VEGF Protects Against Oxidized LDL Toxicity to Endothelial Cells by an Intracellular Glutathione-Dependent Mechanism Through the KDR Receptor

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Abstract—Although the accumulation of vascular endothelial growth factor (VEGF) has been observed in human atherosclerotic lesions, the exact role of this growth factor in atherogenesis remains unknown. We hypothesized that VEGF in the vascular wall might have a preventive effect on endothelial cell damage during atherosclerosis. To test our hypothesis, we examined whether VEGF protects against the toxicity of oxidized low density lipoprotein (Ox-LDL) in cultured endothelial cells derived from bovine aortas (BAECs). Preincubation of BAECs with VEGF prevented Ox-LDL–induced toxicity in a preincubation time– and VEGF concentration– dependent manner. Addition of \( N\)-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, did not reverse the protective effect of VEGF on Ox-LDL toxicity. Incubation of BAECs with VEGF increased intracellular glutathione (GSH) content in a time-dependent manner. Combined addition of VEGF and L-buthionine sulfoximine, a GSH synthesis inhibitor, reversed both GSH levels and the protective effect of VEGF on Ox-LDL–induced cytotoxicity. Placenta growth factor, which ligates to the VEGF Flt-1 receptor but not KDR/Flk-1, failed to prevent Ox-LDL toxicity and had no effect on intracellular GSH levels. An anti-KDR antibody completely blocked these beneficial activities of VEGF. These results suggest that VEGF prevents Ox-LDL–induced endothelial cell damage via an intracellular GSH-dependent mechanism through the KDR/Flk-1 receptor. (Arterioscler Thromb Vasc Biol. 2001;21:765-770.)

Key Words: endothelial cells • atherosclerosis • oxidized LDL lipoproteins • vascular endothelial growth factor • glutathione

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a secreted protein that is a specific mitogen for endothelial cells.\(^1\)\(^,\)\(^2\) This growth factor is an angiogenic factor that has been shown to contribute to the development of the vascular system as well as various pathological process, including tumor growth,\(^3\) wound healing,\(^4\) and diabetic retinopathy.\(^5\) The action of VEGF is regulated by 2 receptors belonging to the tyrosine kinase family, Flt-1 and KDR/Flk-1, both of which are expressed on endothelial cells.\(^2\) Although both of these receptors are essential for the development of the embryonic vasculature, the biological responses mediated through Flt-1 and KDR/Flk-1 are different.\(^6\)\(^,\)\(^7\)

We have reported that vascular smooth muscle cells have the ability to produce VEGF.\(^8\) We recently also observed that oxidized LDL (Ox-LDL) induces VEGF expression and secretion from macrophage and that VEGF is present in human atherosclerotic lesions.\(^9\) In the early atherosclerotic lesion, VEGF is frequently observed in subendothelial macrophage-rich regions. In the atheromatous plaque, VEGF is detected in atheromatous core lesions consisting of lipid-filled macrophages, as well as in basal lesions of the plaque, which contain a predominant population of smooth muscle cells. Although the exact role of VEGF in atherosclerotic lesions remains unknown, a prominent association between VEGF and basal plaque regions rich in microvessels raises the possibility that VEGF may participate in neovascularization of basal atherosclerotic lesions. We previously hypothesized that VEGF in subendothelial macrophage-rich regions adjacent to endothelial cells at the luminal surface may participate in the maintenance and repair of the luminal endothelium.\(^9\)

Several lines of evidence have implicated Ox-LDL in the development and progression of atherosclerosis.\(^10\) Ox-LDL is undoubtedly present in atherosclerotic lesions,\(^11\) and it exhibits a variety of biological properties, including participation in foam cell formation,\(^12\) induction of various kinds of cytokines and growth factors from endothelial cells,\(^13\) and cooperation in a mitogenic effect on smooth muscle cells.\(^14\) In addition, Ox-LDL induces a dramatic cytotoxic effect on vascular endothelial cells.\(^15\)\(^-\)\(^17\)
To address our hypothesis that localized VEGF adjacent to the luminal surface of the vascular wall may participate in the maintenance of the luminal endothelium, we examined whether VEGF has a beneficial effect on the maintenance of vascular endothelial cells after their exposure to Ox-LDL. We report here that VEGF has a protective effect on endothelial cell damage resulting from Ox-LDL via an intracellular glutathione (GSH)-dependent mechanism through the VEGF KDR/Flk-1 receptor.

Methods

Cell Culture

BAECs were established in culture from the thoracic aortas of fetal calves as previously described. The cells were cultured in 10% calf serum plus Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical). Cells at passages 3 to 6 were used for all experiments.

Assessment of Endothelial Cell Damage

Cellular damage was assessed by measuring the amount of lactate dehydrogenase (LDH) released from the cells, as previously described. Confluent BAECs in 48-well plates were pretreated with or without VEGF or placenta growth factor (PIGF) for the indicated periods of time, rinsed with Dulbecco’s phosphate-buffered saline (DPBS) twice, and incubated in a total volume of 250 μL phenol red–free Eagle’s minimal essential medium (Nissui Pharmaceutical) containing Ox-LDL (80 μg/mL) for 10 hours at 37°C. LDH activity in the supernatant was determined spectrophotometrically using NADH oxidation by using a commercially available kit (LDH monotest, Boehringer Mannheim). Each LDH activity value was compared with that released either from Ox-LDL–untreated cells after the addition of minimal essential medium containing Triton X-100 at 0.5% (vol/vol) final concentration (percent of total LDH release) or from control cells treated with Ox-LDL (percent of control). To examine the effect of anti-VEGF receptor–blocking antibodies, indicated concentrations of the mAb (KM1992) were added to the culture 30 minutes before the addition of VEGF. An anti–sialyl Lewis A mAb (KM231) was used as the control mAb.

Measurement of Cellular GSH and GSH Manipulation

Total cellular glutathione (ie, GSH) in BAECs was determined by the method of Tietze as described by Akerboom and Sies by using the enzymatic recycling method with glutathione reductase, NADPH, and 5,5-dithiobis(2-nitrobenzoic acid). A standard curve was generated by using known amounts of glutathione disulfide instead of the sample. GSH manipulation in BAECs was achieved by incubating the cells in Dulbecco’s modified Eagle’s medium containing 10% calf serum with 10 or 100 μmol/L (S,R)-sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, for 24 hours. Treatment of BAECs with 10 or 100 μmol/L BSO for 24 hours reduced the GSH content to 59% and 41%, respectively.

Ox-LDL Preparation

LDL (d=1.019 to 1.063 g/mL) was separated from normal human plasma by preparative ultracentrifugation, dialyzed against PBS containing 0.3 mmol/L EDTA, sterilized by filtration through 0.45-μm Millipore membranes (Millipore), and stored at 4°C. EDTA was removed from LDL by dialysis against PBS before oxidation. LDL was oxidized by incubating 500 to 800 μg/mL LDL in 10 μmol/L CuSO4 for 16 hours at 37°C as described previously.19

Reagents

Nω-nitro-L-arginine methyl ester (L-NNAME), an L-arginine analogue; d-NNAME, a control d-isomer; glutathione reductase; glutathione disulfide; 5,5′-dithiobis(2-nitrobenzoic acid), and BSO were purchased from Sigma Chemical. NADPH was obtained from Boehringer Mannheim. Recombinant human VEGF (VEGF165) and human PIGF were obtained from Genzyme and R&D Systems, respectively. An anti–Flt-1 blocking mAb (KM1750) and an anti-KDR blocking mAb (KM1992) were prepared as previously described.

Statistical Methods

Values are given as mean±SD. Analysis was followed by post hoc testing (Scheffe’s test). A value of P<0.05 was considered significant.

Results

VEGF Attenuates Endothelial Cell Damage Induced by Ox-LDL

Treatment of BAECs with Ox-LDL (80 μg/mL) for 10 hours caused a pronounced increase in toxicity as measured by LDH release. When BAECs were preincubated in 30 ng/mL VEGF for the indicated periods, rinsed with DPBS, and then exposed to Ox-LDL for 10 hours, LDH release from BAECs was attenuated in a preincubation time–dependent manner (Figure 1A). The effect was already observed with as little as 8 hours of preincubation with VEGF. When BAECs were pretreated for 24 hours with the indicated concentrations of VEGF, LDH release induced by Ox-LDL was prevented in a concentration-dependent manner (Figure 1B). Pretreatment with 30 ng/mL VEGF for 24 hours reduced LDH release by 45% compared with control. These results suggested that VEGF enhances the resistance of BAECs against Ox-LDL. When BAECs were pretreated with PIGF, which is known to...
be another ligand for the VEGF receptor Flt-1, for 24 hours, no difference in LDH release arising from Ox-LDL was observed compared with untreated control BAECs (Figure 2).

To obtain more direct evidence that the VEGF receptor, Flt-1 or KDR/Flk-1, is really involved in the protective effect of VEGF against Ox-LDL, BAECs were pretreated with VEGF in the presence of an anti-VEGF receptor–blocking mAb. Although anti–Flt-1 blocking mAb (KM1750) did not neutralize the effect of VEGF on Ox-LDL toxicity up to 100 μg/mL, which was 100-fold higher than the previously observed concentration that completely blocked the VEGF effect through Flt-1 on human endothelial cells, anti-KDR blocking mAb (KM1992) inhibited VEGF effect in a concentration-dependent manner (Figure 2). A control mAb had no effect up to 300 μg/mL.

**Effect of NO on VEGF Action**

The role of nitric oxide (NO) in the protective effect of VEGF on Ox-LDL–induced toxicity to endothelial cells was assessed on BAECs treated with L-NAME, an NO synthase inhibitor, or the control compound D-NAME. When BAECs were pretreated with a combination of VEGF and either L-NAME or D-NAME for 24 hours, rinsed with DPBS, and exposed to Ox-LDL, there was no difference in LDH release among cultures under these conditions (Figure 3A), suggesting that BAECs did not obtain the phenotypic changes through NO during their incubation with VEGF. When BAECs pretreated with VEGF for 24 hours were exposed to Ox-LDL in the presence of L-NAME, again no effect was observed on LDH release compared with that of control as well as BAECs treated with D-NAME (Figure 3B), indicating that the enhanced resistance of BAECs against Ox-LDL by VEGF pretreatment is not due to an increase in NO production via NO synthase.

**Effect of Intracellular GSH**

Previously, we had reported that intracellular GSH plays an important role in the defense of BAECs against Ox-LDL and that the mechanism of Ox-LDL toxicity is related to the depletion of intracellular GSH. Therefore, we next investigated the contribution of intracellular GSH on the VEGF-mediated protection against Ox-LDL toxicity in endothelial cells. The total intracellular GSH level of control BAECs was 2.53 ± 0.37 nmol/mg protein (n = 4). As shown in Figure 4A, VEGF increased intracellular GSH levels in a time-dependent manner between 12 and 48 hours. Conversely, incubation with PIGF for 24 hours had no effect on GSH levels (Figure 4A). Although the anti–Flt-1 mAb (KM1750) and the control mAb had no effect on the VEGF-induced increase in GSH level, the anti-KDR mAb (KM1992) blocked the increase in GSH level in a concentration-dependent manner (Figure 4B). To evaluate the involvement of the increase in intracellular GSH...
GSH levels in the susceptibility of VEGF-treated BAECs to damage resulting from Ox-LDL, the GSH level was manipulated by treating the cells with BSO, an inhibitor of γ-glutamylcysteine synthetase. Incubation of BAECs with VEGF in combination with BSO for 24 hours resulted in a reduction of GSH content in a concentration-dependent manner compared with VEGF alone (Figure 5A). BSO exposure also reversed the protective effect of VEGF pretreatment on Ox-LDL cytotoxicity (Figure 5B).

**Discussion**

In the present study, we have demonstrated that pretreatment of BAECs with VEGF reduced the toxicity of Ox-LDL to BAECs. This effect is dependent on both VEGF incubation time and VEGF concentration. It should be noted that when BAECs were exposed to Ox-LDL in the presence of VEGF but not during preincubation, VEGF also attenuated Ox-LDL–induced toxicity, but the effect was much less (18% reduction of LDH release compared with control) than that with VEGF preincubation. It has been demonstrated that some effects of VEGF, including vascular permeability,22 vasodilation,23 angiogenesis,24 and endothelial cell proliferation,25 are coupled to NO production. However, the preventive effect of VEGF pretreatment is not mediated by NO, because L-NAME did not attenuate the effect of VEGF preincubation on Ox-LDL–induced toxicity.

To date, it is not yet fully understood which of the VEGF receptors, Flt-1 or KDR/Flk-1, is responsible for transmission of the diverse biological responses of VEGF. KDR/Flk-1 displays ligand-dependent phosphorylation in endothelial cells and mediates mitogenesis and chemotaxis in KDR/Flk-1–transfected cells.26,27 In addition, this VEGF receptor has been shown to be involved in NO and prostacyclin production.28 Flt-1 shows minimal tyrosine phosphorylation in response to VEGF, and binding of VEGF does not lead to significant biological roles in transfected cells.26,27 In contrast, others have reported that activation of Flt-1 is correlated with monocyte/macrophage migration and the regulation of embryonic vasculo genesis.7,29 It has also been proposed that the extracellular domain of Flt-1 acts as a negative regulator of VEGF.30 Recent observations with the use of an anti-VEGF receptor–blocking mAb showed that VEGF-induced endothelial cell DNA synthesis is preferentially mediated by KDR/Flk-1 and that Flt-1 regulates cell migration by modulating actin reorganization. KDR also influences cell migration by regulating cell adhesion.20

In the present study, we showed that PIGF, which ligates Flt-1 but not KDR/Flk-1,21 did not affect the toxicity of Ox-LDL. In addition, an anti-KDR mAb blocked the protective effect of VEGF on Ox-LDL–induced cytotoxicity, but an anti–Flt-1 mAb and a control mAb did not. These results indicate the involvement of KDR/Flk-1 in VEGF-induced phenotypic changes in BAECs’ obtaining resistance against Ox-LDL toxicity.

We showed that VEGF increased intracellular GSH in a time-dependent manner. The exposure of BAECs to PIGF failed to elicit an accumulation of GSH, indicating that Flt-1 does not participate in mediating the action of VEGF on BAECs. The anti-KDR mAb suppressed the increase in GSH induced by VEGF, but an anti–Flt-1 mAb and a control mAb did not, supporting the contribution of KDR/Flk-1 to intracellular GSH accumulation in response to...
VEGF. The important role of increased GSH levels in the preventive effect of VEGF treatment is based on the observation that combined treatment with VEGF and BSO, an inhibitor of γ-glutamylcysteine synthetase, reversed the increase in intracellular GSH content and the resistance to Ox-LDL toxicity in endothelial cells. The result is consistent with our previous finding that intracellular GSH levels regulate the susceptibility to Ox-LDL–induced toxicity in endothelial cells.16 These results suggest that GSH-dependent mechanisms in the protective effect of VEGF against Ox-LDL toxicity in endothelial cells are mediated through the VEGF KDR/Flk-1 receptor but not through the Flt-1 receptor. The KDR/Flk-1 receptor mediates many important biological activities, such as VEGF-induced endothelial cell proliferation,26,27 vasculogenesis,6 and NO synthesis.28 The present data provide evidence that the Flk-1/KDR receptor also plays a role in the regulation of intracellular GSH content.

It has been proposed that VEGF acts as a vascular protective factor.31 VEGF is likely able to augment several endothelial functions, including inhibition of vascular smooth muscle hyperplasia,31,32 platelet aggregation, thrombus formation, and leukocyte recruitment to blood vessels.31 NO and prostacyclin production have been reported to be implicated in VEGF-dependent, endothelium-mediated protective effects on the vasculature.31 We propose that endothelial cell GSH content is another key mediator in the vasculoprotective function of VEGF.

We and others have found VEGF in human atherosclerotic lesions.9,33 VEGF in regions rich in microvessels may act as an angiogenic factor. VEGF that is localized in regions adjacent to the luminal surface may serve a repair function after focal endothelial defects, because VEGF is a potent mitogen for vascular endothelial cells.1,2 In the present study, we provided the additional possibility that VEGF may act as an inhibitor of Ox-LDL toxicity to endothelial cells. We demonstrated that this effect is mediated by intracellular GSH-dependent mechanisms via the VEGF receptor KDR/Flk-1. The toxic effect of Ox-LDL on vascular endothelial cells seems to induce focal defects of the integrity of the endothelial cell lining, which are associated with an increase in local permeability, platelet adhesion, and thrombogenicity. The preventive effect of VEGF on Ox-LDL toxicity in endothelial cells may play an important role in the maintenance of luminal endothelial cell morphology as well as endothelial cell function, which may be beneficial in atherogenesis.

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References


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