The Role of Osteoprotegerin and Tumor Necrosis Factor-related Apoptosis-inducing Ligand in Human Microvascular Endothelial Cell Survival

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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 $\alpha_v\beta_3$ integrin ligation, downstream activation of nuclear factor- κ B, 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 $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ -mediated survival in endothelial cells was identified using rat aortic endothelial cells (RAECs) (Scatena *et al.*, 1998). In that study, the $\alpha_v\beta_3$ ligand osteopontin (OPN) protected RAECs from serum deprivation-induced apoptosis by activating a nuclear factor- κ B (NF- κ B)-dependent survival pathway. NF- κ B-dependent, antiapoptotic genes in RAECs were subsequently identified using subtractive hybridization (Malyankar *et al.*, 2000). Os-

teoprotegerin (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- κ B prevented apoptosis in a dose-dependent manner, thus implicating OPG as a downstream mediator of $\alpha_v\beta_3$ -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- κ B ligand (RANKL) and thereby prevents the interaction of receptor activator of nuclear factor- κ B (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; Mizuno *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-

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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 Biomedica Gruppe (Southbridge, MA) and the Seize primary immunoprecipitation kit was purchased from Pierce Chemical (Rockford, IL).

Hoechst Staining

Cells were plated onto 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 μ g/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 μ g 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 [γ -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 μ l 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 μ g/ml leupeptin, 10 μ g/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 $\times$ IP buffer. Antibody (monoclonal anti-TRAIL) or mouse IgG was immobilized onto agarose gel by using sodium cyanoborohydride 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 μ l of buffer containing 0.025 M Tris, pH 7.2, and 0.15 M NaCl and eluted three times in 50 μ l of Immunopure IgG elution buffer. Then, 12.5 μ l of 5 $\times$ sample buffer and 4 μ l 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 horseradish peroxidase-conjugated secondary at a dilution of 1:2500. Membranes were exposed to chemiluminescence reagents for 1 min, and bands were detected by exposing the membrane to x-ray film.

Figure 1. HMEC survival is increased in cells plated on OPN compared with PDL. HMECs were plated on either OPN or PDL in the absence of serum for 3, 6, 12, or 24 h. Nuclei were then stained with Hoechst dye, and the number of apoptotic cells was determined in three fields at 40 $\times$. The percentage of apoptotic cells was then calculated. Cells were considered to be apoptotic on the basis of nuclear condensation and fragmentation. The experiment was repeated three times, and error bars indicate standard deviations for triplicate determinations (analysis of variance, $p < 0.0001$).

Fluorescence-activated Cell Sorting (FACS) Analysis

HMECs were plated on bovine serum albumin (BSA) or OPN for 4 h, trypsinized, and resuspended in PBS/0.2% BSA/0.02% NaN₃ containing 10 μ g/ml anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3, or anti-TRAIL-R4 antibody or 10 μ g/ml goat IgG. The cells were incubated on ice for 30 min and subsequently incubated for 30 min on ice with a 1:50 dilution of a fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat secondary antibody. The cells were then analyzed using flow cytometry at the Cell Analysis Facility (Department of Immunology, University of Washington).

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 $\alpha_v\beta_3$ integrin and the subsequent activation of NF- κ B (Scatena *et al.*, 1998). OPG was subsequently identified as an NF- κ B-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-charge interactions, but cell surface integrins are not engaged, resulting in no NF- κ B activation. HMECs were then stained with Hoechst, and the number of apoptotic cells was determined (Figure 1). At 3 h, 18% of the HMECs plated on PDL were apoptotic (compared with 1.6% plated on OPN). This increased to 49.1% by 24 h, whereas only 8.6% of HMECs plated on OPN were apoptotic. Therefore, consistent with studies in RAECs, OPN promotes the survival of HMECs in the absence of growth factors.

OPN Promotes OPG Synthesis and Secretion

To determine whether OPN 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

Figure 2. OPG RNA and secretion into the media are increased in HMECs plated on OPN. (A) HMECs were plated on either OPN or PDL for 3 and 6 h and the RNA isolated. The samples were run on an agarose gel, and Northern blot analysis was performed. OPG RNA from HMECs plated on OPN was increased at 3 h and was further increased at 6 h. (B) HMECs were plated on either OPN or PDL for 3, 6, 12, or 24 h, and the media were collected and stored at -20°C . The media were concentrated 20-fold by using Microcon

filters, and the amount of OPG was quantitated using a sandwich ELISA. A twofold increase in OPG was found in HMECs plated on OPN at 12 and 24 h. Results are representative of three independent experiments; error bars represent standard deviations (analysis of variance, $p < 0.0001$; three replicates).

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 alternatively 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- κ B 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 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$, the percentage of apoptotic cells was reduced by one-half, and this was further decreased at higher concen-

trations 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. Both TRAIL and OPG were detected in the eluted fractions. Recombinant TRAIL and OPG were loaded as positive controls. This result indicated that TRAIL and OPG form a complex in the lysates of

Figure 3. Recombinant OPG-Fc inhibits apoptosis of HMECs. HMECs were plated on PDL for 10 h in the presence of OPG-Fc concentrations ranging from 0 to 5 $\mu\text{g}/\text{mL}$ and on OPN. Nuclei were stained with Hoechst dye and counted in three fields at $40\times$. The percentage of apoptotic nuclei was then determined. The experiment was repeated three times, and error bars indicate standard deviations (analysis of variance, $p < 0.0001$; three replicates).

Figure 4. TRAIL and OPG coimmunoprecipitate. HMECs were plated on OPN for 16 h, and the lysates were collected in an IP buffer. Mouse IgG and a mAb against TRAIL were each cross-linked to agarose, and each conjugate was then incubated with the cell lysates at 4°C overnight. The bound proteins were eluted off the agarose gel and analyzed by Western blot. Both TRAIL and OPG were detected in the eluted fractions. Recombinant TRAIL and OPG were loaded as positive controls. This result indicated that TRAIL and OPG form a complex that can be immunoprecipitated using an anti-TRAIL antibody. Results are representative of three independent experiments.

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 withdrawal-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 $\alpha_v\beta_3$ 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

Figure 5. Soluble TRAIL receptors and neutralizing antibody against TRAIL promotes HMEC survival. (A) HMECs were plated on OPN or PDL for 18 h. TR1-Fc and TR2-Fc were added at concentrations of 0, 5, and 10 ng/ml to cells plated on PDL. Both TR1-Fc and TR2-Fc inhibited serum withdrawal-induced apoptosis (analysis of variance, $p < 0.003$; three replicates). (B) Treatment of HMECs with a combination of TR1-Fc and TR2-Fc did not result in increased inhibition. (C) Similarly, HMECs were plated on PDL in the presence of a neutralizing monoclonal anti-TRAIL at concentrations of 50 and 100 ng/ml. The percentage of apoptotic cells was determined at 16 h. Treatment of cells with anti-TRAIL resulted in a decrease in the percentage of apoptotic cells. At 100 ng/ml, a threefold difference was seen between IgG and antibody-treated cells (*, Fisher's protected least significant difference, $p < 0.02$). Both soluble TRAIL receptors and an anti-TRAIL mAb inhibited endothelial cell apoptosis. The experiments were repeated three times, and error bars indicate standard deviations.

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. HMECs also were plated on OPN and on PDL in the absence of TRAIL. TRAIL has no effect on the apoptotic rate of HMECs 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, $p < 0.0001$).

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 HMECs plated on OPN either in the presence or absence of PI-PLC (which removes the GPI-linked TRAIL-R3) (our unpublished data). Thus, serum deprivation combined with loss of $\alpha_v\beta_3$ signaling sensitizes HMECs to death induced by exogenous TRAIL.

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.

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 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 μ g/ml goat IgG or 10 μ g/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.

Table 1. Peak fluorescence intensity of TRAIL-receptors in HMECs plated on BSA and OPN

	Mean	Linear fluorescence intensity ^a	Peak fluorescence intensity ^b
BSA IgG	3.5	1.2	
BSA anti-TRAIL-R1	24.0	2.7	2.2
BSA anti-TRAIL-R2	16.2	1.9	1.6
BSA anti-TRAIL-R3	51.4	8.0	6.7
BSA anti-TRAIL-R4	14.3	1.8	1.5
OPN IgG	2.1	1.1	
OPN anti-TRAIL-R1	18.7	2.1	1.9
OPN anti-TRAIL-R2	12.3	1.6	1.4
OPN anti-TRAIL-R3	37.0	4.5	4.1
OPN anti-TRAIL-R4	9.1	1.4	1.3

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 μ g/ml goat IgG or 10 μ g/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. Results are representative of three independent experiments.

^a Linear fluorescence intensity = $2^{(\text{mean}/17)}$.

^b Linear fluorescence intensity TRAILR(1-4)/linear fluorescence intensity IgG.

with 2-methoxyestradiol demonstrated an up-regulation of both TRAIL-R2 and TRAIL, which resulted in increased susceptibility to TRAIL-induced apoptosis (LaVallee *et al.*, 2003). In contrast, one recent paper has suggested that treatment of HUVECs with TRAIL promotes survival after serum reduction through Akt phosphorylation (Secchiero *et al.*, 2003). These authors also found that inhibitors to the PI3K/Akt pathway such as LY294002 were able to sensitize HUVECs to TRAIL-induced apoptosis.

In our study recombinant TRAIL was able to increase the number of apoptotic cells in HMEC plated on PDL but not OPN, suggesting that lack of integrin ligation sensitizes HMECs to endogenous TRAIL. Consistent with this idea, exogenously added TRAIL had no effect on HMEC plated on OPN even after PI-PLC treatment to remove TRAIL-R3 (our unpublished data). Furthermore, no difference was noted in the expression of TRAIL-receptors (R1, R2, R3, and R4) between dying HMECs plated on PDL (or BSA) and surviving HMECs plated on OPN as examined by FACS analysis and Western blot. Only OPG, the third decoy receptor was up-regulated in HMECs on OPN, suggesting that the OPN-induced increase in OPG in endothelial cells may shift the balance toward inhibition of apoptosis by blocking TRAIL function.

Several mechanisms can be proposed to explain these results. First, it might be that the OPG alone, secreted by HMEC in response to OPN, is able to neutralize the exogenously added TRAIL. Second, there may be other pathways induced by integrin ligation that modulate TRAIL-induced apoptosis. Akt has been previously shown to mediate matrix-induced survival of normal epithelial cells (Khwaja *et al.*, 1997) and is activated in response to $\alpha_v\beta_3$ ligation in endothelial cells (Scatena, unpublished observation). Several antiapoptotic pathways have been correlated with resistance to TRAIL. Bcl-x_L an inhibitor of mitochondrial changes associ-

ated with cell death and the short splice form of c-FLIP, an inhibitor of caspase-8 activation were found to be up-regulated in TRAIL-resistant cells (Burns and El-Deiry, 2001). Smac/DIABLO (Deng *et al.*, 2002) and caspase-3-cleaved I κ B α (Kim *et al.*, 2002a) also have been associated with TRAIL sensitivity.

The inhibition of HMECs apoptosis by the soluble TRAIL receptors (TR1-Fc and TR2-Fc) as well as OPG implies that an extrinsic, TRAIL-mediated cell death pathway is responsible for growth factor withdrawal-induced apoptosis in HMECs. Interestingly, a role for death ligands/receptors in triggering loss of anchorage-induced cell death (anoikis) in endothelial and epithelial cells also is supported by findings implicating the activation of the Fas/caspase-8 death pathway (Frisch, 1999; Rytomaa *et al.*, 1999; Aoudjit and Vuori, 2001). The ability of OPG to fully inhibit endothelial cell apoptosis unlike the soluble TRAIL-R (TR1-Fc and TR2-Fc) suggests that these molecules may have different affinities for TRAIL. However, studies using isothermal titration calorimetry have found that at 37°C OPG actually has a lower affinity for TRAIL compared with TRAIL-R1 and TRAIL-R2 (Truneh *et al.*, 2000). An alternate explanation may be that OPG interacts with a second apoptosis-inducing molecule. At this time, the only other known ligand for OPG is RANKL, which is known to mediate osteoclast survival (Lacey *et al.*, 2000). RANKL was found to be angiogenic in a subcutaneous Matrigel assay in mice (Kim *et al.*, 2002b) and inhibited HUVEC apoptosis induced by serum deprivation (Kim *et al.*, 2003), but in our hands it had no effect on HMEC apoptosis (our unpublished data).

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 to 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.

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