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

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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_r 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 tubules 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 VEGF[2] 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 NH_2-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% CO_2 and routinely screened for *Mycoplasma.* Prior to their use in the assays indicated below. HuDMECs were grown to 80% confluency, incubated in DMEM + 1% FCS for 2 h, then harvested with 0.05% trypsin solution, washed twice, and resuspended to the cell density required for each assay (see below).

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 NH_2 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

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[2] The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HuDMEC, human dermal microvascular endothelial cell; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; GF, growth factor.

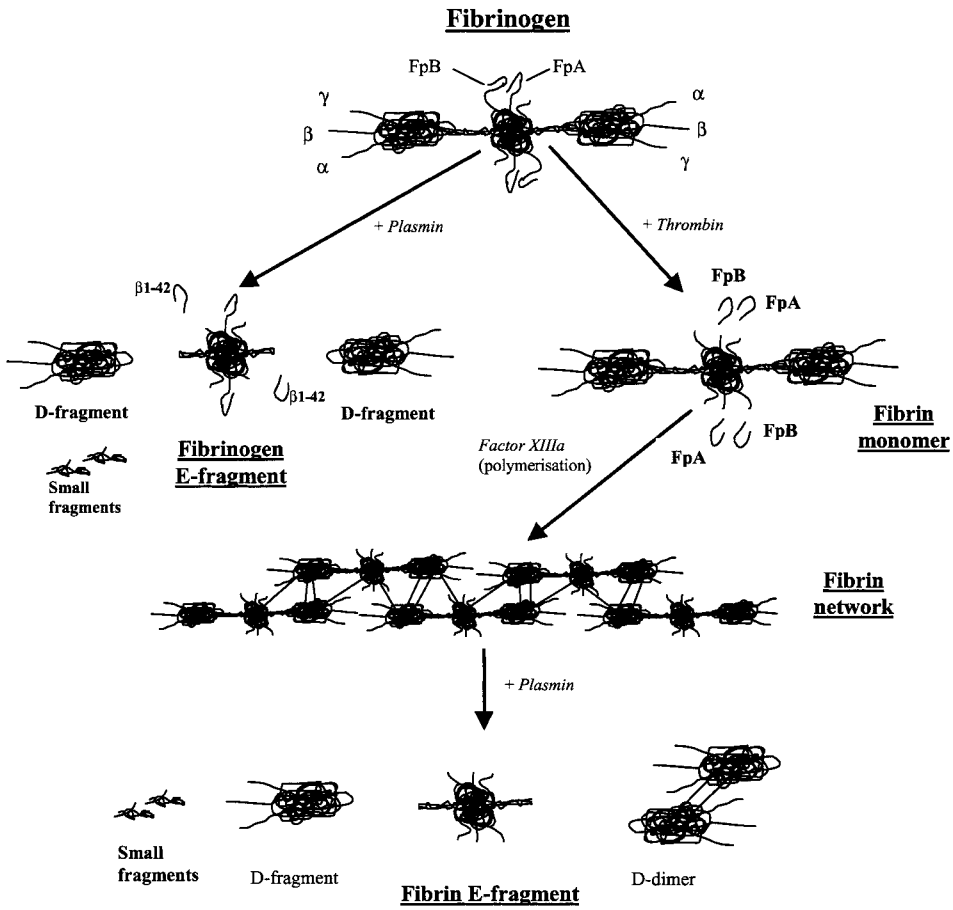

Fig. 1. Schematic illustration of the role of the enzymes, plasmin and thrombin, in the generation of the fibrin(ogen) breakdown products. Fibrinogen consists of two of each of the polypeptide chains, α, β, and γ, joined by disulfide bonds to form a symmetric dimeric structure. The NH_2-terminal regions 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-, β-, and γ-chains), one E-fragment (the NH_2 termini of the α- and γ-chains), and several smaller fragments including a small peptide, β 1-42 (the NH_2 terminus of the β-chain). Cleavage by thrombin releases the two fibrinopeptides A and B (*FpA* and *FpB*) from the NH_2 termini of the α- and β-chains, respectively, exposing polymerization sites that form electrostatic bonds between the E-domain of one molecule and the D-domain of an adjacent one. Factor XIIIa, a transglutaminase, then introduces γ-glutamyl-ε-amino-lysine isopeptide cross-links between D-domains of adjacent fibrin monomers, generating a stable polymer known as fibrin. This can then be broken down by plasmin cleavage in the three-stranded coils found between the D- and E-domains, yielding D-dimer, D-fragment, and fibrin E-fragment (which lacks the fibrinopeptides A and B) and smaller fragments (4).

VEGF or bFGF (R&D Systems Ltd., Abingdon, United Kingdom). The Neuro Probe 48-well microchemotaxis chamber (Neuro Probe, Inc., Cabin John, MD) was used with 8-μm pore size polycarbonate membranes (Neuro Probe, Inc.) coated with 100 μg/ml collagen type IV. Ten ng/ml VEGF or bFGF alone or with various concentrations of fibrinogen, fibrinogen E-fragment, fibrin E-fragment, or fibrinopeptide A were dissolved in DMEM + 1% FCS and placed in the lower wells. The collagen-coated membrane was then placed over this, and 50 μl of 25 $\times$ 10^4 HuDMECs/ml (in DMEM containing 1% FCS) were added to the upper chamber. The chambers were then incubated at 37°C for 4.5 h. After this, chambers were dismantled, the membrane was removed, and nonmigrated cells were scraped from the upper surface. Migrated cells on the lower surface were fixed with methanol, stained with Hema "Gurr" rapid staining kit (Merck, Leics, United Kingdom), and counted using a light microscope ($\times$160) in three random fields/well. Each test condition was carried out in three replicate wells, and each experiment was repeated three times.

Tubule Formation Assay. Twenty-four-well plates were coated with 30 μl/well of GF-reduced Matrigel (Becton Dickinson Labware, Bedford, MA). Endothelial cells plated on this matrix migrate and differentiate into tubules within 6 h of plating as described previously (11). HuDMECs were seeded at a density of 4 $\times$ 10^4 cells/ml and incubated for 6 h in 500 μl of either DMEM + 1% FCS alone (control) or this medium $\pm$ 10 ng/ml VEGF or bFGF in the presence or absence of whole fibrinogen or one of the fibrin(ogen) degradation products. Assessment of tubule formation involved fixing the cell preparation in 70% ethanol at 4°C for 15 min, rinsing in PBS, and staining with H&E. Three random fields of view in three replicate wells for each test condition were visualized under low power ($\times$40), and color images were captured using a Fuji digital camera linked to a Pentium III computer with a frame grabber board. Tubule formation was assessed by counting the number of tubule branches and the total area covered by tubules in each field of view using image analysis software supplied by Scion Image.

Proliferation Assay. The MTT assay was used as described previously (12) to assess HuDMEC proliferation induced by 10 ng/ml VEGF or bFGF in the absence or presence of fibrinogen or a fibrin(ogen) breakdown product. HuDMECs were seeded into 96-well microtiter plates at 3 $\times$ 10^4 cells/ml in DMEM + 1% FCS ($\pm$10 ng/ml VEGF or bFGF in the presence or absence of whole fibrinogen or one of the fibrin(ogen) degradation products) for 4.5 and 6 h. At these time points, a quarter volume of MTT solution (2 mg MTT/ml PBS) was added to each well, and each plate was incubated for 4 h at 37°C, resulting in an insoluble purple formazan product. The medium was aspirated, and the precipitates were dissolved in 100 μl of DMSO buffered at pH 10.5. The absorbance was then read at 540 nm using a Dynex ELISA plate reader.

Cytotoxicity Assay. HuDMECs were seeded at a density of 1–2 $\times$ 10^5 cells/well in a 24-well plate in the absence or presence of fibrinogen or a fibrin(ogen) degradation product. After 6 h, both live cells (after removal by trypsinization) and dead (floating) cells were harvested, and cell viability of all cells present was assessed using propidium iodide staining of 5000 cells in each of triplicate samples per treatment using a FACScan (Becton Dickinson) equipped with a blue laser excitation of 15 mW at 488 nm. The data were collected and analyzed using Cell Quest software (Becton Dickinson).

Statistical Analysis. All experiments were performed at least three times, and data were analyzed using the Mann-Whitney U test, a nonparametric test that does not assume a Gaussian distribution in the data being analyzed. $P \leq 0.05$ was taken as significant.

Results and Discussion

HuDMECs were seen to migrate across collagen-coated filters in the chemotaxis assay and form tubules on GF-reduced Matrigel in the absence of exogenous stimuli (although it should be noted that a residual level of GFs is present even in GF-reduced Matrigel). Both cell activities were significantly ($P < 0.001$) increased when 10 ng/ml of VEGF were added to the medium (Figs. 2, 3, *AI* and *AIII*, and 4*A*). Exposure to fibrinogen E-fragment significantly ($P < 0.001$) inhibited both VEGF-induced migration (Fig. 2*A*) and tubule formation, as

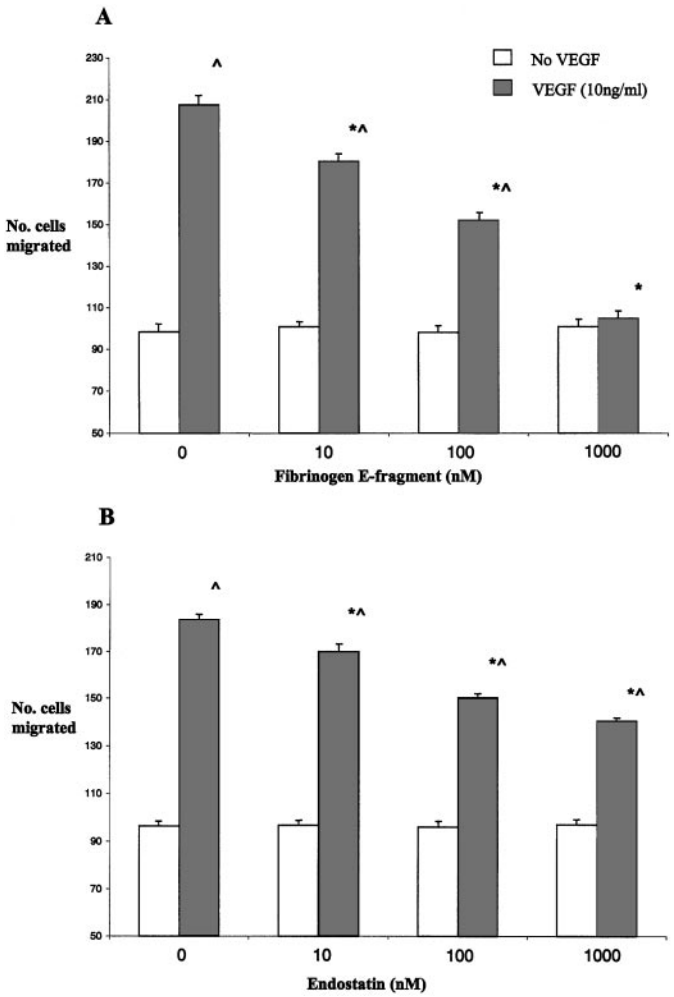

Fig. 2. Mean (*bars*, SE) number of HuDMECs migrating across a collagen-coated filter in response to control medium (no VEGF or bFGF) or medium containing 10 ng/ml VEGF in the absence or presence of various concentrations of fibrinogen E-fragment (*A*) or endostatin (*B*). Representative data from one experiment are given because similar results were obtained in two other identical experiments. *, $P < 0.001$ compared with positive control (VEGF alone); ∧, $P < 0.01$ compared with negative control (no VEGF). Essentially similar results were obtained when VEGF was replaced by 10 ng/ml bFGF (data not shown).

assessed by either total tubule area (Fig. 3, *AII* and *B*) or the number of branches (data not shown) of HuDMECs in a dose-dependent manner. None of the doses of fibrinogen E-fragment tested in this study altered cell migration in the absence of VEGF (Fig. 2*A*). The inhibitory effects of fibrinogen E-fragment were not attributable to a cytostatic or cytotoxic effect of this molecule at 10 and 100 nM, because neither concentration had any notable effect on HuDMEC proliferation or viability in control medium or medium containing 10 ng/ml VEGF. However, the marked decrease in HuDMEC migration and tubule formation evident at the highest dose of fibrinogen E-fragment tested (1 μM) may have been attributable, at least in part, to a cytotoxic effect, because this dose resulted in a marginal but significant ($P < 0.05$) reduction in the viability and proliferation of HuDMECs (data not shown).

Essentially similar results were obtained in these studies when VEGF was replaced by 10 ng/ml bFGF in the Matrigel assay (Fig. 3*C*) or the proliferation, cytotoxicity, and chemotaxis assays (data not shown). This suggests that fibrinogen E-fragment inhibits HuDMEC activity at a postreceptor locus common to both VEGF and bFGF signaling in human endothelial cells. The putative receptor(s) that bind fibrinogen E-fragment on endothelial cells have yet to be defined, although Dejana *et al.* (13) indicated that fibrinogen E-fragment may be capable of binding the fibrinogen receptor *in vitro.* However, RGD motifs in the D-domains of the fibrinogen molecule mediate binding of this protein to the fibrinogen receptor (14). These sites are absent in fibrinogen E-fragment, therefore, binding to the fibrinogen receptor would involve a novel, non-RGD region of this fragment. It is not known whether this receptor is involved in the inhibitory effects of fibrinogen E-fragment demonstrated here, and a distinct receptor/signaling pathway may be involved.

It could be argued that the inhibitory effects of fibrinogen E-fragment are attributable to an indirect rather than a direct effect on endothelial cells, 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 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 HuDMEC 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. 2*B* 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 ($P < 0.001$) increased control and VEGF-induced migration of HuDMECs at doses of 100 nM (Fig. 4*A*). Furthermore, both 100 nM fibrin E-fragment and 100 nM and 1 μM fibrinogen significantly ($P < 0.05$) enhanced basal and VEGF-induced tubule formation (Fig. 4*B*). This accords well with previous reports showing that

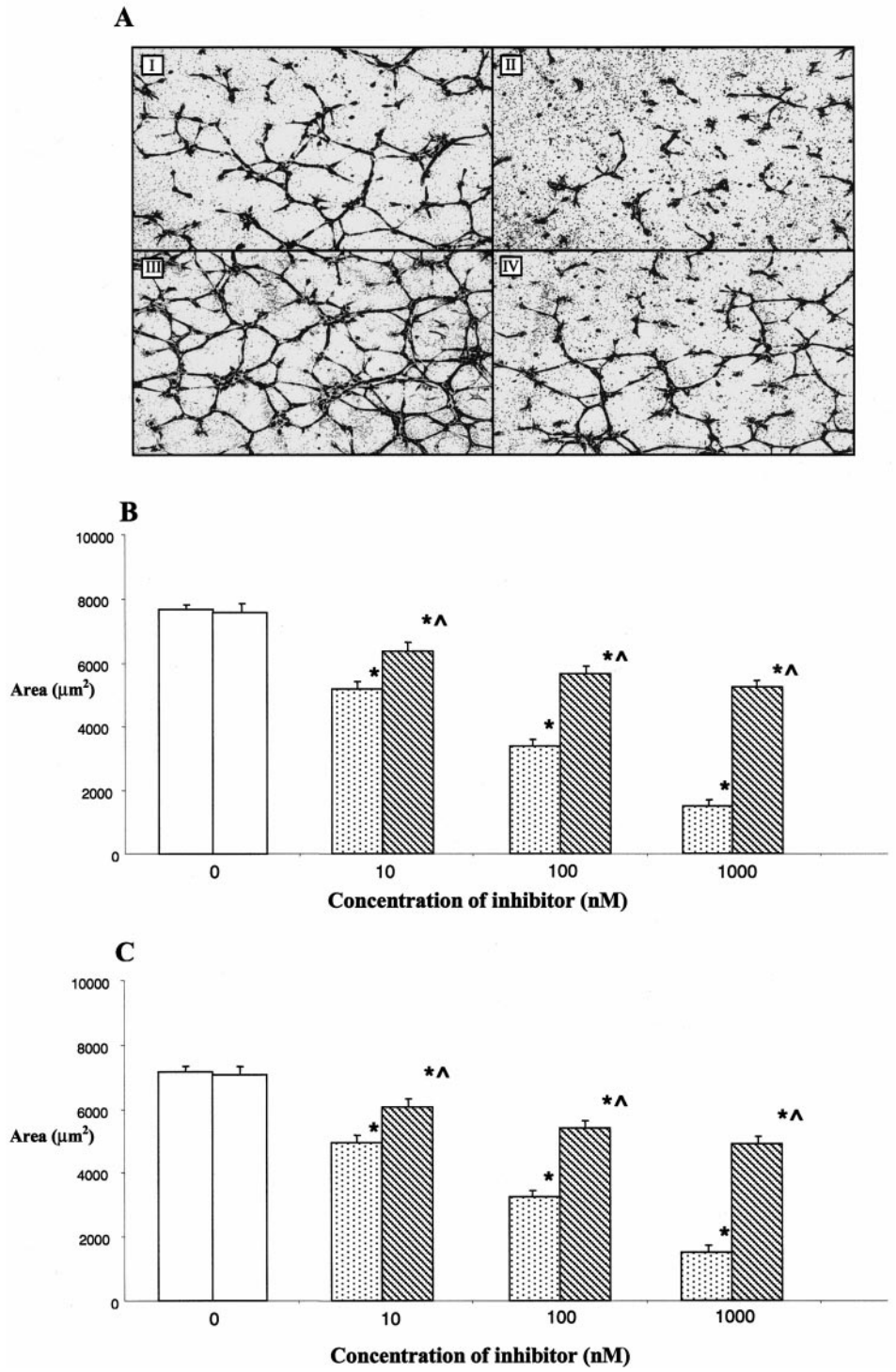

Fig. 3. Comparison of the effects of fibrinogen E-fragment or endostatin on tubule formation by HuDMECs *in vitro*. *A*, tubule formation in the GF-reduced Matrigel assay (×40) in the absence of exogenous factors (control, *I*) or the presence of 100 nM fibrinogen E-fragment (*II*), 10 ng/ml VEGF (*III*), or 100 nM endostatin (*IV*). *B* and *C*, mean (*bars*, SE) area of tubule formation in the absence (□) 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. ∧, $P \leq 0.02$ compared with the same dose of fibrinogen E-fragment. ▨, fibrinogen E-fragment; ▧, endostatin.

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

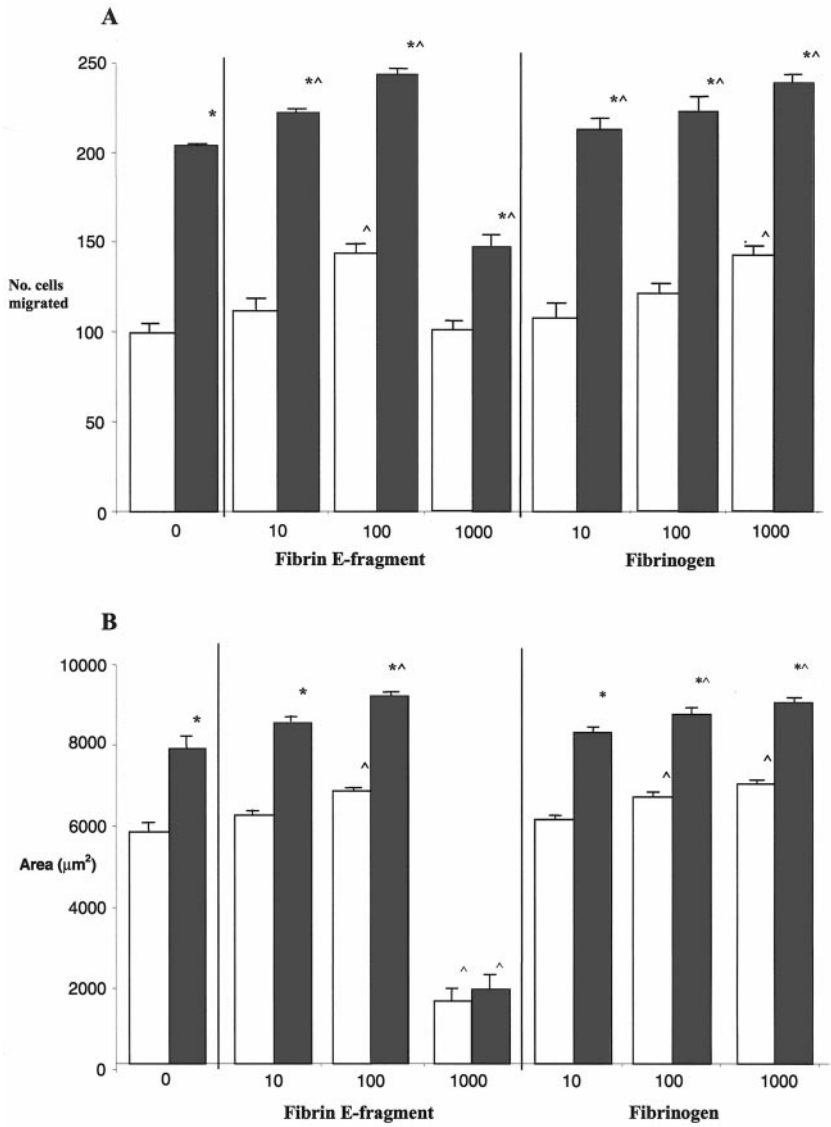

Fig. 4. Effects of various fibrinogen breakdown products; fibrin E-fragment, and whole fibrinogen on HuDMEC migration (*A*) or tubule formation in the GF-reduced Matrigel assay assessed as area (*B*) in the absence or presence of 10 ng/ml VEGF. Data are provided as means (*bars*, SE), and all doses cited are in nM. Representative data from one experiment are given because similar results were obtained in two other identical experiments (and another set of three experiments in which VEGF was replaced by 10 ng/ml bFGF). Each test condition was carried out in three replicate wells, with cell migration or total tubule area measured in three randomly selected fields of view per well (*i.e.*, $n = 9$). $*$, $P < 0.001$ compared with respective group (*i.e.*, either with or without fibrinogen or fibrin E-fragment) with no VEGF. $\wedge$, $P < 0.01$ compared with respective group (*i.e.*, either with or without VEGF) with no fibrinogen or fibrin E-fragment. □, no VEGF; ■, VEGF (10 ng/ml).

in the central E domain of the fibrinogen E-fragment or in the fibrinopeptide A part of the NH_2 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

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