Vascular Endothelial Growth Factor Increases Release of Gelatinase A and Decreases Release of Tissue Inhibitor of Metalloproteinases by Microvascular Endothelial Cells in Vitro

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The present study was designed to determine the influences of vascular endothelial growth factor (VEGF) on cell proliferation and the release of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) from human dermal microvascular endothelial cells. Treatment of cultures with 10 ng/ml or more of VEGF significantly increased cell proliferation. The effect of VEGF treatment on the levels of specific MMPs and TIMPs in the media was subsequently examined in cultures that were treated with 10 ng/ml VEGF. Zymography and Western blot analyses demonstrated that gelatinase A levels in the media were increased by VEGF treatment. Collagenase was detected by Western blots in both VEGF-treated and untreated culture media, but the levels were not significantly increased by the VEGF treatment. An ELISA assay confirmed that VEGF treatment significantly increased gelatinase A levels but did not significantly increase collagenase levels. Western blot and ELISA data showed that VEGF treatment significantly decreased TIMP-1 and TIMP-2 levels compared to untreated cultures. The data suggest that VEGF may modulate endothelial cell-derived MMP activity by: (1) increasing the abundance of gelatinase A; (2) disinhibiting gelatinase A by decreasing the abundance of TIMP-2; and (3) disinhibiting preexisting collagenase by reducing levels of TIMP-1. These actions could contribute to the ability of VEGF to promote endothelial cell invasion of new territory.

Key Words: VEGF; angiogenesis; metalloproteinases; gelatinase; collagenase.

INTRODUCTION

Angiogenesis, also referred to as neovascularization, is the formation of new blood vessels from preexisting blood vessels and is a normal physiological event in development, wound healing, repair of the endometrium, and collateral vessel formation during ischemia.
Undesirable angiogenesis also manifests as a pathological response in arthritis (Peacock et al., 1992), duodenal ulcers (Szabo, 1974), and tumor growth (Folkman, 1995) and during ocular neovascularization such as in diabetic retinopathy (Davis, 1994; Frank, 1984). Initiation of pathological angiogenesis requires at least four events: (1) a hypoxic episode that stimulates the release of mitogens; (2) mitogenic activation of vascular endothelial cells to induce their proliferation; (3) migration of endothelial cells toward the source of a chemotactic stimulus; and (4) degradation of the extracellular matrix (ECM) surrounding the vessel to permit endothelial cell invasion into new territories (Ferrara, 1995; Klagsbrun and D'Amore, 1991). As endothelial cells proliferate and migrate, they form endothelial cell sprouts or cords and eventually form a new capillary network (Pardeuaud et al., 1989). Thus, new vessel formation requires a complex series of events for vascular endothelial cells to proliferate in their traditional location and migrate into sites distant from this location.

In pathologies that display angiogenesis, the initial event that appears to stimulate new vessel growth is hypoxia (Folkman, 1995). Hypoxia is a potent stimulator of angiogenesis, inducing the expression of vascular endothelial growth factor (VEGF) (Brogi et al., 1994). Induction of VEGF production by hypoxia has been observed in tumor angiogenesis (Hlatky et al., 1994; Samoto et al., 1995; Shweiki et al., 1995; Warren et al., 1995; Weindel et al., 1994) and in ocular angiogenesis (Adamis et al., 1994; Aiello et al., 1994; Frank et al., 1994; Hata et al., 1995; Miller et al., 1994). VEGF is therefore important in the hypoxic initiation of endothelial cell proliferation and migration that are required for angiogenesis to occur (Folkman, 1995). Numerous other exogenous signals regulate angiogenesis in vivo. Such other enhancers of angiogenesis include basic fibroblast growth factor (FGF-2), epidermal growth factor, and platelet-derived growth factor. Of these various factors, FGF-2 and VEGF are synergistic endothelial cell mitogens (Goto et al., 1993; Pepper et al., 1992). The coordinated expression of these mitogens may be induced by release of cytokines and other factors by cells in the hypoxic tissue (Brogi et al., 1994).

To accommodate the migration of the proliferating cells, a finely regulated degradation of the basement membrane and surrounding ECM must occur. This degradation of the ECM serves to release the endothelial cells from their basement membrane and to provide a route by which to migrate. Clearance of the basement membrane prior to release and migration requires the production of extracellular matrix-degrading proteinases. One family of such enzymes is the matrix metalloproteinases (MMPs). The MMPs are zinc proteases that cleave components of the ECM. There are three major types of MMPs: the collagenases, the stromelysins, and the gelatinases (Matrisian, 1992). The collagenases (interstitial collagenase or MMP-1, neutrophil collagenase or MMP-8, and collagenase-3 or MMP-13) degrade fibrillar collagens and proteoglycans. There are two gelatinases, termed gelatinase A and B (MMP-2 and -9, respectively), which degrade basement membrane-type collagen (IV) as well as types V, VII, and X collagens. The stromelysins, which include stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), stromelysin-3 (MMP-11), and matrilysin (MMP-7), degrade proteoglycans, collagens X and XI, fibronectin, and laminin. In addition to the common feature that these enzymes degrade at least one component of the extracellular matrix, all are secreted as partially active zymogens that require proteolytic cleavage for maximum activation (Nagase et al., 1992a). This proteolytic loss of amino acids is readily apparent during electrophoretic separation of the enzymes during substrate gel electrophoresis, or zymography. For example, gelatinase A is secreted as a latent 72-kDa proenzyme that upon proteolytic cleavage shifts migration on gelatin zymograms to a 66-kDa partially active proenzyme and a 62-kDa fully active enzyme (Overall et al., 1989). All MMPs exhibit this electrophoretic mobility shift when activated by proteolysis (Nagase et al., 1992b). Increased expression of MMPs has been observed in the vitreous of proliferative diabetic retinopathy patients that exhibit angiogenesis, suggesting a role for MMPs in this ocular pathology (Brown et al., 1994; Sanchez et al., 1991).

Control of MMP activity in vivo is regulated, in part, by tissue inhibitors of metalloproteinases (TIMPs). There are three known TIMPs (TIMP-1, -2, and -3) that bind to latent MMPs to inhibit ECM proteolysis. TIMPs interact with their appropriate proenzyme at the protease’s amino terminus, the region flanking the enzyme’s active site.

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The resulting stearic hindrance effectively blocks the MMP active site from binding to the appropriate ECM components. Under conditions of stasis, there is an equimolar ratio of MMPs and TIMPs outside the cell, with one TIMP binding to one MMP, resulting in a lack of proteolytic activity and stable cell populations (Shapiro et al., 1992). During the induction of cell proliferation and migration, there must be either a shift in the relative protein levels of MMPs and TIMPs or a dissociation of TIMPs from the MMP. Once free of the inhibitor, the enzyme undergoes a two-step activation process that first involves a conformational change in the enzyme followed by proteolytic cleavage of approximately 10 kDa from the amino terminus. The proteolytic cleavage results in an increase in MMP activities (Denhardt et al., 1993).

An apparent correlation between the activity of MMPs and angiogenesis has been described. For example, inhibitors of MMP activity decrease angiogenesis in tumors in vitro (Fan et al., 1995; Moses and Langer, 1991), and TIMP-3 inhibits the chemotaxis of human umbilical vein endothelial cells toward either of the mitogens VEGF or FGF-2 in vitro (Anand-Apte et al., 1997). Similarly, microvascular endothelial cells from bovine retina did not form vessel cords in vitro in the presence of TIMP-3 (Anand-Apte et al., 1997). Further evidence for an MMP role in pathological angiogenesis comes from the discovery that gelatinase levels are directly correlated with the extent of neovascularization in tumors in vivo (Liotta et al., 1991). Together, these data suggest that regulation of MMPs is crucial for inhibition or enhancement of angiogenesis.

In tumor metastasis and several retinopathies, hypoxia and subsequent VEGF production are clearly involved in the induction of angiogenesis. Since release of endothelial cells from their basement membrane and migration through the surrounding ECM may require the release of MMPs, we examined the role of VEGF in regulating endothelial cell proliferation as well as in regulating the levels of MMPs and TIMPs released by microvascular endothelial cells in vitro. We used a variety of techniques (zymography, Western blots, and ELISA) to demonstrate and quantify the changes in the levels of the active and latent forms of specific MMPs and in the levels of TIMPs produced in response to VEGF.

**MATERIALS AND METHODS**

**Cell Culture**

Human microvascular endothelial cells derived from dermis (HMVECd) were purchased from Clonetics (San Diego, CA) and maintained at 37°C, 5% CO2 in endothelial cell basal media containing 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 ng/ml amphotericin B, 0.4% bovine brain extract, and 5% fetal bovine serum (Clonetics). Cells were used during early passage (4–6) and placed on uncoated culture plates.

**Endothelial Cell Proliferation and MMP Release**

To determine the optimal dose of VEGF that would generate proliferation, HMVECd cells were plated onto plastic cell culture plates at a density of ~1250 cells per well, incubated at 37°C and 5% CO2, and allowed to attach to the substrate for 24 h. At the end of the 24-h period, the cell density (number of cells/mm²) was calculated using an ocular grid and inverted microscope (Nikon TMS). Cells were then treated with VEGF (Collaborative Biomedical Products) concentrations ranging from 0 to 100 ng/ml and the cultures were returned to 37°C and 5% CO2 for an additional 24 h. At that time, total cell density was again calculated and the percentage increase in cell density determined.

In separate subsequent experiments designed to determine the effect of VEGF on MMP release, the cells were allowed to grow to approximately 80% confluency, the medium was removed, and the cultures were rinsed with serum-free medium and then incubated for 24 h in serum-free medium with or without 10 ng/ml VEGF, the former of which had been found to produce maximal proliferation in the experiment described above. The use of serum-free medium during rinses and as a vehicle for the treatment of the cells with VEGF was designed to remove endogenous metalloproteinases found in tissue culture serum additive. Serum-free medium incubated without cells was also used in some experiments to confirm the absence of contaminating serum MMPs. Cell morphology was checked visually.
after the 24-h incubation to determine the relative health of the culture. Cells visually appeared to have retained their normal fibroblast-like morphology in the absence of the serum additive, with very few cells exhibiting granulation or vacuole accumulation, both morphological indicators of abnormal cell metabolism. The media were then analyzed using zymography for total gelatinolytic activity and Western blots to determine the presence of both metalloproteinases and TIMPs. To quantify the effect of VEGF on altering MMP levels in vitro, an enzyme-linked immunosorbent assay (ELISA) was performed.

**Zymography**

Gelatin zymography is a sensitive technique that detects activated gelatin-degrading metalloproteinases (gelatinase A, gelatinase B, and a truncated stromelysin-1) (Heussen and Dowdle, 1980). For this study, culture media were collected by aspiration, centrifuged to remove cellular debris, and concentrated using an ultrafiltration device (Centricon 10; Amicon). Total protein concentration was determined by a modified Lowry assay (Bio-Rad DC protein assay). Total protein concentrations were equalized and 30 μg total protein per lane was analyzed by gelatin zymography. This conservative method of equalization compensated for changes due to differences in cell number as a result of VEGF treatment. Zymography detects mainly metalloproteinases that were activated in vivo; however, SDS treatment may have converted some unknown percentage of the latent metalloproteinase to its active form (Overall et al., 1989). We are unable to determine by zymography any changes in the amount of latent metalloproteinase produced by VEGF treatment. Samples were applied without prior boiling to an 8% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and copolymerized with 1 mg/ml gelatin. Samples were electrophoresed at 100 mA, and the gels were rinsed three times for 1 min each in dH2O and finally rinsed three times for 15 min each in Triton wash buffer (25% Triton X-100, 50 mM Tris, pH 7.6, 10 mM CaCl2, and 1 μM ZnCl2). After rinsing, the gels were incubated at 37°C for 20 h in 50 mM Tris, pH 7.6, 10 mM CaCl2, 0.1% NaN3, 1% Triton X-100, and 1 μM ZnCl2. The gels were stained overnight in Coomassie brilliant blue R-250 and then destained twice in 50% methanol, 10% acetic acid for 30 min each, followed by an overnight destain in 10% methanol, 7% acetic acid. Zones of clearing in the gel indicated MMP activity. The apparent molecular weights of the MMPs were compared to prestained standards as well as MMP standards derived from cytokine-treated macrophages. These standards included partially and fully activated forms of gelatinase B and gelatinase A. Gels were run in triplicate from three separate pairs of cultures.

**Western Blot**

Zymography is a sensitive assay for detecting some (such as gelatinases) but not all (e.g., collagenase) metalloproteinases. To determine levels of the enzymes interstitial collagenase, gelatinase A, gelatinase B, and stromelysin-1, as well as levels of two of the tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2), culture media were examined using Western blot analysis. One paired culture (one of the pair treated with 10 ng/ml VEGF and the other untreated) was examined for the presence and levels of the following enzymes and inhibitors: interstitial collagenase, gelatinases A and B, stromelysin-1, and TIMP-1 and -2. The media from these cultures were analyzed by duplicate Western blots. The antigens gelatinase B and stromelysin-1 were not detected in the medium from either the VEGF-treated or the untreated control cultures. Consequently, the presence of these enzymes was not further analyzed in subsequent Western blot detection. Media from two more pairs of cultures (VEGF-treated and untreated controls) were analyzed either once or in duplicate by Western blot for the presence and levels of interstitial collagenase, gelatinase A, TIMP-1, and TIMP-2. Interstitial collagenase was assayed because of its reported involvement in metalloproteinase production by human umbilical vein endothelial cells (Unemori et al., 1992). Gelatinase A was chosen because it was of the approximate size of the band of activity shown in the zymograms. The rationale for testing for the presence of stromelysin-1 (nontruncated form) was that this enzyme shows little gelatinolytic activity and could have been present in the media without being detected by...
gelatin zymography. We also used the Western blot technique to confirm the absence of gelatinase B as indicated by zymography. Experiments in which the primary antibodies were omitted were also performed as controls against nonspecific staining. All such primary omission experiments resulted in no immunostaining of the Western blots.

Samples were reduced with 2-mercaptoethanol and denatured by boiling for 5 min prior to electrophoresis. Equal amounts of total protein for each sample were electrophoresed through a 10% SDS–PAGE gel at 150 V for 1 h. Some gels were stained in Coomassie brilliant blue for total protein to ensure that samples were approximately equivalent in staining. Following electrophoresis, the gel, nitrocellulose, and blotting filters were equilibrated for 20 min in Towbin transfer buffer (25 mM Tris, pH 8.3, 193 mM glycine, 20% methanol) (Towbin et al., 1979). Proteins were transferred electrophoretically using a wet blotting apparatus (Mini TransBlot, Bio-Rad) for 60 min at 100 V and 4°C. Following transfer, the nitrocellulose was blocked in 3% gelatin in Tris-buffered saline with 0.1% Tween 20 (TBST). Following three 5-min washes in TBST, the membranes were incubated in monoclonal primary antibodies directed against human interstitial collagenase, human gelatinase A, human gelatinase B, human stromelysin-1, human TIMP-1, or human TIMP-2 (Oncogene Science) diluted 1:1000 in TBST for 2 h at room temperature. The antibody directed against collagenase recognizes both latent (55 kDa) and active (45 kDa) forms of the enzyme, whereas the antibody directed against gelatinase A recognizes only the latent (72 kDa) form under denatured conditions. As the TIMPs do not have latent and active forms, only one molecular weight species for each antigen was detected (28 kDa for TIMP-1 and 21 kDa for TIMP-2). The denaturation that occurred in sample preparation also freed TIMP–MMP complexes, allowing the monoclonal antibodies to detect the metalloproteinases and TIMPs separated from one another. The primary antibodies were detected using a biotinylated secondary antibody in conjunction with an avidin–biotin complex/alkaline phosphatase kit (Vectastain ABC-AP; Vector Laboratories). The substrate for the alkaline phosphatase was 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in 100 mM Tris, pH 9.5, prepared according to the manufacturer’s instructions.

Densitometry

To determine if VEGF treatment altered levels of enzyme or inhibitor released into the media, zymograms and Western blots were analyzed by densitometry using NIH Image version 1.59 and a gel analysis macro. Triplicates of zymography gels and five blots for each antigen were analyzed by densitometry. Images of the gel or nitrocellulose were scanned with a flatbed scanner (Hewlett-Packard ScanJet I IC) and the image was viewed with a Macintosh Performa computer. The optical density of the lanes containing the bands was measured using NIH Image 1.59 and relative changes in the integrated area under the peak represented relative changes in protein concentration. To further quantify changes in MMP and TIMP levels an ELISA assay was performed on media from VEGF-treated and untreated cultures as detailed below.

Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay was performed using polyclonal antibodies to further determine whether VEGF treatment altered the levels of specific MMPs or TIMPs in the media. The monoclonal antibodies employed in the Western blots were not successful in these ELISA assays. The MMPs and TIMPs may have bound to the plastic plate in such a way as to obscure the antigenic sites against which the monoclonal antibodies were directed. The use of polyclonal primary antibodies in the ELISA assays was thus necessary for antigen detection. Media from six VEGF-treated samples were pooled for the assays, as were the media from six untreated control cultures. Wells of a microtiter plate were coated with 10 μg/ml of medium in 0.2 M sodium carbonate–bicarbonate buffer, pH 9.4, for 1 h at room temperature. Wells were rinsed with modified Dulbecco’s phosphate-buffered saline (DPBS; 8 mM sodium phosphate, 2 mM potassium phosphate, 0.14 M sodium chloride, 10 mM potassium chloride, pH 7.4) containing 0.5% Tween 20 (DPBST). Nonspecific binding sites were blocked with 1% BSA in DPBS for
1 h at room temperature and then rinsed in DPBST. Specific antigens were detected using one of the follow-
ing polyclonal primary antibodies (1:500 in 1% BSA in DPBS): antibodies to interstitial collagenase, gelatinase A, TIMP-1, or TIMP-2 (Chemicon). The polyclonal antibodies recognized both the latent and the active forms of the interstitial collagenase and gelatinase A. Plates containing the primary antibodies were incubated at room temperature for 1 h and then rinsed in DPBST. Detection of the primary antibodies was accomplished by incubation of the antigen–primary complex with anti-rabbit IgG secondary antibodies conjugated with alkaline phosphatase, diluted 1:5000 in 1% BSA in DPBS for 1 h at room temperature, followed by rinses in DPBST. Following the rinses, the enzyme substrate (0.5 mg/ml p-nitrophenyl phosphate in diethanolamine buffer, pH 10.0) was added and the enzyme–substrate reaction was allowed to proceed for 30 min. The resulting colorimetric reaction was read at 415 nm using a microplate reader (MR700; Dynatech).

**RESULTS**

### Dose Response of Endothelial Cell Proliferation to Treatment with VEGF

All cultures exhibited a significant increase in cell density following a 24-h treatment (Table 1), but the proliferation in untreated cultures and in cultures treated with 5 ng/ml VEGF was statistically indistinguishable and therefore likely due to a normal mitotic increase in cell number. For cultures treated with VEGF at concentrations ranging from 10 to 100 ng/ml, the percentage increases in cell density were statistically greater than those for the untreated cultures or cultures treated with 5 ng/ml ($P < 0.001$). Among the VEGF-treated cultures, there were no statistical differences in cell densities between a concentration of 10 mg/ml

#### Statistical Analysis

To compare proliferation rates of HMVECd cells in response to treatment with VEGF, cell densities were analyzed by a repeated-measures ANOVA method. Differences in the effect of various VEGF concentrations on cell proliferation compared to untreated controls are reported. Statistical analysis of the densitometric assessment of band densities in zymograms and Western blots was by use of paired one-tailed $t$ tests. For analysis of the ELISA data, replicates of 10 wells for each antigen tested were used. The absorbance readings of the ELISA assays of VEGF-treated versus untreated control cultures were compared for statistical significance using a $\chi^2$ analysis. The calculated $P$ values are reported for each enzyme or inhibitor. All statistical tests were performed using StatView 512+ for the Macintosh.

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Mean cell density pretreatment ($\pm$SE)</th>
<th>Mean cell density posttreatment ($\pm$SE)</th>
<th>Mean percentage increase</th>
<th>Significance compared to untreated controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>147.5 ± 23.6</td>
<td>186.0 ± 14.8</td>
<td>26.1</td>
<td>n/a</td>
</tr>
<tr>
<td>5</td>
<td>103.5 ± 11.4</td>
<td>140.0 ± 16.7</td>
<td>35.3</td>
<td>0.7231</td>
</tr>
<tr>
<td>10</td>
<td>105.3 ± 11.9</td>
<td>192.8 ± 16.0</td>
<td>83.1</td>
<td>0.0001*</td>
</tr>
<tr>
<td>50</td>
<td>130.5 ± 10.3</td>
<td>232.5 ± 24.4</td>
<td>78.2</td>
<td>0.0003*</td>
</tr>
<tr>
<td>100</td>
<td>109.0 ± 25.5</td>
<td>203.0 ± 12.5</td>
<td>86.8</td>
<td>0.0008*</td>
</tr>
</tbody>
</table>

*Note.* Cell density ($\pm$SE) was calculated after the cells were allowed to attach for 24 h and again 24 h following treatment with VEGF or media alone. Statistical precision is based on an ANOVA repeated-measures analysis comparing VEGF-treated cultures (5–100 ng/ml) to untreated control cultures (0 ng/ml). n/a, not applicable.

* Statistical significance compared to 5 ng/ml, $P = 0.0001$.
* Statistical significance compared to 5 ng/ml, $P = 0.0042$.
* Statistical significance compared to 5 ng/ml, $P = 0.0008$. 

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VEGF and concentrations greater than 10 ng/ml (Table 1). Thus, the data suggested that 10 ng/ml VEGF yielded maximal microvascular endothelial cell proliferation and may have yielded maximum binding of VEGF to available receptors. We therefore used this concentration in all subsequent experiments on the effects of VEGF treatment on MMP and TIMP levels.

**Zymography**

To determine the effect of VEGF on MMP release, the HMVECd were treated with 10 ng/ml VEGF for 24 h and the media collected and assayed for total gelatinolytic activity. When the media from the endothelial cells were examined by zymography, two zones of clearing were seen in the media from VEGF-treated cells and from untreated control cells (Fig. 1). These gelatinolytic bands had apparent molecular weights of 66 and 62 kDa. The molecular weights of these gelatinases were identical with the estimated sizes of partially and fully activated gelatinase A, respectively (Matrisian, 1992). Using gel densitometry to determine relative amount of clearing, and thus protein levels, the gelatinolytic activity (combined partially active and fully active forms) in the media from VEGF-treated HMVECd cells was determined to be 19.7% greater than the control (Fig. 1, Table 2).

**Western Blot Analysis**

The media from the control and VEGF-treated cells were examined by Western blot analysis for the presence of the metalloproteinases interstitial collagenase, gelatinase A, gelatinase B, and stromelysin-1. In both control and VEGF-treated media there was positive immunoreactivity for collagenase, both the latent (55 kDa) and the active (45 kDa) forms (Fig. 2A). There was no statistically significant change in collagenase levels released into the media from VEGF-treated cultures versus untreated control cultures (Table 2; overall 9.1% decrease for the latent and active forms combined, P = 0.1994). The media from both VEGF-treated and untreated control cultures contained latent gelatinase A (72 kDa), with a more intense gelatinase A signal in the media from the VEGF-treated cells (Fig. 2B). Densitometry of the blot probed with anti-gelatinase A antibody exhibited a significant increase in the amount of latent gelatinase in the VEGF-treated cultures compared to the untreated controls (Table 2; 26.6% increase, P = 0.0149). There was no detectable stromelysin-1 or gelatinase B in the media from either the untreated control or the VEGF-treated cells. When the media from the VEGF-treated and untreated control cultures were analyzed for the presence of TIMP-1 and TIMP-2, media were found to be positive for both inhibitors (Figs. 3A and 3B). Densitometric analyses of the blots demonstrated that VEGF treatment of the cultures decreased levels of TIMP-1 and TIMP-2 by 59.8 and 51.6%, respectively (Table 2; P = 0.0029 and 0.0185). To confirm that
TABLE 2
Comparison of Changes in Levels of Metalloproteinase or TIMP Released into Culture Media by Microvascular Endothelial Cells Treated with 10 ng/ml VEGF Compared to Untreated Cultures

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Zymography</th>
<th></th>
<th>Western</th>
<th></th>
<th>ELISA</th>
<th></th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagenase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Latent</td>
<td>nd</td>
<td>—</td>
<td>+8.9</td>
<td>0.8603</td>
<td>nd</td>
<td>—</td>
</tr>
<tr>
<td>Active</td>
<td>nd</td>
<td>—</td>
<td>−22.3</td>
<td>0.3987</td>
<td>nd</td>
<td>—</td>
</tr>
<tr>
<td>Latent + active</td>
<td>nd</td>
<td>—</td>
<td>−9.1</td>
<td>0.7961</td>
<td>+4.0</td>
<td>0.1213</td>
</tr>
<tr>
<td>Gelatinase A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Latent</td>
<td>nd</td>
<td>—</td>
<td>+26.6</td>
<td>0.0299</td>
<td>nd</td>
<td>—</td>
</tr>
<tr>
<td>Active</td>
<td>+19.7</td>
<td>0.0383</td>
<td>nd</td>
<td>—</td>
<td>nd</td>
<td>—</td>
</tr>
<tr>
<td>Latent + Active</td>
<td>nd</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>nd</td>
<td>—</td>
<td>−59.8</td>
<td>0.0059</td>
<td>−69.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>nd</td>
<td>—</td>
<td>−51.6</td>
<td>0.0370</td>
<td>−34.7</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Note. For zymography and Western blot analyses, gels or blots were analyzed by densitometry and band density from VEGF-treated cultures was compared to untreated controls. For ELISA assays, percentage change in protein levels in media from VEGF-treated cultures was compared to levels in media from untreated cultures. Statistical significances reported for zymography are from a two-tailed t test; significances from Western blots are from a paired two-tailed t test; and significances from the ELISA assays are from $\chi^2$. All statistical analyses compare VEGF-treated cultures to untreated controls. nd, not detected by this technique.

the differences in protein levels were the result of VEGF-mediated release into the media, some gels were stained with Coomassie brilliant blue. All gels stained for total protein in this manner gave similar results; that is, the total protein profile was approximately equal in total staining. The results from these stainings suggest that any changes in protein levels observed in the Western blots were due to differences in that protein only and not the result of differential protein loading.

ELISA

To further quantify and confirm the effect of VEGF on levels of MMPs or TIMPs, 10 $\mu$g/ml of total protein from the media from VEGF-treated or untreated controls was assayed for interstitial collagenase, gelatinase A, TIMP-1, or TIMP-2 levels. Examination of concentrations of total interstitial collagenase in VEGF-treated and untreated control media by the ELISA technique confirmed the Western blot observation that VEGF treatment of the endothelial cells did not statistically alter collagenase levels (Table 2; 4.0% increase; $P = 0.1213$). Gelatinase A levels from VEGF-treated cultures were significantly increased (Table 2; 57.8%; $P = 0.0001$) over levels in the media from untreated controls, confirming the observations made with zymography and Western blots. Western blot analyses of the levels of the two TIMPs suggested that VEGF treatment had greatly reduced TIMP levels. Examination of TIMP levels in the media from the VEGF-treated and untreated endothelial cell cultures by ELISA confirmed that the levels of both TIMPs were significantly decreased. TIMP-1 levels were decreased by 69.9% (Table 2; $P = 0.0001$) while TIMP-2 levels were decreased by 34.7% (Table 2; $P = 0.0001$).

DISCUSSION

Effect of VEGF on Endothelial Cell Proliferation

Angiogenesis as part of a pathological response can have detrimental effects, promoting such processes as tumor growth, arthritis, and retinal neovascularization, the last of which can lead to blindness. The process of angiogenesis is complex and requires several steps to both initiate and terminate new vessel growth. Tumor metastasis involves a series of events that includes angiogenesis as a late event that will both promote tumor
and age-related macular degeneration (Davis, 1994; Elman and Fine, 1994; Palmer et al., 1994). In both tumor and retinal angiogenic responses, the onset of new vessel formation is correlated with an increased release of several mitogenic growth factors. The diverse mitogens that initiate the angiogenic response either directly regulate endothelial cell activities or stimulate nonendothelial cells within the vicinity of the target endothelium to produce factors that regulate angiogenesis (Cockerill et al., 1995; Klagsbrun and D’Amore, 1991; Leek et al., 1994; Zagzag, 1995). Of these growth factors, VEGF and FGF-2 appear to specifically activate the endothelium to promote proliferation. We report here that VEGF appears to elicit a mitogenic response in microvascular endothelial cells at doses beginning at 10 ng/ml, which also appears to promote maximal proliferation. This dose falls within the range of concentrations of VEGF needed to stimulate proliferation of large vessel-derived (macrovascular) umbilical vein endothelial cells,

growth and provide nutrition for the existing metastatic lesion (Weinstat-Saslow and Steeg, 1994). Retinal neovascularization is observed in patients with proliferative diabetic retinopathy, retinopathy of prematurity,
which has been reported to be from <0.1 to 50 ng/ml (Unemori et al., 1992). Our data suggest that although treatment with 5 ng/ml VEGF may have elicited a proliferative response, that response was not demonstrably greater than typical mitotic activity with the sample size examined. Addition of VEGF beyond the 10 ng/ml dosage did not appear to significantly further increase the mitogenic response, presumably due to maximal VEGF receptor occupancy.

Effect of VEGF on MMP Release by Endothelial Cells

Collagenase was present in the media from both the control and the VEGF-treated cultures with no significant increase in the amount of collagenase released into the media due to the VEGF treatment. The dose of VEGF used in our study (10 ng/ml) was similar to the concentration used in stimulating macrovascular vein endothelial cells to produce collagenase (Unemori et al., 1992). Interstitial collagenase was not detected in our zymograms due to its poor affinity for the gelatin substrate. If the interstitial collagenase released by microvascular endothelial cells is at basal levels, then increased proteolysis of the ECM by interstitial collagenase must be accomplished by removing TIMP-1 bound to the latent form of this enzyme. We did find that treatment of endothelial cells with VEGF decreases the amount of TIMP-1 found in the media, thus in principle allowing interstitial collagenase to assume a form capable of proteolysis. Zymography and Western blot analyses failed to detect any stromelysin-1 or gelatinase B in either the VEGF-treated or the untreated control media, and as such they were not analyzed by the ELISA assay.

Differences in molecular weight estimates from the gelatinase A bands in the zymogram versus the Western blot were observed. These differences are due to the proteolytic processing required for activation of the enzyme. The Western blot assay demonstrated the presence and relative abundance of the latent (72 kDa) proenzyme form. The gelatin zymograms, conversely, represent the partially activated enzyme (66 kDa) and the fully active form which have undergone proteolysis (62 kDa). Some of the activation of the gelatinase A may have been due to the presence of SDS in the sample buffer. Previous reports have demonstrated that latent progelatinases can be activated in vitro to both partially active and fully active forms by SDS (Overall et al., 1989).

The observed increase in gelatinase A levels was quantified by densitometric analyses of both zymograms and blots as well as by an ELISA. Because the polyclonal antibody employed in the ELISA recognized both the latent and the active forms of the enzyme, the increase observed with the ELISA is likely to be a more complete representation of the relation between VEGF treatment and gelatinase A release into the media. Consistent with this notion, combining the increase in activated gelatinase A seen with zymography (19.7%) and the increase in the latent gelatinase A seen with Western blots (26.6%) approximately yields the increase observed by the ELISA (57.8%). Thus, neither zymography alone nor Western blots alone may have given a full estimate of the gelatinase A increase produced by VEGF. Since the antibody employed in the ELISA technique recognized both latent and active forms of gelatinase A, we believe that data from this technique best represent the actual increase in gelatinase A released into the media. A recent report by Jackson and colleagues also confirms that gelatinase A activity is increased in several types of endothelial cells treated with VEGF or exposed to hypoxia (Jackson et al., 1997). It is therefore likely that an increase in gelatinase A activity is necessary for endothelial cell release from the basement membrane as well as for invasion into new territories during angiogenesis.

Effect of VEGF on TIMP Release by Endothelial Cells

The in vivo regulation of matrix metalloproteinase activity is modulated by the naturally occurring tissue inhibitors of metalloproteinases or TIMPs. There are at least three types of TIMPs, designated TIMP-1, TIMP-2, and TIMP-3. TIMPs bind at the amino terminus of metalloproteinases and block the active sites, thereby denying the enzyme access to substrates (Denhardt et al., 1993). Decreasing TIMP levels leads to an increase in proteolysis of the ECM, thus promoting macrovascular endothelial cell migration (Johnson et al., 1994; Khokha,
In this report, treatment of microvascular endothelial cells with VEGF decreased the levels of TIMP-1 and TIMP-2 released into the media compared to control cells. Coupled with the increase in gelatinase A levels in the same media, this suggests that VEGF may lower the levels of TIMP-2 and increase the levels of the TIMP-2 substrate, gelatinase A, shifting the balance of inhibitor:enzyme toward a stoichiometry that favors ECM degradation and presumably cell migration. Further, lowering levels of TIMP-1 by VEGF treatment appears to favor increasing the activity of collagenase and, therefore, would increase the degradation of collagenase-specific substrates.

**Role of Endothelial Cell Type in Regulating ECM, MMPs, and TIMPs during Angiogenesis**

In a homeostatic state, endothelial cells must operate in such a way as to maintain a stable, nonmigratory existence. Part of this stable existence relies on the synthesis of specific ECM components during normal cell turnover or repair. The synthesis and release of these components organize the cell’s surrounding microenvironment and stabilize the endothelial cell population. There are quantifiable differences in the types of ECM components secreted between large vessel-derived (macrovascular) and small vessel-derived (microvascular) endothelial cells (Iruela-Arispe et al., 1991). The major ECM component that is secreted by macrovascular endothelial cells derived from bovine aorta is type III (interstitial) collagen, while endothelial cells derived from umbilical vein synthesize type IV (basement membrane) collagen (Iruela-Arispe et al., 1991). Microvascular endothelial cells have been observed to secrete type I and type IV collagens during the organization of endothelial cords *in vitro* (Iruela-Arispe et al., 1991). If the microenvironment for the endothelial cells from different origins is unique for that particular cell type, it seems likely that endothelial cells from different sources may also produce different enzymes to manipulate their microenvironment when stimulated to proliferate and migrate. In fact, the components of the ECM do participate in the regulation of MMP release through the endothelial cell’s interactions with the ECM through integrins (Werb et al., 1990). This regulation of MMP release by the ECM components ensures that only the proteases appropriate for the prevailing substrate are released into the environment. Since endothelial cells that are participating in angiogenesis must both release from their basement membrane and migrate through the neighboring ECM, and since MMPs participate in this ECM dissolution and subsequent cell release and migration, it is of importance to determine the possible contributions of MMPs to angiogenesis. Further, it is also necessary to consider the source of the endothelial cell in determining the interactions of ECM, endothelial cell type, and MMPs produced during angiogenesis.

Macrovascular endothelial cells from umbilical vein grown on uncoated cultureware produce interstitial collagenase when treated with 100 ng/ml VEGF (Unemori et al., 1992). When umbilical vein endothelial cells treated with VEGF were assayed for gelatinase A, there was no detectable gelatinase A released into the media, although the gelatinase A mRNA was expressed (Unemori et al., 1992). Likewise, human vein-derived endothelial cells grown on fibronectin constitutively express interstitial collagenase, yet these cells also express abundant levels of gelatinase A (Hanemaaijer et al., 1993). Stimulation of vein macrovascular endothelial cells by phorbol ester, tumor necrosis factor-α, or interleukin-1α enhanced the expression of interstitial collagenase, stromelysin-1, and gelatinase B (Hanemaaijer et al., 1993). It is therefore evident that the microenvironment on which endothelial cells are grown may influence the production of the different MMPs. The presence of fibronectin on the surface of the cultureware may have influenced the macrovascular endothelial cells to produce stromelysin to degrade the fibronectin and influenced gelatinase A to degrade the type IV collagen, whereas macrovascular endothelial cells grown on uncoated surfaces would require release of interstitial collagenase to degrade type III collagen, an ECM component that has been observed to be produced by macrovascular endothelial cells *in vitro* (Iruela-Arispe et al., 1991). Differences in MMP expression have also been observed for microvascular endothelial cells. Human foreskin microvascular endothelial cells grown on fibronectin express interstitial collagenase, gelatinase A, gelatinase B, and stromelysin-1 (Hanemaaijer et al., 1993). Similar to the macrovascular endothelial cells.
grown on fibronectin, these microvascular endothelial cells are likely producing these enzymes to degrade collagen type III (interstitial collagenase), type IV (gelatinases A and B), and fibronectin (fibronectin). Microvascular endothelial cells derived from rabbit brain grown on types I and III collagen secrete both interstitial collagenase and stromelysin-1, but neither of the gelatinases (Herron et al., 1986). The secretion of these enzymes also is likely due to the interaction of the ECM with the endothelial cell as these MMPs are best suited to degrade collagens I and III (Matrisian 1992). Our data indicate that microvascular endothelial cells derived from dermis grown on uncoated cultureware express basal levels of interstitial collagenase and gelatinase A and that the level of gelatinase A can be increased with VEGF treatment. Our data also demonstrate that microvascular endothelial cells stimulated in vitro with VEGF decrease the release of TIMP-1 and -2, thereby increasing the proteolytic activities of both gelatinase A and preexisting interstitial collagenase. By disinhibiting both MMPs, degradation of the ECM is enhanced. An increase in MMP proteolysis would make it possible for the endothelial cell to release from the basement membrane following stimulation by VEGF. As VEGF levels continue to be prevalent during a hypoxic episode, more endothelial cells are stimulated, releasing more MMPs and allowing migration through the surrounding tissue to be possible. The conditions that we describe may apply only to release of the endothelial cell from the basement membrane. As the proliferating endothelial cell contacts new ECM, it is possible that the types and/or levels of MMPs produced would be different.

**Implications on the Mechanisms of Angiogenesis**

Any model of events that may occur in vivo during neovascularization should include the roles of both growth factors and MMPs in endothelial cell proliferation and migration, endothelial cord formation, and invasion of the new vessel into a distant territory. The data presented here suggest a hypothetical scenario that could account for the interaction of VEGF with endothelial cells to initiate angiogenesis. One of the roles of VEGF following its release would be to modulate the activities of two MMPs: gelatinase A and collagenase. Following a hypoxic episode, VEGF is released from one to several cell sources, depending on the tissue and pathology involved. An increase in the levels of VEGF would lead to increased mitogenic stimulation of endothelial cell proliferation. Subsequent to this VEGF stimulation of endothelial cells would be the increase in gelatinase A release, as we have shown. Concurrent with the increase in gelatinase A release, we observed a decrease in the levels of both TIMP-1 and -2. By decreasing levels of TIMP-1, preexisting collagenase would be activated and could begin ECM dissolution. VEGF upregulation of gelatinase A activity coupled with increases in collagenase activity would then allow clearance of the ECM surrounding the endothelium to proceed. This increase in MMP activity would presumably allow the endothelial cells to obtain a path by which to migrate, eventually forming a capillary sprout, vessel cord, and finally a capillary network. At this time, the temporal sequence in which enzyme activation and inhibitor inactivation occur following VEGF treatment of microvascular endothelial cells is not known. Because of the devastating effects of neovascularization during pathological responses, the control of MMP activity offers a means of reducing or retarding the angiogenic response and could therefore be a target of pharmacological inhibition of angiogenesis.

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