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Tumor necrosis factor–related apoptosis-inducing ligand promotes microvascular endothelial cell hyperpermeability through phosphatidylinositol 3-kinase pathway

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Abstract

BACKGROUND: Microvascular hyperpermeability that occurs in hemorrhagic shock and burn trauma is regulated by the apoptotic signaling pathway. We hypothesized that tumor necrosis factor-α (TNF-α)–related apoptosis-inducing ligand (TRAIL) would promote hyperpermeability directly or by interacting with other signaling pathways.

METHODS: Rat lung microvascular endothelial cells (RLMECs) grown on Transwell membranes (Corning Life Sciences, Lowell, MA) were treated with recombinant human TRAIL (10, 50, and 100 ng/mL) for 6 hours or TRAIL (100 ng/mL) + LY294002 (a PI3K inhibitor; 20 μmol/L), Z-DEVD-FMK (a caspase-3 inhibitor; 10 μmol/L), or the inhibitors alone. Fluorescein isothiocyanate (FITC)–albumin flux was an indicator of permeability. Caspase-3 activity was measured fluorometrically. Adherens junction integrity was studied using β-catenin immunofluorescence.

RESULTS: TRAIL + LY294002, but not TRAIL alone, induced monolayer hyperpermeability (P < .05), and caspase-3 activity (P < .05), and disrupted the adherens junctions. Z-DEVD-FMK attenuated hyperpermeability and protected the adherens junctions.

CONCLUSIONS: TRAIL-induced microvascular hyperpermeability is phosphatidylinositol 3-kinase (PI3K)-dependent and may be mediated by caspase-3 cleavage of the endothelial adherens junctional complex.

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Microvascular hyperpermeability is known to be a contributing factor in the pathophysiologic process of hemorrhagic shock, as well as other shock states.1,2 This permeability is multifactorial and can be attributed to derangements in homeostasis such as loss of interendothelial cell-cell junction integrity.

Interendothelial junction integrity, specifically the interactions of the catenins (namely β-catenin), and vascular endothelial cadherin has been shown to be an important regulator of paracellular permeability.3 It is this altered permeability involved with shock states that can lead to deleterious sequelae such as acute respiratory distress syndrome.4

We have previously demonstrated the importance of endothelial cell apoptotic signaling in the pathophysiologic process of microvascular hyperpermeability associated with hemorrhagic shock and burn trauma.5–7 These studies mainly dealt with the mitochondria-mediated intrinsic
apoptotic signaling pathway leading to increased paracellular permeability. The extrinsic apoptotic pathway involves cytokines such as tumor necrosis factor (TNF) and their respective receptors. These receptors, termed death receptors, mediate apoptosis through intracellular machinery that culminates in the activation of effector caspases leading to cellular alterations.8

A novel cytokine was discovered in 1995 after a search of the expressed sequence tag library was performed using homology to a consensus amino acid sequence conserved throughout the TNF family of ligands.9 TNF-related apoptosis-inducing ligand (TRAIL), consisting of 281 amino acids in the human form, is a type II transmembrane protein that also exists in a soluble biologically active form.9 Its study has mainly focused on the fact that it is apoptogenic in tumor cells but not in normal cells, making it attractive as a possible cancer therapeutic adjunct.10

Recently, however, it has been shown that when the antiapoptotic phosphatidylinositol 3-kinase (PI3K) pathway is downregulated or blocked, TRAIL induces vascular endothelial cell apoptosis.11

First described in 1984, the PI3K pathway is a set of enzymes that are found in all eukaryotic cells examined and is linked to a diverse set of major cellular functions, including cell growth, proliferation, motility, differentiation, survival, and intracellular trafficking.12 The PI3K pathway is considered to play a crucial role in the signal transduction pathway leading to human endothelial cell survival.13 PI3K inhibition has been known to promote caspase-3 and -9 activation.14 Caspases cleave the components of cell-cell (β- and γ-catenin) and cell-matrix (focal adhesion kinase and p130 Cas) adherens junctions during apoptosis with dose and time requirements that paralleled those seen in barrier dysfunction and detachment.15,16

We hypothesized that TRAIL would induce damage to the interendothelial adherens junction through activation of the intracellular apoptotic signaling machinery, leading to microvascular hyperpermeability. This hypothesis was derived based on our previous finding that caspase-3 activation leads to the disruption of β-catenin in the adherens junctional complex, resulting in barrier dysfunction. Our data confirm what has been previously demonstrated, that TRAIL alone does not induce caspase-3 activation in endothelial cells. The novelty of our study is that we have shown TRAIL as an inducer of paracellular permeability through disruption of the adherens junctions, which is dependent on PI3K pathway inhibition.

**Methods**

**Cell culture and reagents**

Rat lung microvascular endothelial cells (RLMECs) were obtained from VEC Technologies (Rensselaer, NY). The cells were grown in MCDB-131 complete media (VEC Technologies) on cell culture dishes coated with fibronectin (0.1% solution) from bovine plasma (Sigma-Aldrich, St. Louis, MO). Trypsin-ethylenediaminetetraacetic acid solution (0.25%; Invitrogen-Gibco, Grand Island, NY) was used to detach the cells. The fluorescein isothiocyanate-bovine albumin (FITC-albumin) was obtained from Sigma-Aldrich. The test solution was prepared by dissolving the FITC-albumin in 5 mg/mL of low-serum media. Rabbit anti–β-catenin and FITC-conjugated donkey antirabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). VECTASHIELD mounting medium with 4’,6-diamidino-2-phenylindole was obtained from Vector Laboratories (Burlingame, CA). Recombinant human TRAIL was obtained from R&D Systems (Minneapolis, MN). The Calbiochem PI3K inhibitor LY294002 was obtained from EMD Millipore (Billerica, MA), and the caspase-3 inhibitor Z-DEVD-FMK was obtained from R&D Systems.

**Endothelial cell monolayer permeability**

RLMECs were grown as monolayers in Transwell plates (Corning Life Sciences, Lowell, MA) in complete MCDB-3 media. One hour before the start of the experiment, the monolayers were exposed to fresh media without phenol red dye. A preliminary study was performed in which monolayers were treated with TRAIL alone at increasing doses including 10 ng/mL, 50 ng/mL, and 100 ng/mL for 6 hours. In a separate experiment, monolayers were treated with the PI3K inhibitor LY294002 (20 μmol/L) for 1 hour. After this, the cells were treated with TRAIL (100 ng/mL) for 6 hours. A group was also pretreated with the caspase-3 inhibitor Z-DEVD-FMK (10 μmol/L) for 1 hour before the PI3K inhibitor. An untreated group, a TRAIL-alone group, a PI3K inhibitor–alone group, and a caspase-3 inhibitor–alone group served as controls. FITC-albumin (5 mg/mL) was added to the luminal (upper) chamber of the Transwell and left for 30 minutes. Samples (100 μL) collected from the abluminal (lower) chambers were analyzed for FITC fluorescence using a fluorometric plate reader at an excitation of 494 nm and emission of 520 nm. The data were calculated as percentage of the control (basal) values.

**Caspase-3 assay**

RLMECs were grown on regular dishes (100 mm). The following groups were studied: an untreated control group, a TRAIL-treated (100 ng/mL) group, a TRAIL group pretreated with the PI3K inhibitor LY294002 (20 μmol/L), and a group treated with LY294002 alone. Sixty minutes before the experiments, the monolayers were exposed to fresh media without phenol red dye. The monolayers were treated with LY294002 for 60 minutes followed by treatment with TRAIL for 6 hours. Caspase-3 activity of the cell lysates was determined using a fluorometric caspase-3 assay kit according to the manufacturer’s instructions. The data were calculated as percentage of the control (basal) values.
Endothelial cell adherens junctions

RLMECs were grown on fibronectin-coated chamber slides in complete MCDB-131 media for 24 hours. The following groups were studied: an untreated control group, a TRAIL-treated (100 ng/mL) group, a TRAIL group pretreated with LY294002 (20 μmol/L), a TRAIL group pretreated with LY294002 as well as Z-DEVD-FMK (10 μmol/L), a group treated with LY294002 alone, and a group treated with Z-DEVD-FMK alone. Sixty minutes before the start of each experiment, the cells were exposed to low-serum media. The pretreatments were 60 minutes each and the treatment with TRAIL was 6 hours, as previously stated. After treatment, the cells were washed in phosphate-buffered saline, fixed with 4% paraformaldehyde, and made permeable with Triton X-100 (Sigma-Aldrich). The cells were then blocked with 2% bovine serum albumin in phosphate-buffered saline and exposed to rabbit polyclonal antibody against β-catenin overnight at 4°C. The cells were exposed to FITC-conjugated donkey antirabbit secondary antibodies for 1 hour. The cells were then washed and mounted using antifade reagent containing 4',6-diamidino-2-phenylindole and visualized using a confocal laser scanning microscope at ×60.

Statistical analysis

All data are expressed as mean ± standard of error. The comparisons between groups were made using analysis of variance followed by the Bonferroni post-test for multiple comparisons. Each experimental value was compared with the initial baseline value and expressed as percentage change. A P value of less than .05 was considered to indicate a statistically significant difference.

Results

TRAIL induces endothelial cell monolayer hyperpermeability in the presence of PI3K inhibitor

In RLMEC monolayers, treatment with TRAIL (10-100 ng/mL) for 6 hours resulted in no significant change in FITC-albumin fluorescent intensity compared with the controls (Fig. 1). This indicates that TRAIL has no direct effect on endothelial cell monolayer permeability. The endothelial cell monolayers pretreated with the PI3K inhibitor LY294002 (20 μmol/L) when exposed to TRAIL (100 ng/mL), showed a significant increase in permeability compared with the control, TRAIL-alone, and the LY294002-alone groups (P < .05) (Fig. 2). Pretreatment of monolayers with the caspase-3 inhibitor Z-DEVD-FMK resulted in a significant decrease in hyperpermeability compared with the TRAIL + LY294002 group (P < .05) (Fig. 2).

TRAIL induces caspase-3 activity in the presence of a PI3K inhibitor

In RLMECs, treatment with TRAIL alone showed no significant change in caspase-3 activity compared with the untreated control group. The cells pretreated with the PI3K inhibitor LY294002 (20 μmol/L) when exposed to TRAIL (100 ng/mL) for 6 hours, showed a significant increase in caspase-3 activity compared with the control, TRAIL-alone, and LY294002-alone groups (P < .05) (Fig. 3).

TRAIL induces disruption of the endothelial cell adherens junctions in the presence of the PI3K inhibitor

RLMECs grown on chamber slides and subjected to immunofluorescence showed intact adherens junctional complexes evidenced by the strong and continuous presence of β-catenin in the cell-cell junctions. Cells treated with TRAIL alone showed no visible change in β-catenin localization, suggesting intact cell-cell junctions. When exposed to TRAIL (100 ng/mL), the cells pretreated with the PI3K inhibitor LY294002 (20 μmol/L) showed a decrease and discontinuity of β-catenin in the cell-cell junctions, indicating loss of β-catenin in the cell-cell junctions and subsequent barrier dysfunction. The cells pretreated with the caspase-3 inhibitor Z-DEVD-FMK and the PI3K inhibitor LY294002 before exposure to TRAIL showed a strong and continuous presence of β-catenin in the cell-cell junctions comparable to that of the untreated control cells. The cells treated with LY294002 alone or Z-DEVD-FMK alone showed no visible change in β-catenin localization (Fig. 4).

Comments

The deleterious sequelae of microvascular hyperpermeability associated with shock states have been well documented.
Our laboratory has demonstrated the importance of apoptotic signaling in the development of microvascular hyperpermeability.\textsuperscript{5,6} In this report we show that TRAIL, in the circumstance of PI3K inhibition, is capable of inducing microvascular hyperpermeability as evidenced by the increased flux of FITC-albumin across an endothelial monolayer when exposed to the combination of TRAIL and LY294002. This is in contrast to TRAIL alone having no effect on monolayer permeability.

Recent work has shown that in a rodent model of traumatic hemorrhage the PI3K pathway is downregulated in certain cell lines and that this downregulation is specific to the pathologic condition; they showed that a hypoxia model did not induce downregulation of PI3K.\textsuperscript{17} Also, studies have shown that in proinflammatory models, the production of TRAIL is increased.\textsuperscript{18} In a particular model, human volunteers were given an intravenous injection of endotoxin and had a rapid 10-fold increase in the blood level of TRAIL.\textsuperscript{19} From these data we can project a proinflammatory situation such as hemorrhagic shock or sepsis (in which the PI3K pathway is inhibited and TRAIL is upregulated) that could induce vascular endothelial apoptotic signaling and lead to microvascular hyperpermeability. It has been previously shown by us, as well as others, that the adherens junction proteins (in this case \(\beta\)-catenin) are substrates for caspase cleavage, and when this occurs loss of endothelial integrity ensues.\textsuperscript{15} The mechanism by which TRAIL is able to induce apoptotic signaling and effect hyperpermeability seems to be PI3K inhibition–dependent caspase-3–mediated damage to the interendothelial cell–cell junction. In our study, a significant increase in caspase-3 activation in the cells treated with the combination of TRAIL and the PI3K inhibitor LY294002 was observed, whereas TRAIL alone did not induce caspase-3 activation compared with untreated controls. Using immunofluorescence localization of \(\beta\)-catenin, we have demonstrated that TRAIL induces damage of the adherens junction complex in the circumstance of PI3K inhibition in that there is loss of \(\beta\)-catenin staining and loss of interendothelial cell–cell junction integrity. This damage is abrogated when treatment with the caspase-3–specific inhibitor Z-DEVD-FMK is instituted. Our data unite when one views the hyperpermeability induced by TRAIL through PI3K inhibition, which can be attenuated with caspase-3 inhibition, in the light of the adherens junction damage.

A stable cell–cell adherens junction is important for normal barrier functions and requires the close interaction

\[\text{Figure 2}\] Pretreatment with a specific PI3K inhibitor, LY294002 (20 \(\mu\)mol/L), and then treatment with TRAIL (100 ng/mL) induced significant hyperpermeability in RLMEC monolayers compared with control (\(*P < .05\); \(n = 6\)). This was significantly attenuated with the addition of a specific caspase-3 inhibitor, Z-DEVD-FMK (10 \(\mu\)mol/L) (†\(P < .05\); \(n = 6\)). Treatment with either LY294002 or Z-DEVD-FMK alone did not induce significant hyperpermeability compared with control cells. Change in permeability is expressed as percentage of the basal fluorescence.

\[\text{Figure 3}\] TRAIL (100 ng/mL) alone did not induce caspase-3 activity in RLMECs, whereas pretreatment with the PI3K inhibitor LY294002 (20 \(\mu\)mol/L) showed significantly increased caspase-3 activity (\(*P < .05\); \(n = 4\)).
Proteolytic cleavage of β-catenin occurs after the activation of procaspase-3, -6, or -8. Cleavage of β-catenin has been found to be caspase dependent, and 5 cleavage products of β-catenin were identified after cleavage by caspase-3. The absence of β-catenin significantly reduces the capacity of the cells to maintain intercellular contacts, which results in fluid leakage. Our results suggest that TRAIL, under a condition of PI3K inhibition, leads the caspase-3-mediated proteolytic cleavage of β-catenin and compromises endothelial cell barrier integrity. Since TRAIL’s discovery, effort in its study has focused on the observation that TRAIL seems to affect apoptosis in tumor or virus-infected cells while sparing normal cells. This has made it attractive as a potential cancer therapy, and rightly so, as in vivo tumor models have shown suppression with TRAIL treatment. The fact that TRAIL along with its receptors is expressed across many cell types, including the vascular endothelium, points to the conclusion that it likely has a complex physiologic role. In fact, a study found that TRAIL actually promoted survival of human umbilical vein endothelial cells through PI3K activation, but when PI3K was inhibited apoptotic signaling ensued. TRAIL-mediated activation of the apoptotic signaling pathway has been shown to vary greatly depending on the cell and tissue type.

As referenced earlier, recent studies have demonstrated the downregulation of the PI3K pathway after trauma and hemorrhage and the restoration of the PI3K pathway with estradiol treatment. We too have recently shown the protective effect of estradiol both on blocking apoptotic signaling and attenuating microvascular hyperpermeability after hemorrhagic shock. It is a possibility, then, that with the restoration of the PI3K pathway, the apoptotic signaling induced by TRAIL could be prevented. The role of TRAIL in maintaining normal endothelial cell physiology, in some instances augmenting survival while in others leading down the pathway of apoptosis, is complex and warrants further study.

Figure 4  Immunofluorescence localization of β-catenin in RLMECs reveals damage of the interendothelial cell-cell junction in the group treated with TRAIL (100 ng/mL) and the PI3K inhibitor LY294002 (20 μmol/L), as evidenced by loss of barrier continuity (arrows). This continuity is restored in the group treated with TRAIL (100 ng/mL) and the addition of the specific caspase-3 inhibitor Z-DEVD-FMK (10 μmol/L). Treatment with TRAIL, LY294002, or Z-DEVD-FMK alone did not affect the barrier continuity when compared with controls. Images were obtained at 60×.
In our study, we have shown the ability of TRAIL to induce apoptotic signaling, leading to caspase-3 activation, microvascular endothelial adherens junction damage, and effectual microvascular hyperpermeability in a PI3K pathway-dependent manner. Our hope is that this will lead to a better understanding of the complex physiology involved in maintaining endothelial integrity and avenues to guard this in pathophysiologic states.

Conclusions

Microvascular hyperpermeability that occurs in hemorrhagic shock and burn trauma is regulated by the apoptotic signaling pathway. We hypothesized that TNF-α-related apoptosis-inducing ligand (TRAIL) would promote hyperpermeability directly or by interacting with other signaling pathways. Our study shows that TRAIL-induced microvascular hyperpermeability is PI3K dependent and may be mediated by caspase-3 cleavage of the endothelial cell adherens junctional complex.

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9. Foster FM, Traer CJ, Abraham SM, et al. The phosphoinositide (PI)-3-kinase/Akt pathway-dependent manner. Our hope is that this will lead to a better understanding of the complex physiology involved in maintaining endothelial integrity and avenues to guard this in pathophysiologic states.
