminus of the β-chain). Cleavage by thrombin releases the two fibrinopeptides A and B (FpA and FpB) from the NH₂ termini of the fibrinogen molecule, when cleaved by plasmin, releases two D-fragments (the COOH termini of the α- and β-chains), one E-fragment (the NH₂ termini of the α- and γ-chains), and several smaller fragments including a small peptide, β 1-42 (the NH₂ terminus of the β-chain). Cleavage by thrombin releases the two fibrinopeptides A and B (FpA and FpB) from the NH₂ termini of
study altered cell migration in the absence of VEGF (Fig. 2). None of the doses of fibrinogen E-fragment tested (100 nM) significantly decreased VEGF-induced migration of HuDMECs in a dose-dependent manner. None of the doses of fibrinogen E-fragment tested (100 nM) may have been attributable, at least in part, to a cytotoxic effect because there is no effect seen on nonstimulated endothelial cells. For example, fibrinogen has recently been shown to be capable of binding to such proangiogenic factors as bFGF (15) and could thereby block the proangiogenic function(s) of such cytokines. It is not known, however, whether fibrinogen E-fragment can also bind VEGF or whether fibrinogen E-fragment, like its parent molecule, can bind either GF. It was also possible that fibrinogen E-fragment may bind nonspecifically to the filter in the chemotaxis assay and/or constituents of the Matrigel matrix in the tubule formation assay, thereby reducing endothelial cell adhesion and function. Because one or both of these could, in theory, have been responsible, wholly or in part, for the inhibition of HuDMECs migration and tubule formation by fibrinogen E-fragment recorded in this study, we repeated these studies but this time preexposed endothelial cells to fibrinogen E-fragment prior to their use in the migration or tubule formation assay. Exposure of HuDMECs to 10 and 100 nM fibrinogen E for 1 h prior to the assays was sufficient to cause similar levels of inhibition in VEGF/bFGF-induced migration and tubule formation as that seen when fibrinogen E-fragment was present throughout the assay (data not shown). To assess the antiangiogenic potential of fibrinogen E-fragment, the level of endothelial cell inhibition was compared with that elicited by the well-characterized antiangiogenic agent, endostatin. Others have reported that 700 ng/ml (35 nM) endostatin is highly effective in blocking angiogenesis in vitro (16); therefore, various concentrations in this range were used in the present study. Fibrinogen E-fragment produced similar or greater levels of inhibition than seen by any concentration of endostatin (Figs. 2B and 3, AIV and 3, B and C). This finding suggests that, whatever the mechanism subserving its effect, fibrinogen E-fragment is a potent, new antagonist of angiogenic growth factors in vitro.

It may be important to note that the effects of fibrinogen E-fragment are not confined to endothelial cells. This polypeptide is known to also inhibit the migratory activity of neutrophils (17), stimulate fibrinogen release by hepatocytes (18), and enhance the release of interleukin 6 by macrophages (19). Further studies are required to see whether these and possibly other effects of fibrinogen E-fragment, as yet undefined, will result in limiting side effects during or after its administration in vivo.

The antiangiogenic effects of fibrinogen E-fragment contrast with results obtained using equimolar amounts of fibrinogen, fibrin E-fragment, and fibrinopeptide A. To compare the effects of these proteins/peptides to those of fibrinogen E-fragment (and thus accurately establish their structure-function relationships), most of the experiments we report in the paper were performed in the same experiments (where the dimensions of the assay/equipment would allow this). Both fibrinogen and fibrin E-fragment significantly increased control and VEGF-induced migration of HuDMECs at doses of 100 nM (Fig. 4A). Furthermore, both 100 nM fibrin E-fragment and 100 nM and 1 μM fibrinogen significantly enhanced basal and VEGF-induced tubule formation (Fig. 4B). This accords well with previous reports showing that
fibrinogen stimulates endothelial cell migration (13). Fibrin E-frAGMENT has also been shown to be proangiogenic, possibly because of conformational changes induced within the fragment by thrombin cleavage of fibrinopeptide A. Ten and 100 nM fibrinogen E-frAGMENT appeared to increase the proliferation rate of HuDMECs. However, as with fibrinogen E-frAGMENT, the highest dose (1 μM) of fibrin E-frAGMENT tested was cytotoxic for HuDMECs and triggered a significant (P < 0.001) decrease in cell viability and proliferation (data not shown). This in turn caused marked reductions in HuDMEC migration and tubule formation in our assays systems (Fig. 4). Similar results were obtained when VEGF was replaced by 10 ng/ml bFGF in these assays.

Fibrinogen E- and fibrin E-frAGMENTS differ mainly in that the latter is denuded of fibrinopeptide A by thrombin cleavage. We, therefore, investigated whether the antiangiogenic function of fibrinogen E-frAGMENT resides in this part of the molecule by testing the effects of equimolar amounts of fibrinopeptide A alone on HuDMEC migration and tubule formation. This fragment did not exert a significant effect on either HuDMEC activity in either assay (data not shown), suggesting that the active site resides either

---

**Fig. 3.** Comparison of the effects of fibrinogen E-frAGMENT or endostatin on tubule formation by HuDMECs in vitro. **A,** tubule formation in the GF-reduced Matrigel assay (×40) in the absence of exogenous factors (control, I) or the presence of 100 nM fibrinogen E-frAGMENT (II), 10 ng/ml VEGF (III), or 100 nM endostatin (IV). **B and C,** mean (bars, SE) area of tubule formation in the absence (I) or presence of various concentrations of fibrinogen E-frAGMENT or endostatin (shaded columns). HuDMECs were grown on GF-reduced Matrigel in DMEM + 1% FCS with either VEGF (10 ng/ml; B) or bFGF (10 ng/ml; C). Each test condition was carried out in three replicate wells, with total tubule area measured in three randomly selected fields of view per well (i.e., n = 9). Representative data from one experiment are given because essentially similar results were obtained in three identical experiments. *, P < 0.05 compared with control group. @, P ≤ 0.02 compared with the same dose of fibrinogen E-frAGMENT. □, fibrinogen E-frAGMENT; ■, endostatin.
in the central E domain of the fibrinogen E-fragment or in the fibrinopeptide A part of the NH₂ terminus of the α chain, but is only held in the correct confirmation for biological activity when it is attached to the rest of the fragment.

In summary, this report is the first to demonstrate the antiangiogenic activity of fibrinogen E-fragment in vitro. Moreover, the fact that it inhibits VEGF and bFGF-induced changes in endothelial cell function but leaves cells unaffected in the absence of these angiogenic stimuli bids well for the likely specificity of its effects in vivo. It remains to be seen whether the in vivo effects of fibrinogen E-fragment are confined to areas of such growth factor-induced angiogenic activity as occurs in tumors or whether it also disrupts the activity of cells lining quiescent, normal vessels.

Acknowledgments

We thank Drs. Douglas Thompson and Chris Stirk for helpful advice in the preparation of the manuscript.

References


Fibrinogen E-fragment Inhibits the Migration and Tubule Formation of Human Dermal Microvascular Endothelial Cells in Vitro


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/17/4719

Cited articles
This article cites 16 articles, 4 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/17/4719.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
/content/60/17/4719.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.