Disruption of Cadherin-Related Junctions Triggers Autocrine Expression of Vascular Endothelial Growth Factor in Bovine Aortic Endothelial Cells
Effects on Cell Proliferation and Death Resistance

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Abstract—The mechanisms involved in the blockade of proliferation in confluent endothelial cells are insufficiently understood. In this regard, the continuity of intercellular junctions appears to be critical to the regulation of endothelial monolayer cell growth. The present study examined the hypothesis that the disruption of the intercellular adherens junctions will trigger both endothelial cell proliferation and autocrine production of growth factors. With this purpose, we assessed the changes in growth, death resistance, and expression of vascular endothelial growth factor (VEGF) under conditions of disruption of the intercellular junctions between endothelial cells. Disruption of cell junctions was produced by means of a specific anti–vascular endothelial cadherin monoclonal antibody, EGTA, or cytochalasin D. Our results disclosed that these maneuvers induce an increase in VEGF mRNA production, with transcription of the 121-, 165-, and 189–amino acid isoforms of VEGF. Further evidence of the relationship between endothelial cells monolayer continuity and VEGF protein expression was obtained by the demonstration of an increase in VEGF protein, as determined by Western blot, induced by the aforementioned maneuvers, as well as by immunocytochemical detection of increased VEGF staining in the areas surrounding a mechanical endothelial injury and in endothelial cells at subconfluence. In functional terms, the autocrine expression of VEGF was associated with growth-promoting and cytoprotective effects, as assessed by [3H]thymidine uptake, 51Cr release, and flow cytometry. In conclusion, our results reveal that disruption of homophilic interendothelial junctions induces VEGF expression. Under these conditions, autocrine VEGF appears to have a relevant role in death inhibition and proliferation of endothelial cells. (Circ Res. 1999;85:1132-1138.)

Key Words: vascular endothelial growth factor ■ vascular endothelial cadherin ■ endothelial cell ■ cytoprotection ■ proliferation

Endothelial cells grow following a monolayer pattern, in a process that involves a host of regulatory growth factors and hormones. This behavior implies the existence of contact inhibition, which blocks further growth on cell confluence. The mechanisms leading to this phenomenon remain, however, largely unknown, but they should be critical for understanding different situations, from physiological vascular repair to pathological neovascularization. The monolayer, growth-inhibited condition observed in vivo persists in vitro in endothelial cells in confluent cultures. Endothelial integrity implies the existence of cell-to-cell interactions, which involve different types of junctions between endothelial cells. Among them, adherens junctions, which are constituted by a rather complex system, including the endothelial cell–specific vascular endothelial (VE) cadherin, catenins, plakoglobin, and α-actin, are instrumental for the integrity of the monolayer structure. Cadherins are a multiple-gene family of Ca2+-dependent cell adhesion molecules with a typical single-spanning transmembrane structure and with homophilic binding specificity. Cadherins localize at adherens junctions and mediate physiological interactions involving cytoplasmic anchoring molecules, catenins, and the actin-based cytoskeleton network. The signaling system by which these junctions participate in sensing the integrity of the endothelial layer are, however, insufficiently characterized. In research done in the early 1980s, Selden et al described how agents that disrupt microtubule structures of the cytoskeleton can trigger endothelial cell proliferation. However, no specific mechanisms of this effect were identified at that time, and these remain undefined.

The phenomenon of contact inhibition implies the absence of effect of growth factors participating in endothelial cell
proliferation. The main sources for endothelial growth factors are paracrine. A relevant example of this is the production of vascular endothelial growth factor (VEGF) by vascular smooth muscle cells submitted to either in vivo or in vitro injuring conditions.9–12 VEGF is an endothelial cell–specific mitogen, which appears to be a crucial mediator of angiogenesis in different conditions.13 However, despite the role of paracrine VEGF, the development of capillary networks may require the existence of autocrine growth stimulation. In this regard, there is solid evidence supporting the existence of autocrine production of VEGF by endothelial cells in several conditions, of which hypoxia is the most representative.14–18 Nevertheless, no data are available to ascertain whether autocrine VEGF expression may be triggered in other situations, specially when the endothelial cell monolayer is injured and disrupted. Injured endothelia, either in vivo or in vitro, tend to reorganize and cover the denuded surfaces, therefore indicating that a repair program is activated by the injury. Accordingly, we hypothesized that the interruption of endothelial cell monolayer continuity will trigger both endothelial cell proliferation and autocrine production of growth factors. Based on this hypothesis, the aim of the present study was to assess the proliferative response and the autocrine VEGF expression on endothelial cells, under conditions of interruption of cell-to-cell adhesion. The main attention was focused on the production of VEGF.

Materials and Methods

Endothelial Cell Culture

Bovine aorta endothelial cells (BAECs) were obtained and cultured as described.19–24

Electron Microscopy (EM)

Confluent BAECs were treated as described.22

DNA Synthesis Assay

Uptake of [3H]thymidine was measured as described.19

Analysis of the mRNA Expression of VEGF

Total RNA was isolated from confluent monolayers 3 hours after the different treatments (see below). Reverse transcriptase–polymerase chain reaction (PCR) and Northern blot were performed as described.20 Oligonucleotide primers of human VEGF were synthesized according to established sequences.23 The PCR products were analyzed by automatic sequencing (373 DNA sequencer, Applied Biosystems). GAPDH was used as a control gene.

Immunocytochemistry of VEGF Expression

Confluent and subconfluent BAECs grown on glass chamber slides (Nalge Nunc) were submitted to the different experimental maneuvers, washed with PBS, and incubated in MEM d-valine without FCS for an additional 24 hours and then fixed in 95% ethanol. Immunostaining was performed by a modification of described methods, using a specific anti-VEGF antibody raised in our institution.24

Western Blot Analysis of VEGF

Confluent BAECs were submitted to the different experimental maneuvers, washed with PBS, and incubated in MEM d-valine without FCS for an additional 24 hours. Then, cells were extracted with 0.5% (octylphenox)polyethoxyethanol (Igepal CA-630; Sigma) in Tris-buffered saline containing protease inhibitors. Samples separated by SDS-PAGE and transferred onto nitrocellulose (Bio-Rad Laboratories) were probed with anti-VEGF monoclonal antibody (mAb; 1/250; Sigma) and probed with a mouse monoclonal anti–α-tubulin to normalize to equivalent gel loading.

Flow Cytometry

These experiments were done as described,25 using an EPICS-XL MCL cytometer (Coulter) and the Mcycle program (Coulter) for evaluating cell death.

Assay of Cytotoxicity and Measurement of Cytosolic Ca2+ Concentration ([Ca2+]i) by Fura-2

Cytotoxicity and [Ca2+]i, were measured as described, by 51Cr release and fura-2, respectively.20,26

Experimental Maneuvers

Intercellular adhesion of BAECs was disrupted by treatment with EGTA (2 mmol/L, 30 minutes), cytochalasin D (CytD; 0.2 μg/mL, 60 minutes; Sigma) or VE-cadherin mAb (VE-mAb; 0.25 μg/mL, 60 minutes; Transduction Laboratories), as described.27–30 Monoclonal anti-PTHrP (0.25 μg/mL) and nonspecific rabbit IgG (0.25 μg/mL) were used as controls without effects on VE-cadherin. BAECs were also exposed to antibodies against CD31 (0.25 μg/mL) and CD34 (0.25 μg/mL) antigens, which are highly expressed in endothelial cells but are not involved in VE-cadherin junctions and do not produce a significant [Ca2+]i signal (M.A. Castilla et al, unpublished results, 1998). When indicated, a specific anti-VEGF mAb (0.5 mg/mL, 1:500; Sigma) was used to block VEGF effects.20 To assess the triggering of VEGF expression by other means, a mechanical endothelial wound was done by using a sterile 200-μL pipette tip.

Statistics

Results are expressed as mean±SEM. Unless otherwise stated, each value corresponds to a minimum of 5 triplicate experiments. Comparisons were done by 1-way ANOVA and Scheffé test, Kruskal Wallis and Dunn tests, and paired and unpaired Student t and Bonferroni tests when appropriate. P was considered significant at ≤0.05.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Disruption of Intercellular Adhesion Between BAECs

The effects of the VE-mAb on intercellular junctions are shown in the EM images in Figure 1A and 1B. Similar disruptive images were observed on BAECs treated with EGTA or CytD (data not shown). The images shown in Figure 1A and 1B are representative of at least 10 samples showing similar changes. On average, 10±3 junctions were studied in each set of microphotographs. Cytotoxicity was not present with the incubation times and concentrations used in this series of experiments (percentage 51Cr release at 24 hours by BAECs incubated in no FBS; 2 mmol/L EGTA, 21±1.1%; 0.2 μg/mL CytD, 22.9±0.9%; 0.25 μg/mL VE-mAb, 21.4±2.1%; 20% FBS, 21.5±1.6; untreated control, 29.2±1.3 [P<0.05 with respect to the untreated control, P=NS between EGTA, CytD, VE-mAb, and 20% FBS]). Rather, as shown in the values above, a protective effect was observed with the 3 maneuvers, at a level similar to that obtained under conditions of maximal cytoprotection with 20% FBS. In the same line of evidence about cell viability, BAECs treated with short-term incubations with EGTA, CytD, or VE-mAb had normal [Ca2+]i, transients when stimulated with ATP (10−4 mol/L) (P=NS between untreated BAECs with respect to BAECs treated with any of the 3
maneuvers). Moreover, no differences in [Ca\(^{2+}\)]\(_{i}\) transient response were detected between BAECs incubated with VE-mAb (0.25 µg/mL) during 1, 3, or 24 hours (P=NS between untreated BAECs with respect to BAECs treated with VE-mAb at any of the 3 incubation times).

**Effects of Disruption of Intercellular Adhesion on VEGF mRNA Expression and VEGF Production**

A moderate, albeit consistent basal expression of VEGF mRNA corresponding to 3 isoforms was present in BAECs (Figure 2); this expression appeared to be enhanced, particularly for the 165–amino acid isoform, with the 3 maneuvers disrupting BAEC intercellular adhesion. For more accurate quantification, Northern blot experiments were performed. These experiments revealed an increased VEGF mRNA expression in BAECs treated with the disruption maneuvers. An image of a representative Northern blot is depicted in Figure 3 (top). Quantification of VEGF mRNA expression against 28S cDNA (Figure 3, bottom) demonstrated a significant increment with respect to the control, with all 3 maneuvers. Separate experiments revealed that the simultaneous treatments with EGTA and VE-mAb were nonadditive (P=NS between the expression of VEGF mRNA in BAECs treated with either 2 mmol/L EGTA or 0.25 µg/mL VE-mAb alone versus 2 mmol/L EGTA+0.25 µg/mL VE-mAb). No effect on VEGF expression was detected with the non–VEGF-specific antibodies; these antibodies did not provoke changes in [Ca\(^{2+}\)], (data not shown), therefore suggesting the absence of intracellular signaling produced by the antibody itself.

A similar stimulating effect was obtained when VEGF protein production was assessed by Western blotting. As shown in Figure 4, lanes 4 through 6, an increased VEGF band intensity was detected with EGTA, CytD, and VE-mAb. In quantitative data corresponding to the mean of 3 experiments showing similar results, we have found the following values of VEGF protein increment with respect to the baseline: EGTA, 5.3±0.6 times (P<0.01); CytD, 4.2±0.5 times (P<0.01), VE-mAb, 16.2±0.6 times (P<0.001), anti-CD31, 1.4±0.3 times (P=NS); and anti-CD34, 1±0.2 times (P=NS). In the same line of evidence, an increased VEGF immunostaining was detected in the areas surrounding an endothelial wound (Figure 5A). Furthermore, to assess whether the increased expression of VEGF was due to the disruption or to the lack of formation of the intercellular junctions, BAECs were examined by immunocytochemistry at the subconfluent state. Of interest, a marked VEGF immunostaining was evident in subconfluent BAECs (Figure 5B). The latter experiments were the only performed in nonconfluent cells. Although these results are not shown, because they provide a similar kind of information as the Western blot results showing an increase in VEGF protein, the exposure of BAECs to EGTA, CytD, and VE-mAb (similar concentrations as above) increased VEGF immunocytochemical staining in a diffuse pattern on the entire preparation.

**Functional Implications of the Stimulation of VEGF Production by Loss of the Adherens Junctions**

A major effect of the 3 treatments was observed on cell protection against FBS deprivation. This effect was assessed by flow cytometry (Figure 6A through 6C). As can be seen,
the 3 treatments afforded a relevant degree of protection against cell death within a range close to that obtained by incubation with 20% FBS. This cytoprotective effect was completely inhibited in the presence of anti-VEGF mAb (Figure 6A through 6C). No effects of the anti-VEGF mAb by itself were observed (percentage of cell death, 37.6±1.9, P=NS with respect to baseline; untreated cells, n=4). Of interest, the simultaneous incubation of BAECs with CytD and anti-VEGF mAb did actually produce an increment in cell death with respect to the baseline (Figure 6B). Finally, the treatment with 2 of the 3 maneuvers, namely CytD and VE-mAb, increased endothelial cell DNA synthesis, as assessed by [3 H]thymidine uptake and cell number (Figure 7A and 7B). As can be observed, EGTA incubation differed from the other 2 maneuvers in that it did not increase DNA synthesis in the same proportion. In any case, the magnitude of the increase in [3 H]thymidine with CytD and VE-mAb was substantially smaller when compared with the stimulation reached with 20% FBS (70.6±4 times increase in [3 H]thymidine uptake, P<0.0001 between 20% FBS versus CytD and VE-mAb).

Discussion
The present study discloses that the disruption of intercellular junctions between endothelial cells triggers both VEGF expression and production. This VEGF expression results in resistance to death provoked by serum deprivation and in increased proliferation of endothelial cells. Therefore, the present results link the state of endothelial cell-to-cell junc-
tions with the autocrine expression and action of a major angiogenic growth factor, namely, VEGF. The use of different types of maneuvers, all with disrupting effects on the VE-cadherin system, albeit with different mechanisms of action and different degrees of specificity, provides further support to the findings. Two additional findings add meaningful information on the regulation of VEGF expression in endothelial cells. First, the fact that a mechanical injury induces VEGF accumulation localized to the area surrounding the endothelial wound suggests that the disruption of endothelial cell junctions by means other than chemical is also capable of triggering VEGF expression. Second, the evidence that subconfluent BAECs markedly expressed VEGF protein suggests that the absence of intercellular junctions may be the critical factor in the mechanism that stimulates VEGF production, no matter whether this absence has occurred by monolayer disruption or because of a preconfluent state. Of additional interest, the absence of VEGF protein expression in areas of BAEC monolayers far from the wounded area (see Figure 5) suggests that VEGF expression may be set off on reaching confluence. In this regard, recent data from our laboratory24 obtained in a particular form of vascular outgrowth, namely, pyogenic granuloma, suggest that in this particular condition, VEGF expression by groups of endothelial cells become suppressed when the vessels reach a more mature state.

Reports from other investigators provided the theoretical background for the present study. Previously published data have related the disruption of microtubule structures with cell proliferation.2 More recently, Inger et al31 ascertained the importance of actin-containing microfilaments in shape-dependent growth control by culture cells in the presence of CytD that, in short-term incubations, produced mitogenic effects. Our results not only confirm the reports of Inger et al31 in terms of the growth-promoting effect of brief exposures to CytD, but also provide an explanation, based on the effects of the stimulated VEGF production, for the previously unexplained induction of endothelial cell proliferation by CytD. In addition, the present results demonstrate that the effects of CytD to promote endothelial cell gene expression,
which were already described for the endothelin 1 gene.\textsuperscript{29} involve also VEGF expression. In a second set of previous data related to our findings, recent studies demonstrated that cell proliferation becomes inhibited after transfecting cadherin-lacking cells with VE-cadherin.\textsuperscript{32} Our results using the VE-mAb, therefore, mirror the aforementioned effects of VE-cadherin transfection. Functionally, the present experiments demonstrate that the attack of the VE-cadherin junctions with the VE-mAb triggers cytoprotection and DNA synthesis on endothelial cells. Giving further pathophysiological significance to this finding, an increased VEGF expression and vessel growth has been observed in blistering skin diseases, which are causally related to autoantibodies directed to members of the cadherin gene superfamily.\textsuperscript{6,33,34} Taken together, this information suggests that a relationship exists between cadherin system integrity, VEGF expression, and endothelial cell growth. The association found in pemphigus of immune disruption of intercellular adhesion and VEGF expression is coincident with our present in vitro findings, albeit in cells other than endothelial.

In mechanistic terms, the fact that in the present experiments the disruption at the level of either the extracellular or the intracellular domain of the adherens system, as obtained by means of the VE-mAb or EGTA or by means of CytD, respectively, triggered similar responses in BAECS suggests that both parts of the system are relevant to the regulation of growth and VEGF expression. Furthermore, the absence of additive effects between the different maneuvers in terms of VEGF expression, \([1^H]\)thymidine incorporation, or flow cytometry further suggest that similar pathways are involved. The differences found in the effect of EGTA exposure on \([1^H]\)thymidine incorporation and cell count indicate that the effect of EGTA in these circumstances occurred predominantly through cell protection without stimulating DNA synthesis. This is not surprising, taking into account the role of EGTA as a mitogenic inhibitor.\textsuperscript{35,36} However, the effects of the 3 maneuvers on \([1^H]\)thymidine incorporation were of small magnitude compared with the effect of 20% FBS. Taken together, these data suggest that the effects observed were more based on the promotion of cell survival rather than on stimulation of DNA synthesis; the latter probably was just a marginal, albeit statistically significant, phenomenon. In addition, the present data provide a sound basis for the importance of autocrine VEGF production as a survival mechanism under conditions of loss of integrity of the endothelial cell layer. A recent communication by Gerber et al\textsuperscript{37} has given support to the existence of an antiapoptotic role of VEGF on endothelial cells involving bcl-2 expression. Of special interest, the appearance of an augmented cell death in BAECS exposed to CytD in the presence of the VEGF-specific antibody uncovers the existence of CytD-related toxic effects on BAECS even after short exposures and strongly emphasizes the importance of VEGF as a cytoprotective mediator.

Our finding that endothelial cells have a small, but significant, basal VEGF mRNA expression are in agreement with reports from other groups using cultured endothelial cells of diverse origins.\textsuperscript{14,16} There is solid evidence demonstrating that VEGF production by endothelial cells is markedly stimulated in hypoxia and other hypoxia-like stimuli.\textsuperscript{38} These results indicate that autocrine and paracrine VEGFs may take part in the proliferation and death resistance of endothelial cells.\textsuperscript{15}

In summary, our results provide further insight in the mechanisms by which endothelial cells react to the absence of their normal cell-to-cell anchorage. The consequences of this phenomenon may be of considerable magnitude under in vivo conditions, and the present findings may be useful to our understanding of the pathways initiating endothelial growth in several situations of vascular injury, as well as in neangiogenesis. The triggering of autocrine VEGF expression on the disruption of interendothelial junctions may help explain the proliferative behavior of endothelial cells under conditions of loss of continuity of the monolayer, as occurs, eg, in angioplastic procedures.

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