Endothelial cell survival and antiapoptotic pathways, including those stimulated by extracellular matrix, are critical regulators of vasculogenesis, angiogenesis, endothelial repair, and shear-stress-induced endothelial activation. One of these pathways is mediated by αvβ3 integrin ligation, downstream activation of nuclear factor-κB, and subsequent up-regulation of osteoprotegerin (OPG). In this study, the mechanism by which OPG protects endothelial cells from death was examined. Serum-starved human microvascular endothelial cells (HMECs) plated on the αvβ3 ligand osteopontin were protected from cell death. Immunoprecipitation experiments indicated that OPG formed a complex with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in HMECs under these conditions. Furthermore, inhibitors of TRAIL, including recombinant soluble TRAIL receptors and a neutralizing antibody against TRAIL, blocked apoptosis of serum-starved HMECs plated on the nonintegrin attachment factor poly-D-lysine. Whereas TRAIL was unable to induce apoptosis in HMECs plated on osteopontin, the addition of recombinant TRAIL did increase the percentage of apoptotic HMECs plated on poly-D-lysine. This evidence indicates that OPG blocks endothelial cell apoptosis through binding TRAIL and preventing its interaction with death-inducing TRAIL-receptors.

INTRODUCTION

Endothelial apoptosis is an important regulator of angiogenesis, vasculogenesis, vascular pruning, and shear stress-induced endothelial activation (Dimmeler et al., 1996; Dimmeler and Zeiher, 2000). Angiogenesis, the formation of capillaries from preexistent blood vessels is an essential process in development, reproduction, and tissue repair but also occurs in the adult under pathological conditions such as ischemic disease, arthritis, and the growth of solid tumors. Many angiogenic factors, including vascular endothelial growth factor, angiopoietin-1, and basic fibroblast growth factor, act in part by promoting endothelial cell survival or inhibiting endothelial cell apoptosis (Alon et al., 1995; Karsan et al., 1997; Hayes et al., 1999; Kwak et al., 1999). The interaction of endothelial cells and extracellular matrix through integrins also has been found to be important for cell survival (Meredith et al., 1993). The ligation of αvβ3 integrin has been implicated in angiogenesis because studies using neutralizing antibodies or cyclic peptide antagonists induced endothelial cell apoptosis and thereby blocked angiogenesis (Brooks et al., 1994a,b; Friedlander et al., 1996). A potential mechanism for αvβ3-mediated survival in endothelial cells was identified using rat aortic endothelial cells (RAECs) (Scatena et al., 1998). In that study, the αvβ3 ligand osteopontin (OPN) protected RAECs from serum deprivation-induced apoptosis by activating a nuclear factor-κB (NF-κB)-dependent survival pathway. NF-κB-dependent, antiapoptotic genes in RAECs were subsequently identified using subtractive hybridization (Malyankar et al., 2000). Osteoprotegerin (OPG) was identified as one of the induced genes and was shown to have increased mRNA and protein levels in RAECs plated on OPN. The addition of recombinant OPG to RAECs with inactive NF-κB prevented apoptosis in a dose-dependent manner, thus implicating OPG as a downstream mediator of αvβ3-mediated survival.

OPG is a secreted glycoprotein that exists as both a 60-kDa monomer and a 120-kDa disulfide-linked dimer and is a soluble member of the tumor necrosis factor (TNF) receptor superfamily (Simonet et al., 1997). In bone, OPG inhibits osteoclastogenesis by binding receptor activator of nuclear factor-κB ligand (RANKL) and thereby prevents the interaction of receptor activator of nuclear factor-κB (RANK) and RANKL (Lacey et al., 1998; Yasuda et al., 1998). Consistent with this, transgenic mice overexpressing OPG have decreased numbers of osteoclasts and a corresponding increase in the amount of bone (Simonet et al., 1997). Likewise, OPG-deficient mice have decreased bone density (Bucay et al., 1998; Mizonu et al., 1998; Yun et al., 2001). OPG also was found to regulate B cell maturation and development; populations of peripheral B cells are elevated in OPG null mice and OPG null dendritic cells (ex vivo) have an increased ability to stimulate T cells (Yun et al., 2001). Most relevant to the present studies, OPG has been implicated as a mediator of cell survival. Indeed, OPG has been shown to bind TNF-related apoptosis-inducing ligand (TRAIL) and thereby inhibit TRAIL-induced apoptosis of Jurkat cells (Emery et al., 1998).

In the present study, we investigated the mechanism by which OPG acts as a survival factor in endothelial cells. Specifically, we tested the hypothesis that OPG binds TRAIL and thereby prevents apoptosis of serum-starved human microvascular endothelial cells (HMECs). Our studies suggest that OPG inhibits serum starvation-induced endothelial apoptosis by binding TRAIL and preventing TRAIL recep-
tor-induced death. Furthermore, the studies suggest that endothelial cells are sensitized to TRAIL-induced death by serum and adhesion deprivation.

MATERIALS AND METHODS

Materials

Human dermal microvascular endothelial cells and EGM-2-MV media were purchased from Cambrex Bio Science Walkersville (Walkersville, MD). Recombinant rat OPN was prepared as described previously (Martin et al., 2003). Antibodies against OPG and TRAIL and the soluble TRAIL-R1 and TRAIL-R2 fusion proteins were purchased from R&D Systems (Minneapolis, MN). Recombinant TRAIL was purchased from Upstate Biotechnology (Waltham, MA). Polyvinylidene difluoride membranes and Renaissance chemiluminescence reagents were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Zetaprobe GT membranes were purchased from Bio-Rad (Hercules, CA). The enzyme-linked immunosorbent assay (ELISA) kit was purchased from Pierce Chemical (Rockford, IL).

Hoechst Staining

Cells were plated on four-well chamber slides at ~75% confluence. A 1 in 10 dilution was made from a concentrated stock of Hoechst dye (4 mg/ml). Fifty microliters of the diluted dye was added to each well to make a final concentration of 4 μg/ml in the media. The dye was incubated on the cells for 30 min at 37°C. The media were then removed, the cells rinsed with phosphate-buffered saline (PBS) and fixed for 10 min in 4% paraformaldehyde. The cells were then washed three times with PBS, coverslipped with Vectashield mounting media, and sealed with varnish. The slides were then examined by fluorescence microscopy for punctate nuclear staining and rounded nuclei indicative of apoptosis.

Northern Blot Analysis

Total RNA was isolated from HMECs plated on poly-D-lysine (PDL) and OPN for 3 and 6 h. Northern blot analysis was carried out by electrophoretic separation of 12.5 μg of total RNA by using formaldehyde-agarose gels and subsequent transfer to a Zetaprobe GT membrane. The OPG cDNA insert was labeled using the Multiprime kit (Amersham Biosciences, Piscataway, NJ) and [α-32P]dCTP, whereas the 18S probe was end-labeled using T4 kinase (New England Biolabs, Beverly, MA) and [α-32P]dATP; hybridization was performed as described previously (Giachelli et al., 1991).

OPG ELISA

Media from HMEC plated on PDL or OPN for 3, 6, 12, and 24 h was collected and concentrated 20-fold by using Microcon filters (10-kDa molecular weight cutoff) (Amicon, Beverly, MA). A volume of 50 μl of concentrated media was added to each well, and the plate was incubated overnight at 4°C. Substrate and conjugate were added according to the manufacturer’s instructions, and the absorbance was read at 450 nm. OPG concentrations were determined by interpolation from a standard curve generated with recombinant human OPG.

Immunoprecipitation

HMECs were plated on OPN or PDL coated plates for 24 h. The cells were then lysed in an immunoprecipitation (IP) buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5% NP-40, 0.5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride. Media also were collected, the cells spun down at 15,000 rpm for 5 min, and added to 10× IP buffer. Antibody (monoclonal anti-TRAIL) or mouse IgG was immobilized onto agarose gel by using sodium cyanoxyrrolyxide as directed by the Seize primary immunoprecipitation kit instructions. The antibody conjugate was washed and stored overnight. Immunoprecipitation was carried out in IP buffer; samples were rotated overnight at 4°C. Samples were washed four times with 500 μl of buffer containing 0.025 M Tris, pH 7.2, and 0.15 M NaCl and eluted three times in 50 μl of Immunopure IgG elution buffer. Then, 12.5 μl of 5X sample buffer and 4 μl of 1 M dithiothreitol were added to each sample before boiling for 5 min followed by separation on 12.5% SDS-PAGE gels.

Western Blot Analysis

SDS-PAGE (12.5%) gels were run and subsequently transferred to polyvinylidene difluoride membranes. The membranes were then blocked in 10% milk in Tris-buffered saline Tween 20 overnight at 4°C. Primary antibodies were added for 2 h at room temperature followed by the appropriate horseshad peroxidase-conjugated secondary antibody and then exposed to chemiluminescence reagents for 1 min, and bands were detected by exposing the membrane to x-ray film.

RESULTS

Osteopontin Promotes the Survival of HMECs in the Absence of Serum

Previous studies have demonstrated that OPN protects RAECs from serum deprivation-induced apoptosis through its interaction with the αvβ3 integrin and the subsequent activation of NF-κB (Scatena et al., 1998). OPG was subsequently identified as an NF-κB–dependent survival factor for the RAECs (Malyankar et al., 2000). To determine whether HMECs also survived on osteopontin through the production of OPG, HMECs were plated on PDL and OPN for 3, 6, 12, and 24 h. HMECs bind to PDL through charge interactions, but cell surface integrins are not engaged. Therefore, consistent with studies in RAECs, OPG promotes the survival of HMECs in the absence of growth factors.

OPG Promotes OPG Synthesis and Secretion

To determine whether OPG promotes the survival of HMECs through the production of OPG, cells were plated on PDL and OPN for 3 and 6 h, and RNA was isolated. Northern blot analysis was carried out using a probe for OPG (Figure 2A). Increased OPG RNA was detected from
HMECs plated on OPN at 3 h, and a further increase was found at 6 h. This increase in OPG RNA suggests that OPG may act to promote the survival of these cells in the absence of growth factors.

Because OPG functions as an extracellular molecule, we investigated the amount of OPG secreted into the media at 3, 6, 12, and 24 h of incubation on OPN. The media were collected and concentrated 20-fold by using Microcon filters. HMECs plated on OPN secreted two times more OPG into the media as cells plated on PDL (Figure 2B). Using the standard curve to interpolate the absorbance of the samples at 24 h, it was found that HMEC plated on OPN secreted 5.7 pmol/l OPG compared with 2.4 pmol/l on PDL. Although the levels of OPG are increased in HMECs plated on PDL at 24 h, these levels do not seem to be protective. Several explanations are possible; the increased levels of OPG may be too late to inhibit the progression of apoptosis or alternately OPN may initiate other synergistic survival pathways not induced on PDL.

**Recombinant OPG Prevents Apoptosis of HMECs Plated on PDL in the Absence of Serum**

Recombinant OPG inhibits RAEC apoptosis when NF-κB is inactive (Malyankar et al., 2000). HMECs were plated on PDL for 10 h in the presence of OPG-Fc at concentrations ranging from 0 to 5 μg/ml and on OPN (Figure 3). Apoptotic nuclei were stained and counted as described previously, and the percentage of apoptotic cells was calculated. At 0.5 μg/ml, the percentage of apoptotic cells was reduced by one-half, and this was further decreased at higher concentrations of OPG-Fc. Thus, OPG-Fc inhibits apoptosis of HMECs in a dose-dependent manner.

**TRAIL and OPG Are Coimmunoprecipitated by a Monoclonal Antibody (mAb) against TRAIL**

Because OPG has been found to bind to TRAIL and inhibit the apoptosis of Jurkat cells (Emery et al., 1998), we were interested in determining whether OPG bound TRAIL in HMECs and thereby inhibited serum-induced apoptosis. The amount of TRAIL protein was measured by Western blot in HMECs plated on OPN compared with HMECs plated on PDL and was not found to be changed (our unpublished data), demonstrating that only OPG is up-regulated by OPN adhesion. FACS analysis using an anti-TRAIL antibody also indicated that no differences existed in the amount of cell surface TRAIL between surviving HMECs (plated on OPN) and apoptotic HMECs (plated on BSA) (our unpublished data).

To isolate the complex of OPG and TRAIL, HMECs were plated on OPN for 16 h, and the lysates were collected in an IP buffer. A mAb against TRAIL was cross-linked to agarose gel by using sodium cyanoborohydride. This antibody conjugate was then incubated with the cell lysates at 4°C overnight and subsequently washed with IP buffer. The proteins were eluted off the agarose gel in three fractions. Each fraction was loaded separately onto a 12.5% SDS-PAGE gel and then analyzed by Western blot. Recombinant TRAIL and OPG were loaded as positive controls. This result indicated that TRAIL and OPG form a complex in the lysates of
HMECs plated on OPN, which can be immunoprecipitated using an anti-TRAIL antibody (Figure 4).

**Soluble TRAIL-Receptors and Anti-TRAIL Neutralizing Antibody Inhibit HMEC Apoptosis Induced by the Absence of Serum**

Because OPG, a TRAIL-binding molecule, inhibited the apoptosis of HMECs due to serum deprivation, we used two different approaches to investigate the role of TRAIL in HMEC apoptosis. First, the effect of recombinant soluble TRAIL receptor-Fc fusion proteins for TRAIL-R1 and TRAIL-R2 (designated TR1-Fc and TR2-Fc respectively) was investigated. HMECs were plated on PDL for 18 h; TR1-Fc and TR2-Fc were added at 0, 5, and 10 ng/ml to cells plated on PDL (Figure 5A). Both TR1-Fc and TR2-Fc inhibited serum-induced apoptosis of HMECs. Treating HMEC with a combination of TR1-Fc and TR2-Fc did not result in increased inhibition (Figure 5B).

Next, the effect of a neutralizing anti-TRAIL antibody on HMEC apoptosis was determined. HMECs were plated on PDL for 16 h in the presence of anti-TRAIL or mouse IgG at 0, 50, and 100 ng/ml. The addition of an anti-TRAIL neutralizing antibody to HMECs plated on PDL for 16 h decreased the percentage of apoptotic cells (Figure 5C). Although no difference in apoptosis was noted at 50 ng/ml, the percentage of apoptotic cells decreased from 7.3 to 1.9% at 100 ng/ml. Therefore, TRAIL is at least partly responsible for the apoptosis of HMECs plated on PDL.

**Serum Deprivation Combined with Loss of α₅β₃ Signaling Sensitizes HMECs to Death Induced by Exogenous TRAIL**

The addition of recombinant TRAIL induces apoptosis in a number of cell lines but was thought to have no effect on normal cells (reviewed in Degli-Esposti, 1999). However, recent studies have questioned this assumption because TRAIL induced apoptosis in normal human hepatocytes (Jo et al., 2000), keratinocytes (Leverkus et al., 2000), and in human brain slices (damage was noted in neurons, oligodendrocytes, astrocytes, and microglial cells) (Nitsch et al., 2000). In addition, several studies have found that TRAIL can induce apoptosis in endothelial cells; in one study HMECs and human umbilical vein endothelial cells (HUVECs) were resistant to TRAIL-induced apoptosis unless the glycosyl-phosphatidylinositol (GPI)-linked decoy receptor TRAIL-R3 was removed through pretreatment with phospholipase C (Sheridan et al., 1997). A second study was unable to find TRAIL expression in HUVECs but found a small increase (10–20%) in cell lysis in HUVECs that had been exposed to TRAIL overnight (Gochuico et al., 2000). An additional study indicated that both HUVECs and HMECs are susceptible to TRAIL-induced apoptosis and that cell death was increased by cotreatment with cycloheximide (Li et al., 2003). Thus, under some conditions, endothelial cells may become sensitized to death induced by TRAIL.

To determine whether TRAIL could increase the rate of apoptosis; HMECs were plated on PDL and 200 ng/ml recombinant human TRAIL was added 30 min later. The
DISCUSSION

In the present study, recombinant OPG promoted the survival of HMECs under conditions of serum deprivation, and HMECs plated on the extracellular matrix protein OPN had increased OPG RNA and protein secretion into the media. These results confirm our previous observations that OPG is up-regulated in response to adhesion on OPN and promotes survival in RAECs (Malyankar et al., 2000) and extends these findings to primary HMECs. OPG has been found to neutralize the cell death mediator TRAIL (Emery et al., 1998), making it a logical candidate in our system. Indeed, immunoprecipitation of OPG with an anti-TRAIL mAb demonstrated that OPG and TRAIL form a complex in the lysate of HMECs plated on OPN. To further test the hypothesis that TRAIL was mediating HMEC cell death, we specifically neutralized TRAIL with soluble TR1-Fc and TR2-Fc molecules and with a specific TRAIL neutralizing antibody. We found that these molecules were able to prevent apoptosis. Finally, we were able to show that TRAIL enhanced apoptosis of HMECs plated on PDL. These findings indicate that OPG protects HMECs against serum starvation-induced cell death, in part, by binding TRAIL and blocking TRAIL-R induced apoptosis.

TRAIL is a type II transmembrane protein with a molecular mass of 33 kDa and is a member of the tumor necrosis family of ligands (Wiley et al., 1995). TRAIL shares the highest homology with FasL with 28% amino acid identity at the C-terminal sequence. There are five TRAIL receptors, including OPG. Two receptors, TRAIL-R1 and TRAIL-R2 (Pan et al., 1997; Schneider et al., 1997; Walczak et al., 1997), contain sequences homologous to the death domains of Fas and tumor necrosis factor receptor-1 in their cytoplasmic regions and are able to induce apoptosis via caspase activation pathways. TRAIL-R3, which lacks a cytoplasmic domain and is linked to the cell membrane through a glycosphospholipid anchor (Pan et al., 1997; Schneider et al., 1997; Sheridan et al., 1997), and TRAIL-R4, which contains a truncated death domain (Degli-Esposti et al., 1997; Pan et al., 1998), are considered decoy receptors for TRAIL along with OPG. Both OPG and TRAIL have been implicated in vascular pathology; OPG expression was increased in vascular smooth muscle after balloon injury (Zhang et al., 2002), and the gene for TRAIL was associated with endothelial apoptosis in thrombotic thrombocytopenic purpura (Kim et al., 2001).

TRAIL has emerged as a cytotoxic factor for a variety of transformed cells, but it was originally not found to induce death in normal cells (Wiley et al., 1995; Pan et al., 1997). However, recent studies have shown that normal hepatocytes and keratinocytes are susceptible to specific versions of recombinant TRAIL (Jo et al., 2000; Leverkus et al., 2000; Lawrence et al., 2001; Qin et al., 2001). The level of expression of each TRAIL receptor has been proposed as the mechanism by which cells may be protected from TRAIL-mediated cytotoxicity, but this may be cell type dependent. For example, in various melanoma cell lines little correlation was found between expression of death-inducing receptors (TRAIL-R1 and TRAIL-R2) or decoy receptors (TRAIL-R3 and TRAIL-R4) and relative susceptibilities to TRAIL (Zhang et al., 1999). However, another study in endothelial cells, considered to be resistant to TRAIL-mediated cytotoxicity, found that expression of TRAIL-R3 decoy receptor seems to be important in protection against TRAIL-induced apoptosis. Indeed, removal of the GPI-linked TRAIL-R3 receptor sensitized HUVECs to TRAIL (Sheridan et al., 1997; Zhang et al., 2000). Moreover, HUVECs that were treated

**Figure 6.** TRAIL enhances apoptosis of HMECs plated on PDL. HMECs were plated on PDL and recombinant TRAIL was added 30 min later at a concentration of 200 ng/ml. The percentage of apoptotic cells was determined at 3, 6, 10, and 24 h. Treatment of cells plated on PDL with TRAIL resulted in a two- to threefold increase in the percentage of apoptotic cells (Figure 6). HMECs also were plated on OPN and on PDL in the absence of TRAIL. TRAIL has no effect on the apoptotic rate of HMEC plated on OPN (our unpublished data). The results shown are representative of three independent experiments; error bars indicate standard deviations for triplicate determinations (analysis of variance, p < 0.0001).

TRAIL-R Expression on the Cell Surface Is Unchanged in HMECs under Conditions of Survival

Expression of TRAIL-R has been suggested to regulate the susceptibility of cells to TRAIL-induced apoptosis (Sheridan et al., 1997). However, a number of studies have challenged such a correlation (Zhang et al., 1999). To determine whether modulation of TRAIL-R levels could explain the enhanced susceptibility of HMECs on PDL, HMECs were plated under conditions of apoptosis (cells kept in suspension by plating on BSA) and survival (OPN) for 4 h, trypsinized, and incubated with anti-TRAIL-R antibodies. The cells were then incubated with an FITC-labeled secondary antibody and analyzed by flow cytometry (Figure 7). The peak fluorescence intensity was calculated for each TRAIL-R and the values are shown in Table 1. FACS analysis of cell surface TRAIL-R demonstrated that the amount of TRAIL-R expression on the surface of surviving (plated on OPN) versus apoptotic (plated on BSA) cells was unchanged. Western blot analysis of HMEC lysates plated on PDL or OPN for 3, 6, 12, and 24 h confirmed that there were no changes in TRAIL-R expression (our unpublished data). Thus, modulation of TRAIL-R on the surface of HMECs does not explain the enhanced susceptibility of these cells to exogenous TRAIL-induced death under conditions of serum and adhesion deprivation. Furthermore, these results confirm that up-regulation of OPG is the only change that occurs in the expression of the TRAIL-R as a result of adhesion to OPN.
Figure 7. TRAIL-receptor expression on the cell surface is unchanged in HMECs plated on BSA and OPN. The expression of TRAIL-R on the cell surface was examined under conditions of survival (OPN) and apoptosis (cells kept in suspension by plating on BSA). HMECs were plated on OPN or BSA for 4 h and resuspended in a buffer containing 10 μg/ml goat IgG or 10 μg/ml antibody against one of TRAIL-R1, TRAIL-R2, TRAIL-R3, or TRAIL-R4. The cells were then incubated with a FITC-labeled anti-goat antibody and analyzed by flow cytometry. No changes were found in the expression of TRAIL-R between HMECs plated on BSA and those plated on OPN. Results are representative of three independent experiments.
The expression of TRAIL-R on the cell surface was examined under conditions of survival (OPN) and apoptosis (cells kept in suspension by plating on BSA). FACS analysis of HMEC-plated on OPN or BSA and subsequently resuspended in a buffer containing 10 µg/ml goat IgG or 10 µg/ml of antibody against one of TRAIL-R1, TRAIL-R2, TRAIL-R3, or TRAIL-R4 is shown in Figure 7. Linear fluorescence intensity (LFI) and peak fluorescence intensity values were then calculated. No changes were found in the expression of TRAIL-R between HMECs plated on OPN and those plated on BSA.

<table>
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<tr>
<th>Antibody</th>
<th>Mean</th>
<th>Linear fluorescence intensity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peak fluorescence intensity&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>BSA IgG</td>
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<td>1.3</td>
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<sup>a</sup> Linear fluorescence intensity = 2<sup>(mean/17)</sup>.<br>
<sup>b</sup> Linear fluorescence intensity TRAIL-R(1-4)/linear fluorescence intensity IgG.

In conclusion, we have found that OPG acts as a survival factor for HMECs plated on OPN in the absence of serum due to its ability of bind and block TRAIL-induced apoptosis. The mechanism by which TRAIL induces apoptosis is not fully understood, but it is thought to be mediated by one or more downstream molecules that result in caspase-8 and caspase-3 activation. These downstream molecules and events are also likely to be regulated because the susceptibility of HMECs to TRAIL does not seem to be solely determined by TRAIL-R expression. The interaction of TRAIL and OPG in endothelial cells under conditions of serum deprivation may represent a mechanism of survival that occurs under ischemic conditions. Further elucidation of the function of these two molecules and the downstream effects of their interaction will result in a more complete understanding of endothelial cell survival and angiogenesis under pathological conditions.

REFERENCES


