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VASCULAR ENDOTHELIAL CELL GROWTH FACTOR EXPRESSION IN ENDOTHELIAL CELLS IS INDUCED BY MECHANICAL WOUNDING

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ABSTRACT

Endothelial cell motility is central to several biological processes including angiogenesis during wound healing, reendothelialization of vessel walls after damage and neovascularization of tumors. However, control mechanisms that stimulate and inhibit cell movement are not known. Our objective is to understand the signals that initiate movement of endothelial cells. To examine these questions, we used an in vitro wound model of quiescent pulmonary artery endothelial cell monolayers which were stimulated to move by mechanical injury. Ca^{2+} signaling at the time of wounding produces long lasting effects on cell movement. We investigated whether new gene transcription after wounding might also stimulate endothelial cell movement. Specifically, we examined transcriptional activation of vascular endothelial cell growth factor (VEGF) after injury. While many studies have reported that tumor and epithelial cells produce VEGF, there is conflicting evidence for VEGF synthesis by endothelial cells. We found that RNA transcripts for 121 and 165 amino acids VEGF isoforms were expressed in quiescent endothelial cells. These transcripts were also produced by ovarian cancer cells which induce angiogenesis in vivo. Surprisingly, after wounding, additional RNA transcripts encoding the 189 amino acid VEGF isoform were induced. VEGF might self-stimulate endothelial cells since exogenous, recombinant VEGF accelerated cell motility as much as basic fibroblast growth factor. Our data suggest that expression of the 189 amino acid VEGF isoform is upregulated in response to extravascular signals such as mechanical wounding. VEGF might act in an autocrine or paracrine manner to stimulate movement after wounding.

Cell motility is a critical aspect of successful wound healing, yet its regulation is not well understood. To facilitate studies of motile cells, we focused on control of motility during activation of quiescent cells in vitro. Initiation of motility is of special interest since essential regulatory signaling pathways are likely to be revealed during the transition to a motile state. To model initiation of cell movement, we examined confluent endothelial cell monolayers. Movement was initiated in cultured monolayers upon release from contact inhibition by mechanical scraping. Release from contact inhibition, while necessary, was not sufficient to initiate cell movement. We found that stimulatory wound factors derived from injured cells were required for movement in addition to removal of inhibitory cell-to-cell contacts[1]. The wound factors include soluble ligands that mobilize intracellular free Ca^{2+} ($[Ca^{2+}]_i$).[1] Briefly increasing or decreasing Ca^{2+} increased or decreased the rate of motility for hours, suggesting that transient signaling events had long term effects[2]. These results suggest that motility is actively initiated by intracellular messengers. Although Ca^{2+} is important to motility, the mechanism of regulating motility by Ca^{2+} remains unknown. We also found that inducing motility of quiescent endothelial cells required new gene expression since actinomycin D treatment within the first hour postwounding prevented subsequent cell movement. Interestingly, maintenance of cell motility did not require mRNA synthesis since inhibition of gene expression one hour after wounding had no effect on wound closure (Sammak et al., unpublished observations). Therefore, we propose that transcripts encoding protein products essential for motility are lacking in quiescent cells and are manufactured within the first hour after wounding.

Our long term goal is to determine whether endothelial cell motility is initiated by intracellular messenger-stimulated gene expression. One candidate that could affect motility is vascular endothelial cell growth factor (VEGF). VEGF expression has been shown to be stimulated by Ca^{2+} [3,4] and stimulation of motility by Ca^{2+} might be mediated, in part, by synthesis of VEGF. VEGF is known to have a specific function in vascular development.[5-7] Deletion of a single allele is lethal in utero because of deficient blood vessel ingrowth during implantation.[8] Control of VEGF by $[Ca^{2+}]_i$ might mediate, in part, endothelial cell motility during angiogenesis and vasculogenesis. We report here that mRNA for one isoform of VEGF is selectively synthesized in response to wounding and that a pulse of exogenous VEGF stimulates motility for hours.

MATERIALS AND METHODS

Calf pulmonary artery endothelial cells (CPAE; line CCL 209, ATCC, Rockville, MD), were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Sciences, Grand Island, NY) supplemented with 5 mg/mL penicillin, 5 mg/mL streptomycin and 10% fetal bovine serum, (FBS, Hyclone Laboratories, Logan, UT). Transformed OVCAR-3 epithelial ovarian carcinoma cells (NCI Frederick Facility, Frederick, MD) were cultured in RPMI 1640 with 15% FBS and antibiotics. Cells were grown to confluence (2 - 3 days). For motility experiments, cells were starved (serum free medium) for 2 days in DMEM containing 0.1% bovine serum albumin (Sigma, St. Louis, MO) with penicillin and streptomycin to reduce effects of exogenous growth factors on wound response.

To measure gene expression induced by wounding, we collected cells stimulated by nearby wounding, but not themselves fatally damaged. A confluent, starved 100 mm plate of cells was wounded with a comb designed so that all cells that remained intact were stimulated by nearby wounds. Dead cells were scraped off and were washed away before collecting RNA from surviving cells. Total RNA was isolated from endothelial cell monolayers one hour after mechanical injury using Trizol per

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manufacturer's instructions (Gibco Life Sciences, Grand Island, NY). Reverse transcriptase-polymerase chain reaction (RT-PCR) was done using oligonucleotide primers (5' GGGCTCGAGCA CCCATGGCAGAAGGA 3' and 5' GGGGGATCCCTGCCCG GCTCACCGCCTCGG 3') specific for VEGF as previously described.[9] The flt-1 specific primers were (5'CGCGGTC TTGCCTTACGCGCT 3' and 5'CCATTTATGGGCTGCTTCC CCCCTGCA3') as previously described.[5]

To measure motility during healing, wounds were made by scratching monolayers with an 18-g needle (8 μm diameter), which removed a 4 to 6 cell-wide swath (about 100 - 200 μm). Hank's balanced salt solution (HBSS), with or without recombinant VEGF (165 amino acid isoform),[10] was added 0.5 minutes before through 4.5 minutes after wounding. After wounding, dishes were drained, washed with HBSS, refilled with serum-free DMEM, and reincubated. Dead cells, distinguishable in phase contrast microscopy by their lucidity, were limited to the first row of cells at the wound edge.[1] Most dead cells rounded up and floated away before the first measurement at 1 hour. At one, two and three hours postwounding, wound area was measured using a Nikon TMS microscope equipped with a 10X objective and a Cohu CCD camera that provided direct video input into a Macintosh video-capable computer. Areas were calculated with the public domain program, NIH Image (available by FTP from zippy.nimh.nih.gov). Distances were calibrated with a stage micrometer (Fisher Scientific, Pittsburgh, PA). From these measurements, changes in wound width between one and three hours postwounding were calculated. Wound closure was proportional to the net rate of cell movement and spreading, averaged over 100 cells. Cell proliferation did not occur during the initial three hours postwounding.

Results

We determined whether VEGF was expressed in CPAE cells using RT-PCR, with oligonucleotide primers specific to VEGF and its receptor, flt-1. RNAs encoding VEGF were detected in total RNA isolated from CPAE endothelial cells before wounding and at one hour after wounding. As a control, total RNA from transformed OVCAR-3 epithelial ovarian carcinoma cells was also analyzed. Lanes A and B show that nonwounded CPAE cells express mRNA for two isoforms of VEGF (121 and 165 amino acids) and the flt-1 receptor, respectively (fig 1). Since CPAE cells express flt-1, it is likely that these cells are responsive to VEGF. Transformed OVCAR-3 cells also express isoforms for these two VEGF peptides (Lane C), but not the receptor (Lane D), and are not responsive to VEGF (not shown). Surprisingly, RT-PCR using RNA isolated from CPAE cells one hour after wounding demonstrated mRNA encoding the 189 amino acid isoform of VEGF in addition to transcripts for the 121 and 165 amino acid isoforms (Lane E).

VEGF has been reported to stimulate angiogenesis in vivo and proliferation of CPAE cells in vitro.[10] However, effects of VEGF on cell motility have not been well described. We applied VEGF for 5 minutes during mechanical wounding and measured the width of wounds at 1 and 3 hours post wounding. The rate of wound closure during this period was stimulated by VEGF in a concentration-dependent manner (Table 1). The average speed at low doses (29.1 μm/hr at 0.01 - 1 ng/mL) differed from that at high concentrations (35.2 μm/hr at 10 - 100 ng/mL; $p < 0.05$). At higher concentrations, cells rounded while moving (1 μg/mL). Note that VEGF was applied for only 5 minutes during wounding and that motility was measured for hours. VEGF increased wound closure rates as much as 10 ng/mL bFGF (increased movement by about 50%; from 23 to 35 μm/hr) and stimulated movement more than insulin (applied continuously stimulated movement by about 20%; from 20 to 26 μm/hr) (Table 1).

Discussion

Whether or not VEGF is synthesized by endothelial cells is controversial. Most reports suggest that VEGF is synthesized by epithelial but not endothelial cells.[11-13] The target for VEGF action is endothelial not epithelial tissues since VEGF receptors are expressed by endothelium.[14] Therefore, most studies support a paracrine function of epithelial-derived VEGF acting on vascular endothelium. In contrast, a few studies support an autocrine function of endothelial-derived VEGF.[15,16] The variability among reports about VEGF production by endothelium might be accounted for by the difference between normal and activated endothelium. VEGF might not be present in nonproliferative vascular endothelium but might be induced by stress or other stimuli.

We have found that RNA transcripts for one VEGF isoform was specifically expressed in endothelial cells only after mechanical injury. Serum starved endothelial cells produced transcripts for the 165 and 121 amino acid isoforms of VEGF peptide and for the VEGF receptor, flt-1 (fig 1). Upon wounding, expression of transcripts encoding the 189 amino acid VEGF isoform also were induced. It is not known whether these isoforms have different effects on motility. Exogenous recombinant VEGF (165 amino acid isoform) stimulated endothelial cell motility in a concentration-dependent manner as much as bFGF and more than insulin (Table 1). This suggests that wound-derived signals might set up an autocrine control of cell movement by induction of VEGF expression. An autocrine role of VEGF for initiation of motility might not be found in quiescent endothelial cells under other conditions.

In other studies, we found that immediate-early genes, *c-fos* and *c-jun*, are induced minutes after wounding, and that Ca^{2+} differentially induces *c-fos*, but not *c-jun*.[17] These factors

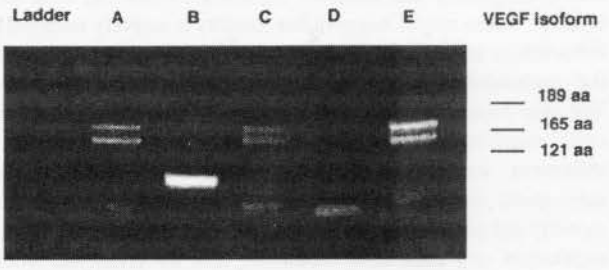

Figure 1. RT-PCR detection of VEGF and its receptor, flt-1, in endothelial cells. RT-PCR, using primers for VEGF and flt-1 was performed on total RNA isolated from CPAE endothelial cells and transformed OVCAR-3 ovarian carcinoma cells. Lane A shows transcripts for two VEGF isoforms (121 and 165 amino acids; aa) from nonwounded CPAE cells. Lane B shows transcripts for flt-1 receptor from these same cells. Transcripts for the two VEGF isoforms (Lane C), but not the receptor (Lane D), were found in transformed OVCAR-3 cells. One hour after wounding, CPAE cells contained transcripts for three VEGF isoforms (189, 121 and 165 aa; Lane E).

Table 1: Stimulation of wound closure (μm/hr) by VEGF, bFGF, and Insulin

Growth Factor[1]		VEGF					bFGF	Insulin
concentration		0.01	0.1	1	10	100 ng/mL	10 ng/mL	10 μM
Control[2]	23.1 ± 1.4						23.0 ± 1.7	20.0 ± 2.6
Treated[3]		27.4	31.0	29.2	35.8	34.8		
		29.2 ± 1.8*			35.2 ± 1.0*†		35.0 ± 6.6*	26.0 ± 3.4*

[1] VEGF applied for 5 minutes, bFGF for 2 minutes, insulin continuously.
[2] Data are means ± 1 sd; n = 4
[3] VEGF dose-response series are data representative of two replicates (two wounds each, 60 cells/wound).
* $p < 0.05$ vs control; † $p < 0.05$ vs 0.01 - 1 ng/mL VEGF

dimerize to form the activator protein-1 (AP-1) complex involved in VEGF expression.[18] VEGF expression also can be induced by Ca^{2+},[3,4] protein kinase C and cAMP.[4,14] Thus, wound-induced signals such as $[Ca^{2+}]_i$, *c-fos* and *c-jun* might stimulate VEGF synthesis. Yet, in hypoxia, VEGF synthesis also is induced independent of AP-1.[19] It remains to be determined whether VEGF induction during wounding is mediated by AP-1 factors.

Our working hypothesis is that transient mobilization of $[Ca^{2+}]_i$ triggers long term stimulation of movement by a sustained autocrine mechanism. A signaling cascade beginning with $[Ca^{2+}]_i$ (lasting seconds) might initiate primary genes, such as *c-fos* and *c-jun* (lasting minutes) which, in turn, might activate the secondary gene VEGF (lasting hours). Sustained production of VEGF, then, could maintain cell motility at a rate that was defined by its level of expression.

How might VEGF stimulate cell movement? The specificity and potency of VEGF for motility of endothelial cells has been studied. One effect is to increase expression of urokinase-type plasminogen activator and $\alpha_v\beta_3$ integrin which speed movement and lamella formation.[20,21] One hypothesis for effects of urokinase is competition for plasminogen activator inhibitor which can slow cell movement by competing with vitronectin for binding to $\alpha_v\beta_3$ integrin.[22] Adherence of pulmonary artery endothelial cells depends on mobilizing $\alpha_v\beta_3$ in a $[Ca^{2+}]_i$-dependent manner.[2] Thus, VEGF might alter $\alpha_v\beta_3$-dependent cell adhesion. Investigation of the effects of VEGF on motility, cell adhesion and lamella formation is needed to resolve these questions.

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