THE ROLE OF OSTEOPROTEGERIN
AND TNF-RELATED APOPTOSIS INDUCING LIGAND
IN HUMAN MICROVASCULAR ENDOTHELIAL CELL SURVIVAL

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Abstract

Endothelial cell survival and anti-apoptotic 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-kappa B (NF-κB), and subsequent upregulation of osteoprotegerin (OPG). In this study, the mechanism by which OPG protects endothelial cells from death was examined. Serum-starved human microvascular endothelial cells (HMEC) plated on the $\alpha_v\beta_3$ ligand, osteopontin (OPN), were protected from cell death. Immunoprecipitation experiments indicated that OPG formed a complex with TNF-related apoptosis-inducing ligand (TRAIL) in HMEC under these conditions. Furthermore, inhibitors of TRAIL including recombinant soluble TRAIL receptors and a neutralizing antibody against TRAIL blocked apoptosis of serum-starved HMEC plated on the non-integrin attachment factor, poly-D-lysine (PDL). While TRAIL was unable to induce apoptosis in HMEC plated on OPN, the addition of recombinant TRAIL did increase the percentage of apoptotic HMEC plated on PDL. 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 VEGF, angiopoietin-1, and bFGF act in part by promoting endothelial cell survival or inhibiting endothelial cell apoptosis (Alon et al., 1995; Hayes et al., 1999; Karsan et al., 1997; Kwak et al., 1999). The interaction of endothelial cells and extracellular matrix through integrins has also been found to be important for cell survival (Meredith et al., 1993). The ligation of αvβ3 integrin has been implicated in angiogenesis as studies using neutralizing antibodies or cyclic peptide antagonists induced endothelial cell apoptosis and thereby blocked angiogenesis (Brooks et al., 1994a; Brooks et al., 1994b; Friedlander et al., 1996). A potential mechanism for αvβ3-mediated survival in endothelial cells was identified using rat aortic endothelial cells (RAEC) (Scatena et al., 1998). In that study the αvβ3 ligand osteopontin (OPN), protected rat aortic endothelial cells (RAEC) from serum-deprivation induced apoptosis by activating an NF-κB dependent survival pathway. NF-κB dependent, anti-apoptotic genes in RAEC 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 RAEC plated on OPN. The addition of recombinant OPG to RAEC 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 which exists as both a 60 kDa monomer and a 120 kDa disulfide linked dimer and is a soluble member of the tumor necrosis factor receptor (TNFR) 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; Mizuno et al., 1998; Yun et al., 2001). OPG was also found to regulate B cell maturation and development; populations of peripheral B cells are elevated in OPG null mice and OPG -/- 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 HMEC. Our studies suggest that OPG inhibits serum starvation-induced endothelial apoptosis by binding TRAIL and preventing TRAIL receptor-induced death. Furthermore the studies suggest that endothelial cells are sensitized to TRAIL-induced death by serum- and adhesion-deprivation.

Methods

Materials
Human dermal microvascular endothelial cells and EGM-2-MV media were purchased from Clonetics (Walkersville, MD). Recombinant rat OPN was prepared as previously described (Martin et al., 2003). Antibodies against OPG and TRAIL and the soluble TRAIL-R1 and TRAIL-R2 fusion proteins were purchased from R&D (Minneapolis, MN). Recombinant TRAIL was purchased from Upstate Biotech (Waltham, MA). PDVF membranes and Renaissance chemiluminescence reagents were purchased from NEN Life Science (Boston, MA). Zetaprobe GT membranes were purchased from Biorad (Hercules, CA). The ELISA kit was purchased from Biomedica Gruppe (Southbridge, MA) and the Seize Primary Immunoprecipitation kit was purchased from Pierce Biotechnology (Rockford, IL).

**Hoechst Staining**

Cells were plated onto four well chamber slides at about 75% confluence. A 1 in 10 dilution was made from a concentrated stock of Hoechst dye (4 mg/mL). Fifty microlitres 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 was then removed, the cells rinsed with 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 HMEC plated on PDL and OPN for 3 and 6 hours. Northern blot analysis was carried out by electrophoretic separation of 12.5 µg of total RNA using formaldehyde-agarose gels and subsequent transfer to a Zetaprobe GT membrane. The OPG cDNA insert was labelled using the Multiprime kit (Amersham Bioscience, Piscataway, NJ) and \((\alpha-^{32}P)dCTP\); whereas the 18S probe was end-labelled using \(T_4\) kinase (New England Biolab, Beverley, MA) and \((\gamma-^{32}P)dATP\); hybridization was performed as previously described (Giachelli et al., 1991).

**OPG ELISA**

Media from HMEC plated on PDL or OPN for 3, 6, 12 and 24 hours was collected and concentrated 20 fold using Microcon filters (10 kDa molecular weight cut off) (Amicon, Beverley, 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 manufacturers 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**

HMEC were plated on OPN or PDL coated plates for 24 hours. The cells were then lysed in an IP buffer containing 10 mM Tris HCl pH 7.4, 100 mM NaCl, 0.5% NP-40, 0.5 µg/mL leupeptin, 10 µg/mL aprotinin and 0.2 mM PMSF. Media was also collected, the cells spun down at 15 000 rpm for 5 min and added to 10x IP buffer. Antibody (monoclonal anti-TRAIL) or mouse IgG was immobilized onto agarose gel
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 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. 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**

12.5% SDS PAGE gels were run and subsequently transferred to PDVF membranes. The membranes were then blocked in 10% milk in TTBS overnight at 4°C. Primary antibodies were added for two hours at room temperature followed by the appropriate HRP-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.

**FACS Analysis**

HMEC were plated on BSA or OPN for 4 hours, trypsinized and resuspended in PBS/0.2% BSA/0.02% NaN₃ containing 10 µg/mL of an anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3 or anti-TRAIL-R4 antibody or 10 µg/mL of 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 FITC-labelled 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 HMEC in the absence of serum*

Previous studies have demonstrated that OPN protects RAEC from serum deprivation induced apoptosis through its interaction with the $\alpha_v\beta_3$ 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 RAEC (Malyankar et al., 2000). To determine whether HMEC also survived on osteopontin through the production of OPG, HMEC were plated on poly-D-lysine (PDL) and OPN for 3, 6, 12 and 24 hours. HMEC bind to PDL through charge-charge interactions but cell surface integrins are not engaged resulting in no NF-κB activation. HMEC were then stained with Hoechst and the number of apoptotic cells was determined (Figure 1). At 3 hours 18% of the HMEC plated on PDL were apoptotic (compared with 1.6% plated on OPN). This increased to 49.1% by 24 hours whereas only 8.6% of HMEC plated on OPN were apoptotic. Therefore, consistent with studies in RAEC, OPN promotes the survival of HMEC in the absence of growth factors.

*OPN promotes OPG synthesis and secretion*

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

Since OPG functions as an extracellular molecule, we investigated the amount of OPG secreted into the media at 3, 6, 12, and 24 hours of incubation on OPN. The media was collected and concentrated 20 fold using Microcon filters. HMEC 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 hours 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 HMEC plated on PDL at 24 hours, these levels do not appear 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 HMEC plated on PDL in the absence of serum**

Recombinant OPG inhibits RAEC apoptosis when NF-κB is inactive (Malyankar et al., 2000). HMEC were plated on PDL for 10 hours 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 previously described and the percentage of apoptotic cells calculated. At 0.5 µg/mL the percentage of apoptotic cells was reduced by half and this was further decreased at higher concentrations of OPG-Fc. Thus, OPG-Fc inhibits apoptosis of HMEC in a dose-dependent manner.
TRAIL and OPG are coimmunoprecipitated by a monoclonal antibody against TRAIL

Since OPG has been found to bind to TRAIL and inhibit the apoptosis of Jurkat cells (Emery et al., 1998) we were interested in determining if OPG bound TRAIL in HMEC and thereby inhibited serum induced apoptosis. The amount of TRAIL protein was measured by Western blot in HMEC plated on OPN compared with HMEC plated on PDL and was not found to be changed (our unpublished results) demonstrating that only OPG is upregulated 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 HMEC (plated on OPN) and apoptotic HMEC (plated on BSA) (our unpublished results).

In order to isolate the complex of OPG and TRAIL, HMEC were plated on OPN for 16 hours and the lysates collected in an IP buffer. A monoclonal antibody against TRAIL was cross-linked to agarose gel 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 HMEC 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
Since OPG, a TRAIL-binding molecule, inhibited the apoptosis of HMEC 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) were investigated. HMEC were plated on PDL for 18 hours; 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 HMEC. 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. HMEC were plated on PDL for 16 hours 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 HMEC plated on PDL for 16 hours 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 HMEC plated on PDL.

Serum deprivation combined with loss of $\alpha_v\beta_3$ signaling sensitizes HMEC 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 since 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 HMEC and HUVEC 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 HUVEC but found a small increase (10-20%) in cell lysis in HUVEC that had been exposed to TRAIL overnight (Gochuico et al., 2000). An additional study indicated that both HUVEC and HMEC 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.

In order to determine if TRAIL could increase the rate of apoptosis; HMEC were plated on PDL and 200 ng/mL of recombinant human TRAIL was added 30 minutes later. The percentage of apoptotic cells was determined at 3, 6, 10, and 24 hours. Treatment of cells plated on PDL with TRAIL resulted in a 2-3 fold increase in the percentage of apoptotic cells (Figure 6). HMEC were also plated on OPN and on PDL in the absence of TRAIL. TRAIL has no effect on the apoptotic rate of HMEC plated on OPN either in the presence or absence of PI-PLC (which removes the GPI-linked TRAIL-R3) (our unpublished results). Thus, serum deprivation combined with loss of \( \alpha_\text{v}\beta_3 \) signaling sensitizes HMEC to death induced by exogenous TRAIL.

\textit{TRAIL-R expression on the cell surface is unchanged in HMEC 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 HMEC on PDL, HMEC were plated under conditions of apoptosis (cells kept in suspension by plating on BSA) and survival (OPN) for 4 hours, trypsinized and incubated with anti-TRAIL-R antibodies. The cells were then incubated with a FITC-labelled 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) vs. apoptotic (plated on BSA) cells was unchanged. Western blot analysis of HMEC lysates plated on PDL or OPN for 3, 6, 12 and 24 hours confirmed that there were no changes in TRAIL-R expression (our unpublished results). Thus, modulation of TRAIL-R on the surface of HMEC 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 upregulation of OPG is the only change which 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 HMEC under conditions of serum deprivation and HMEC 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 upregulated in response to adhesion on
OPN and promotes survival in RAEC (Malyankar et al., 2000) and extends these findings to primary HMEC. 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 monoclonal antibody demonstrated that OPG and TRAIL form a complex in the lysate of HMEC 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 HMEC plated on PDL. These findings indicate that OPG protects HMEC 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 weight 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 TNFR-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 glycoprophospholipid 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 wide variety of transformed cells but was originally not found to induce death in normal cells (Pan et al., 1997; Wiley et al., 1995). However, recent studies have shown that normal hepatocytes and keratinocytes are susceptible to specific versions of recombinant TRAIL (Jo et al., 2000; Lawrence et al., 2001; Leverkus et al., 2000; 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-medicated 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 appears to be important in protection against TRAIL-induced apoptosis. Indeed, removal of the GPI-linked TRAIL-R3 receptor sensitized human umbilical vein endothelial cells (HUVEC) to TRAIL (Sheridan et al., 1997; Zhang et al., 2000). Moreover, HUVEC that were treated with 2-methoxyestradiol demonstrated an upregulation 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 HUVEC with TRAIL promotes survival after serum reduction through Akt phosphorylation (Secchiero et al.,
These authors also found that inhibitors to the PI3K/Akt pathway such as LY294002 were able to sensitize HUVEC 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 HMEC 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 results). Furthermore, no difference was noted in the expression of TRAIL-receptors (R1, R2, R3, and R4) between dying HMEC plated on PDL (or BSA) and surviving HMEC plated on OPN as examined by FACS analysis and Western blot. Only OPG, the third decoy receptor was upregulated in HMEC on OPN, suggesting that the OPN-induced increase in OPG in endothelial cells may shift the balance towards 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 which 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 αvβ3 ligation in endothelial cells (Scatena, unpublished observation). Several anti-apoptotic pathways have been correlated with resistance to TRAIL. Bcl-xL, an inhibitor of mitochondrial changes associated with cell death and the short splice form of c-FLIP, an inhibitor of caspase-8 activation were found to be upregulated in TRAIL-resistant cells (Burns and El-Deiry, 2001). Smac/DIABLO (Deng
et al., 2002) and caspase-3 cleaved IκBα (Kim et al., 2002a) have also been associated with TRAIL-sensitivity.

The inhibition of HMEC 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 HMEC. Interestingly, a role for death ligands/receptors in triggering loss of anchorage-induced cell death (anoikis) in endothelial and epithelial cells is also supported by findings implicating the activation of the Fas/caspase-8 death pathway (Aoudjit and Vuori, 2001; Frisch, 1999; Rytomaa et al., 1999). 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 had no effect on HMEC apoptosis (our unpublished results).

In conclusion we have found that OPG acts as a survival factor for HMEC 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 is thought to be mediated by one or more downstream molecules which result in
caspase-8 and caspase-3 activation. These downstream molecules and events are also likely to be regulated as the susceptibility of HMEC to TRAIL does not appear 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 which 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:


Figure Legends:

**Figure 1:** *HMEC survival is increased in cells plated on OPN compared with PDL*
HMEC were plated on either OPN or PDL in the absence of serum for 3, 6, 12 or 24 hours. Nuclei were then stained with Hoechst dye and the number of apoptotic cells was determined in three fields at 40x. 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 (ANOVA P<0.0001).

**Figure 2:** *OPG RNA and secretion into the media is increased in HMEC plated on OPN*
A) HMEC were plated on either OPN or PDL for 3 and 6 hours and the RNA isolated. The samples were run on an agarose gel and Northern blot analysis was performed. OPG RNA from HMEC plated on OPN was increased at 3 hours and was further increased at 6 hours. B) HMEC were plated on either OPN or PDL for 3, 6, 12 or 24 hours and the media collected and stored at -20°C. The media was concentrated 20 fold using Microcon filters and the amount of OPG quantitated using a sandwich ELISA. A two fold increase in OPG was found in HMEC plated on OPN at 12 and 24 hours. Results are representative of three independent experiments; error bars represent standard deviations (ANOVA P<0.0001; 3 replicates).

**Figure 3:** *Recombinant OPG-Fc inhibits apoptosis of HMEC*
HMEC were plated on PDL for 10 hours in the presence of OPG-Fc concentrations ranging from 0-5 µg/mL and on OPN. Nuclei were stained with Hoechst dye and counted in three fields at 40x. The percentage of apoptotic nuclei was then determined. The experiment was repeated three times and error bars indicate standard deviations (ANOVA P<0.0001; 3 replicates).

**Figure 4:** *TRAIL and OPG co-immunoprecipitate*
HMEC were plated on OPN for 16 hours and the lysates collected in an IP buffer. Mouse IgG and a monoclonal antibody 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 which can be immunoprecipitated using an anti-TRAIL antibody. Results are representative of 3 independent experiments.

**Figure 5: Soluble TRAIL receptors and neutralizing antibody against TRAIL promote HMEC survival**

A) HMEC were plated on OPN or PDL for 18 hours. 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 (ANOVA P<0.003; 3 replicates). B) Treatment of HMEC with a combination of TR1-Fc and TR2-Fc did not result in increased inhibition. C) Similarly, HMEC 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 hours. Treatment of cells with anti-TRAIL resulted in a decrease in the percentage of apoptotic cells. At 100 ng/mL a 3 fold difference was seen between IgG and antibody treated cells (*Fisher's PLSD 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 HMEC plated on PDL**

HMEC were plated on PDL and recombinant TRAIL was added 30 minutes later at a concentration of 200 ng/mL. The percentage of apoptotic cells was determined at 3, 6, 10 and 24 hours. Treatment of cells plated on PDL with TRAIL resulted in a 2-3 fold increase in the percentage of apoptotic cells. HMEC were also 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 results). The results shown are representative of three independent experiments; error bars indicate standard deviations for triplicate determinations (ANOVA P<0.0001).
Figure 7: *TRAIL-receptor expression on the cell surface is unchanged in HMEC 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). HMEC were plated on OPN or BSA for four hours and 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. The cells were then incubated with a FITC-labelled anti-goat antibody and analyzed by flow cytometry. No changes were found in the expression of TRAIL-R between HMEC plated on BSA and those plated on OPN. Results are representative of three independent experiments.
Table 1:  Peak fluorescence intensity of TRAIL-receptors in HMEC 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). 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 and peak fluorescence intensity values were then calculated. No changes were found in the expression of TRAIL-R between HMEC plated on OPN and those plated on BSA. Results are representative of three independent experiments.

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<td>1.3</td>
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* Linear fluorescence intensity = $2^{\text{mean}^{1/2}}$

** Peak fluorescence intensity = LFI(sample)/LFI(negative control)
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A) Percentage Apoptotic Cells

B) Percentage Apoptotic Cells

C) Percentage Apoptotic Cells

Concentration (ng/mL)

Concentration of TR1-Fc + TR2-Fc (ng/mL)

Concentration (ng/mL)
Figure 6
Figure 7