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The Counteradhesive Proteins, Thrombospondin 1 and SPARC/Osteonectin, Open the Tyrosine Phosphorylation-Responsive Paracellular Pathway in Pulmonary Vascular Endothelia

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Abstract

The counteradhesive proteins are a group of genetically and structurally distinct multidomain proteins that have been grouped together for their ability to inhibit cell-substrate interactions. Three counteradhesive proteins that influence endothelial cell behavior include thrombospondin (TSP)1, SPARC (Secreted Protein Acidic and Rich in Cysteine), also known as osteonectin, and tenascin. More recently, these proteins have been shown to not only regulate cell-matrix interactions but cell-cell interactions as well. TSP1 increases tyrosine phosphorylation of components of the cell-cell adherens junctions or zonula adherens (ZA) and opens the paracellular pathway in human lung microvascular endothelia. The EGF-like repeats of TSP1 activate the epidermal growth factor receptor (EGFR) and ErbB2 and these two receptor protein tyrosine kinase (PTK)s participate in ZA protein tyrosine phosphorylation and barrier disruption in response to the TSP1 stimulus. For the barrier response to TSP1, EGFR/ErbB2 activation is necessary but insufficient. Protein tyrosine phosphatase (PTP)μ counter-regulates phosphorylation of selected tyrosine residues within the cytoplasmic domain of EGFR. Although tenascin, like TSP1, also contains EGF-like repeats and is known to activate EGFR, whether it too opens the paracellular pathway is unknown. In addition to TSP1, tenascin, and the other TSP family members, there are numerous other proteins that also contain EGF-like repeats and participate in hemostasis, wound healing, and tissue remodeling. EGFR not only responds to direct binding of EGF motif-containing ligands but can be transactivated by a wide range of diverse stimuli. In fact, several established mediators of increased vascular permeability and/or lung injury, including thrombin, tumor necrosis factor-α, platelet-activating factor, bradykinin, angiopoietin, and H_2O_2, each transactivate EGFR. It is conceivable that EGFR serves a pivotal signaling role in a final common pathway for the pulmonary response to selected injurious stimuli. SPARC/Osteonectin also increases tyrosine phosphorylation of ZA proteins and opens the endothelial paracellular pathway in a PTK dependent manner. The expression of the counteradhesive proteins is increased in response to a wide range of injurious stimuli. It is likely that these same molecules participate in the host response to acute lung injury and are operative during the barrier response within the pulmonary microvasculature.

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Keywords

Counter adhesive proteins; Thrombospondin 1; SPARC/Osteonectin; Tyrosine phosphorylation; Zonula adherens; Vascular endothelial-cadherin; Catenins; Epidermal growth factor receptor; Protein tyrosine phosphatase-mu; Paracellular pathway

Introduction to the Counteradhesive Proteins

The counteradhesive proteins are a group of structurally dissimilar multidomain proteins that have been grouped together solely on a functional basis (Sage et al., 1991; Chiquet-Ehrismann 1991; Chiquet-Ehrismann 1995; Murphy-Ullrich 1995). Each of these proteins contains multiple domains, some of which can recognize and bind to multiple receptors on a given host cell. These receptor-ligand interactions can induce overlapping, or at times, conflicting biological responses. By definition, each of these so-called counteradhesive proteins, at least under certain conditions, can alter cell-substrate adhesion. The action of these counteradhesive, matricellular proteins stimulates the intermediate adhesive phenotype, which is defined as an adaptive state. This is characterized by a spread cell which lacks central actin-containing stress fibers and vinculin-containing focal adhesions (FA)s (Murphy-Ullrich 2001). Talin and integrin remain associated with these altered adhesive structures. Cells exposed to these counteradhesive proteins can either be prevented from forming focal adhesions and stress fibers or alternately, focal adhesion disassembly can be stimulated in fully adherent cells. Cells in this intermediate adhesive state are generally more migratory. This emphasis on single-cell morphology and on the cell-matrix interface that requires specialized techniques such as interference reflection microscopy, has encouraged studies of cells under subconfluent conditions. More recently, it has been appreciated that the counteradhesive proteins may also influence cell-cell interactions (Goldblum et al., 1994; Goldblum et al., 1999; Young et al., 1998; Garg et al., 2007). Two principal members of the counteradhesive protein group that exert biological effects on the endothelial cell (EC) and the endothelial paracellular pathway are thrombospondin (TSP)1 and SPARC (Secreted Protein Acidic and Rich in Cysteine), also known as osteonectin. Since extensive reviews of these two proteins are available in the literature (Lane and Sage, 1994; Lawler, 1986; Mosher, 1990; Bornstein, 1992; Adams and Lawler, 1993; Lahav, 1993; Frazier, 1995; Bornstein and Sage, 1995; Bornstein, 1995, Bornstein, 2001; Sid et al., 2004; Bonnefoy et al., 2008), only that information most relevant to endothelial barrier function will be emphasized here. Within this context, data are available for TSP1, and less so for SPARC. Accordingly, the discussion focuses on these two proteins with some speculation on a third counteradhesive protein, tenascin (Erickson and Bourdon, 1989; Erickson, 1993; Jones and Jones, 2000; Chiquet-Ehrismann and Chiquet, 2003; Chiquet-Ehrismann, 2004; Hsia and Schwarzbauer, 2005; Orend and Chiquet-Ehrismann, 2006).

The Tyrosine Phosphorylation-Responsive Paracellular Pathway in Pulmonary Vascular Endothelia

Multiple stimuli that increase tyrosine phosphorylation of proteins within EC-EC intercellular junctions and/or the actin cytoskeleton profoundly alter their organization (Bannerman and Goldblum, 1997; Ayalon and Geiger, 1997; Esser et al., 1998; Tsukita et al., 1991; Hamaguchi et al., 1993). Several established mediators of increased vascular permeability also increase tyrosine phosphorylation of components of the zonula adherens (ZA) (Bannerman and Goldblum, 1997; Esser et al., 1998), zonula occludins (Van Itallie et al., 1995), and gap junctions (Lidington et al., 2002). In fact, the counteradhesive proteins, TSP1 and SPARC, increase tyrosine phosphorylation of ZA proteins as well (Goldblum et

al, 1994; Young et al., 1998). In the case of ZA proteins, increases in their tyrosine phosphorylation can coincide with uncoupling of ZA proteins with their binding partners, disruption of the ZA-actin cytoskeleton linkage, and reduction of homophilic adhesion between opposing VE-cadherin ectodomain expressed on the surface of neighboring cells (Hamaguchi et al., 1993). The evidence that such a mechanism(s) explains the reversible and physiological regulation of the paracellular pathway is incomplete. The operative protein tyrosine kinase (PTK)s, protein tyrosine phosphatase (PTP)s, and their substrates are only partially understood. Taken together, these combined data suggest that modulation of the tyrosine phosphorylation states of selected ZA and perhaps other junctional and signaling proteins regulates paracellular pathway function. Evidence exists to support perturbation of the tyrosine phosphorylation-responsive pathway by one or more counteradhesive proteins.

Thrombospondin 1

TSP1 Structure

Thrombospondin (TSP)1 is a ~420 kDa trimeric glycoprotein composed of three identical 145 kDa polypeptide chains linked by disulfide bonds (Lawler et al., 1985; Mosher, 1990; Lahav, 1993; Bornstein, 1995). Each subunit of TSP1 contains the following structural elements: an NH_2-terminal globular domain of the laminin G domain and concanvalin A-like lectin/glucanase superfamily; an α-helical region that presumably forms a parallel homotrimeric coiled coil as in matrilin-1; a von Willebrand factor type C module; three TSP type 1 (TSR) repeats homologous to both properdin and a malarial coat protein, each of which is elongated and consists of a novel, antiparallel three-stranded fold; and the TSP “signature piece” that is made up of three epidermal growth factor (EGF)-like TSP type 2 repeats, 7 calcium-binding type 3 repeats, the last of which contains an RGD sequence, and a COOH-terminal domain that forms a lectin-like β-sandwich (Carlson et al., 2008). The elements of the “signature piece” interact extensively to form three structural regions termed the stalk, wire, and globe, and are further stabilized by disulfide bonds and bound calcium (Carlson et al., 2005). TSP1 is one of five family members (Carlson et al., 2008). All five vertebrate TSPs contain the “signature piece”. TSP3, TSP4, and TSP5 have a pentameric coiled coil (Adams, 1993; Malashkevich et al., 1996; Adams, 2001), lack the von Willebrand factor C domain and TSRs, and have an extra EGF-like repeat (Bornstein P., 1995; Tan et al., 2006).

Tissue Sources

TSP1 was first demonstrated in the releasate of thrombin-stimulated platelets in 1971 (Baenziger et al, 1971). TSP1 is secreted by numerous host tissues including ECs, and is present in the extracellular matrix (ECM) (Mosher, 1990; Lahav, 1993; Bornstein, 1995). In the lung, TSP1 is also expressed by type II alveolar epithelial cells, alveolar macrophages, and interstitial lung fibroblasts (Lahav, 1993; Zhou, 2004). TSP1 is not only expressed in tissues relevant and anatomically proximal to the vasculature and lung, but is also present within the intravascular compartment as a circulating plasma protein (Lahav, 1993) and in PMNs, monocytes, and the α-granules of platelets (Mosher, 1990; Lahav, 1993; Leung, 1984; Jaffe, 1985; Kreis, 1989). These cells continuously traffic through the microvasculature where they intimately interact with the endothelial surface. ECs can both produce (Mosher et al., 1982) and respond (Goldblum et al., 1999) to TSP1. TSP1 may be presented to the pulmonary microvascular endothelium, *in vivo*, through an endocrine, paracrine, and/or autocrine pathway.

Multidomain TSP1 Engages Multiple EC Receptors

TSP1 recognizes multiple EC receptors and induces multiple and sometimes opposing biological responses (Mosher, 1990; Lahav, 1993; Bornstein, 1995; Lawler et al., 1988; Gao

et al., 1996; Swerlick et al., 1992; Tsao and Mousa, 1995; Mikhailenko et al., 1995). It contains an NH_2-terminal heparin-binding domain that recognizes heparan sulfate proteoglycans including syndecans-1 and 4 (Adams et al., 2001; Nunes et al., 2008), calreticulin (39–41), and low density lipoprotein (LDL) receptor related protein (LRP)s on the EC surface (Mikhailenko et al., 1995), as well as several integrins $\alpha_6\beta_1$, $\alpha_4\beta_1$,$\alpha_9\beta_1$, and $\alpha_3\beta_1$ (Calzada et al., 2004). The TSP type 1 repeats contain a CSVTCG motif that recognizes CD36 (Asch et al., 1992), as well as a β_1 integrin binding site (Calzada et al., 2004; Short et al., 2005). The COOH-terminal signature domain binds both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins and CD47 through the COOH-terminal, RFYVVM and FIRVVM sequences (Lindberg et al., 1993; Reinhold et al., 1995). CD36 is an ~88 kDa transmembrane glycoprotein expressed in microvascular ECs (Swerlick et al., 1992; Greenwalt et al., 1992). LRP, also known as CD91 or the α_2-macroglobulin receptor, is a 600 kDa transmembrane protein that can function as a co-receptor for the Ca^{2+}-binding protein, calreticulin, in transducing FA disassembly in response to TSP1 (Goicoechea et al., 2000; Goicoechea et al., 2002; Orr et al., 2003). CD47 is an unusual Ig family member with an extracellular domain, a domain with 5 putative membrane-spanning helices, and a short cytoplasmic tail of 16 residues that can be expressed in 4 alternatively spliced forms (Lindberg et al., 1993; Reinhold et al., 1995). This ~50 kDa constitutively-expressed, cell surface glycoprotein often physically and functionally associates with β_3 and other integrins (Gao et al., 1996).

Increased TSP1 Expression in Response to Injury

TSP1 expression is increased in response to multiple injurious stimuli. The TSP1 gene contains glucose responsive elements (Holmes et al., 1997; Bhattacharyya et al., 2008) and its expression is increased in response to oxidative stress, hypoxia, hyperoxia, nitrative stress, complement complexes, and heat shock (Ketis et al., 1988; Kermorvant-Duchemin et al., 2005; Wang et al., 2003; Favier et al., 2005; Thakar et al., 2005; Gao et al., 2006; Zhou et al., 2006; Sezaki et al., 2005). In the context of vascular and lung injury, TSP1 is increased during platelet aggregation and release (Leung, 1984), as well as in atherosclerotic lesions (Riessen et al., 1998). TSP1 is reportedly operative in the adhesion of both sickled erythrocytes (Brittain et al., 1993) and erythrocytes parasitized with malaria (Cook et al., 1994; Siano et al., 1997) to the endothelial surface. TSP1 expression is elevated in the settings of pulmonary hypertension (Botney et al., 1992), pulmonary fibrosis (Kuhn and Mason, 1995; Yehualaeshet et al., 2000; Ide et al., 2008), and perhaps most relevant to this discussion, in the bronchoalveolar lavage fluids of ARDS patients (Idell et al., 1989).

TSP1 Increases Endothelial Paracellular Permeability

In bovine and human lung EC systems, human platelet-derived TSP1 increases movement of ^{14}C-bovine serum albumin (BSA) across EC monolayers cultured on gelatin-impregnated polycarbonate filters mounted in chemotactic chambers (Goldblum et al., 1999; Garg et al., 2007). In bovine pulmonary artery ECs, TSP1 at ≥1.0 μg/ml (≥7 nM) increased transendothelial ^{14}C-BSA flux in a concentration-dependent manner (Goldblum et al., 1999). These concentrations are well within the range of TSP1 levels detected in the whole blood of healthy human subjects (Dawes et al, 1983). In this same system, we used 1) a sensitive ^{51}Cr-release assay to exclude EC injury, 2) serum deprivation, polymixin B preadsorption, and a neutralizing anti-TSP1 antibody to exclude LPS contamination, and 3) TSP1 depleted of TGFβ, anti-TGFβ antibody, and a peptide known to block TSP1 activation of latent TGFβ to exclude TGFβ bioactivity (Goldblum et al., 1999). TSP1-induced increments in albumin flux were evident after 2–6h. We asked whether during this prolonged stimulus-to-response lag time, TSP1 could induce EC synthesis of a second protein mediator(s) that could alter barrier function. Pretreatment of EC monolayers with cycloheximide which inhibited >95% protein synthesis, failed to block the TSP1-induced increment in albumin flux (0.062 ± 0.001 pmol/h, n=7 *vs* 0.073 ± 0.008 pmol/h, n=5)

(Goldblum et al., 1999), indicating that the TSP1 effect could not be explained through *de novo* EC synthesis of a second autocrine/paracrine factor. In human lung microvascular ECs, after 6h, TSP1 at ≥15 μg/ml (≥0.107 μM) increased ^{14}C-BSA flux compared to the simultaneous media control (Garg et al., 2007). The maximum mean (±SE) ^{14}C-BSA flux of 0.067±0.005 pmol/h was seen with TSP1 30 μg/ml (214 nM), at which point the TSP1-induced effect had begun to plateau or saturate. Again, the TSP1 effect on endothelial barrier function was time-dependent but in the human EC system, the TSP1 effect was evident as early as 10 min with further time-dependent increases at 2–8h (Garg et al., 2007). The more rapid onset of action of human TSP1 in a human microvascular EC system may be due to higher receptor-ligand species compatibility and/or differential responsiveness of ECs from large *vs* small caliber vessels (Gebb and Stevens, 2004), possibly due to differential CD36 (Swerlick et al., 1992; Greenwalt et al., 1992) and/or EGFR (SEG-personal communication) expression. It should also be noted that in Akt−/− mice TSP1 and 2 expression is reduced whereas vascular permeability was enhanced (Chen et al., 2005). These leaky vessels displayed marked reduction of basement membrane thickness, laminin content, and recruitment of mural cells. Although TSP1 increased permeability across wild-type endothelial monolayers, it decreased permeability across Akt−/− cells. These studies indicate that, over the long term, TSP1 regulates vascular integrity, most likely through collagen matrix assembly.

Effect of PTK Inhibition on TSP1-Induced Opening of the Endothelial Paracellular Pathway

To determine whether TSP1 might open the paracellular pathway through a PTK-dependent mechanism, TSP1 was presented to postconfluent bovine pulmonary artery EC monolayers in the presence of either of 2 structurally and functionally dissimilar broad-spectrum PTK inhibitors, herbimycin A or genistein (Goldblum et al., 1999). In the barrier function assay, prior PTK inhibition with either herbimycin A or genistein protected against TSP1-induced increments in ^{14}C-BSA flux by >80% and >50%, respectively. When postconfluent EC monolayers were exposed to TSP1 +/− herbimycin A and stained with the F-actin probe, fluorescein-phalloidin (Molec. Probes, Eugene, OR), isolated ellipsoid disruptions within the F-actin lattice occurred exclusively at the cell-cell interface (Goldblum et al., 1999). Prior PTK inhibition prevented intercellular gap formation. In human ECs, prior broadspectrum PTK inhibition with erbstatin (5 μM) completely protected against the TSP1-induced increase in ^{14}C-albumin flux (Garg et al., 2007). These combined data indicate that PTK inhibition in tightly confluent ECs blocks TSP1-induced formation of intercellular gaps and increments in paracellular permeability.

Identification of Substrates for TSP1-Induced Protein Tyrosine Phosphorylation

As a first step to determine which EC proteins might be tyrosine phosphorylated in response to TSP1, bovine PA ECs cultured to confluence on filters were exposed to TSP1 (20 μg/ml) or media alone and probed with fluorescein isothiocyanate (FITC)-conjugated antiphosphotyrosine antibody (5 μg/ml) (UBI) (Goldblum et al., 1999). At both 10 min and 1h, TSP1-exposed ECs displayed enhanced fluorescence signal predominately restricted to intercellular boundaries compared to the media control (Goldblum et al., 1999). TSP1 induces comparable findings in HMVEC-Ls (Garg et al., 2007). These data suggest that TSP1, after exposure times as brief as 10min, preferentially stimulates tyrosine phosphorylation of proteins that are either enriched to or upon phosphorylation translocate to cell-cell junctions in confluent EC monolayers. On the basis of gel mobility and subcellular localization, we adopted an immunoprecipitation immunoscreening strategy to determine whether ZA proteins are substrates for TSP1-induced tyrosine phosphorylation. In the human EC system, TSP1 increased tyrosine phosphorylation of the ZA proteins, VE-cadherin, γ-catenin, and p120^{ctn} but not β-catenin (Garg et al., 2007). Further, we have

identified EGFR and ErbB2 as substrates in TSP1-treated ECs; these 2 phosphoproteins likely explain the ~170 and ~185 kDa phosphotyrosine-containing bands (Garg et al., 2007).

EGFR/ErbB2 Expression in Human Lung Microvascular Endothelia

Prior broadspectrum PTK inhibition protects against TSP1-induced barrier disruption (Goldblum et al., 1999). TSP1 contains EGF-like repeats (Mosher, 1990; Lahav, 1993; Bornstein, 1995), and increases ZA protein tyrosine phosphorylation in bovine and human endothelia (Goldblum et al., 1999; Garg et al., 2007), an event known to occur downstream of EGFR/ErbB2 activation (Hoschuetzky et al., 1994; Shibamoto et al., 1994; Mariner et al., 2003). We asked whether EGFR/ErbB2 might be the operative PTK(s). In HMVEC-Ls, we applied a stringent RT-PCR approach to detect mRNA for the 4 ErbB family members (Garg et al., 2007). EGFR and ErbB2 mRNA but neither ErbB3 nor ErbB4 mRNA was detected. Only after prior enrichment through immunoprecipitation of large quantities of EC protein, could we detect EGFR or ErbB2 protein (Garg et al., 2007). We then tested HMVEC-Ls for a proliferative response to the EGFR-specific ligand, EGF; EGF at 100 ng/ml increased ^{3}H-thymidine incorporation >2-fold within 24h (424.5 ± 137.9 dpms *vs* 204.7 ± 18.3 dpms; $p<0.05$). These combined data indicate that EGFR and ErbB2 are expressed at extremely low levels in HMVEC-Ls where they respond to an authentic high-affinity EGFR ligand.

TSP1 Activates EGFR/ErbB2

To determine whether TSP1 can activate EGFR/ErbB2, increasing concentrations of TSP1 were first presented for increasing exposure times to high EGFR/ErbB2-expressing A431 cells (Liu et al., 2007). TSP1 increased EGFR (Y1068) autophosphorylation in a time-dependent manner and activation was inhibitable by the EGFR-selective tyrphostin, AG1478, siRNA-mediated knockdown of EGFR, and by an EGFR ectodomain blocking antibody. In these same cells, baculovirus-derived recombinant TSP1 domains that correspond to overlapping sequences from the full-length protein, each at a concentration equimolar to TSP1 30 μg/ml, i.e. 214 nM, were tested for EGFR Y1068 phosphorylation. Only the 3 recombinant domains containing EGF-like repeats 1–3 activated EGFR, and the EGF-like repeats 1–3 alone, produced the highest level of Y1068 phosphorylation (Liu et al., 2007). This same recombinant TSP1 domain activated EGFR in a dose- and time-dependent manner. In low EGFR-expressing HMVEC-Ls, using reciprocal co-immunoprecipitation assays, TSP1 induced EGFR/ErbB2 heterodimerization at 10 and 30 min (Garg et al., 2007). Coincident with this heterodimerization, TSP1 activated EGFR/ErbB2 as shown by their tyrosine transautophosphorylation (Garg et al., 2007). These combined data indicate that 1) TSP1 induces EGFR/ErbB2 heterodimerization and autotransphosphorylation, 2) the EGF-like repeats alone are sufficient for EGFR/ErbB2 activation, and 3) native TSP1-induced EGFR/ErbB2 activation is temporally proximal to opening of the paracellular pathway.

TSP1 Opens the Endothelial Paracellular Pathway through EGFR/ErbB2 Activation

TSP1 activates EGFR/ErbB2 (Liu et al., 2007), increases tyrosine phosphorylation of ZA proteins (Goldblum et al., 1999; Garg et al., 2007), and prior broad-spectrum PTK inhibition protects against TSP1-induced loss of endothelial barrier function (Goldblum et al., 1999; Garg et al., 2007). We asked whether either EGFR or ErbB2 catalytic activity might be required. Prior PTK inhibition with either the EGFR-selective tyrphostin, AG1478, or the ErbB2-selective tyrphostin, AG825, blocked TSP1-induced EGFR/ErbB2 activation (Liu et al., 2007), ZA protein tyrosine phosphorylation (Garg et al., 2007) and opening of the endothelial paracellular pathway (Garg et al., 2007). Interestingly, the ErbB2-selective tyrphostin, AG825, selectively blocked ErbB2 activation whereas AG1478 blocked EGFR and ErbB2 activation, suggesting that ErbB2 activation was mediated through EGFR

transactivation. In other experiments, preincubation of EC monolayers with an antibody that blocks EGF binding to the ligand-binding portion of the EGFR ectodomain protected against TSP1-induced opening of the paracellular pathway whereas a species- and isotype-matched irrelevant antibody control did not (Garg et al., 2007). These combined data support the concept that EGFR/ErbB2 PTK catalytic activities are required for TSP1-induced opening of the paracellular pathway.

Failure of EGFR Ligands to Open Paracellular Pathway

We then tested the portion of TSP1 that activates EGFR/ErbB2, the EGF-like repeats, in the barrier function assay. Exposure to increasing concentrations of the recombinant EGF-like repeats (up to 5 μM) failed to increase transendothelial ^{14}C-BSA flux (Garg et al., 2007). Therefore, the EGF-like repeats at concentrations up to ~50-fold greater than those required for native TSP1 to open the paracellular pathway failed to increase ^{14}C-BSA flux. Although this baculovirus-derived recombinant domain can clearly activate EGFR (Liu et al., 2007), we asked whether an altered tertiary structure might explain its inability to open the paracellular pathway. We tested 3 high-affinity EGFR ligands, EGF, TGFα, and amphiregulin; each, at concentrations up to 10,000 ng/ml (i.e. 1.7 μM, 1.8 μM, and 0.9 μM, respectively), failed to open the endothelial paracellular pathway (Garg et al., 2007). These combined data indicate that engagement and activation of EGFR alone is necessary but insufficient to open the endothelial paracellular pathway.

Overexpression of EGFR Increases HMVEC-L Responsiveness

This failure of recombinant TSP1 EGF-like repeats or authentic high-affinity EGFR ligands to open the endothelial paracellular pathway could be explained by the extremely low levels of EGFR and ErbB2 expression in HMVEC-Ls and/or the requirement of one or more other TSP1 domains in addition to the type 2 repeats. To address the issue of low EGFR expression, EGFR was overexpressed in HMVEC-Ls prior to exposure to recombinant TSP1 EGF-like repeats (Garg et al., 2007). Infection of HMVEC-Ls with adenoEGFR at multiplicities of infection (MOIs) ≥10 dramatically increased levels of EGFR expression. In these same infected ECs, EGFR Y1068 autophosphorylation also was evident at MOIs ≥10 whereas barrier function was lost only at MOIs ≥200. In HMVEC-Ls infected with adenoEGFR at MOI = 150, the cells became barrier responsive to EGF (100 ng/ml) as well as recombinant TSP1 EGF-like repeats whereas those infected with an equivalent MOI of control adenovirus did not. Therefore, in unmanipulated HMVEC-Ls, in which EGFR protein is expressed at low, almost undetectable levels, EGFR activation alone is insufficient to open the paracellular pathway. This finding indicates that under physiological conditions, one or more TSP1 domains outside of the EGF-like repeats are also required for the barrier-disrupting effect. However, after EGFR expression is sufficiently elevated, activation of the receptor opens the endothelial paracellular pathway. Whether EGFR expression in other endothelia might reach levels adequate for EC responsiveness to EGFR ligands is not known.

Participation of PTPs in the TSP1 Biological Effect

To determine which EC proteins might be tyrosine phosphorylated in response to TSP1, ECs were exposed to TSP1 or media alone and processed for phosphotyrosine immunoblotting as we have described (Goldblum et al., 1999). Exposure of ECs to a range of TSP1 concentrations for varying time intervals demonstrated no consistent increases in protein tyrosine phosphorylation. However, in the presence of 2 structurally and functionally dissimilar PTP inhibitors, vanadate and PAO, exogenous TSP1 clearly increased tyrosine phosphorylation of EC proteins in a dose- and time-dependent manner (Goldblum et al., 1999). To determine whether one or more PTPs might participate in TSP1-induced, PTK-dependent opening of the paracellular pathway, ECs were exposed to TSP1 in the presence

and absence of either vanadate or PAO at concentrations that in themselves, did not alter barrier function compared to simultaneous media controls (Goldblum et al., 1999). Prior PTP inhibition with either vanadate or PAO enhanced the TSP1 effect 62% and 120%, respectively, implicating one or more counter-regulatory PTPs in the EC response to TSP1.

PTPμ Counter-Regulates EGFR Activation

A number of receptor and nonreceptor PTPs are known to dephosphorylate tyrosine residues within receptor PTKs including EGFR (Lee et al., 1998; Deb et al). One receptor PTP, PTPμ, is highly expressed in human lung endothelia, including pulmonary artery and microvascular ECs, where it is predominantly localized to intercellular boundaries (Sui et al., 2005). PTPμ directly associates with the ZA protein, VE-cadherin, and restrains tyrosine phosphorylation of both VE-cadherin and a second ZA protein, $p120^{ctn}$. Finally, siRNA-induced depletion of PTPμ opens the paracellular pathway in human lung microvascular endothelia. These findings indicate that PTPμ counter-regulates both the tyrosine phosphorylation state(s) of one or more ZA proteins and paracellular pathway function in pulmonary vascular endothelia. Since EGFR is expressed at such low levels in ECs, making it more difficult to study, we asked whether PTPμ might influence EGFR autophosphorylation in lung epithelial cells (Anglin et al., 2007). High PTPμ-expressing confluent A549 cells were transfected with either PTPμ-targeting or control siRNAs. Full-length PTPμ and its proteolytically processed forms were all reduced ≥95% relative to control siRNA transfected cells. After transfection with PTPμ-targeting or control siRNAs, A549 cells were exposed for 10 min to EGF (100 ng/ml) or media alone, after which they were processed for phosphoEGFR immunoblotting with antibodies that recognize specific EGFR phosphotyrosines (PY845, PY992, PY1045, PY1068, PY1086, PY1148, and PY1173). Under unstimulated conditions, PTPμ knockdown increased phosphorylation of Y992 and Y1068. In response to the EGF stimulus, PTPμ knockdown increased phosphorylation of Y845, Y992, Y1068, and Y1086 but not of Y1148 or Y1173. These combined data indicate that in lung epithelial cells, PTPμ selectively restrains EGFR autophosphorylation of Y845, Y992, Y1068, and Y1086. How this distinct pattern of EGFR tyrosine dephosphorylation impacts on downstream signaling and biological responses is currently under study (Anglin et al., 2007). It is also conceivable that PTPμ may indirectly influence EGFR autophosphorylation or downstream signaling events through its targeting of relevant substrates including src, p190 RhoGAP, Akt, and RhoA (Singleton et al., 2008).

Other Counter-Adhesive Proteins that Contain EGF-Like Repeats

Like TSP1, the other TSP family members, TSP2-5, each contains multiple EGF-like repeats (Adams and Lawler, 1993). In fact, we have demonstrated in A431 cells that baculovirus-derived recombinant EGF-like repeats of TSP2 and TSP4 also activate EGFR (Y1068 phosphorylation) (Liu et al., 2007). Although TSP3 and TSP5 have yet to be tested, it is possible that they too can activate EGFR. Another genetically distinct counteradhesive protein, tenascin, also contains multiple copies of the EGF-like repeat (Erickson and Bourdon, 1989; Erickson, 1993). In one report, tenascin-C increased clustering and tyrosine phosphorylation of EGFR in vascular smooth muscle cells (Swindle et al., 2001). These findings were explained, in part, through tenascin C-$\alpha_v\beta_3$ integrin interactions. Whether more than one member of the TSP family, together with tenascin, like TSP1, also open the paracellular pathway in pulmonary vascular endothelia is unknown. Other proteins that contain EGF-like repeats include the LDL receptor (Apella et al., 1988), decorin, a small leucine-rich proteoglycan (Iozzo et al., 1999), tissue plasiminogen activator (tPA), coagulation factors VII, IX, X, and XII, protein C, and the ECM protein, laminin (Apella et al., 1988; Engel, 1989). In fact, laminin-5 displays low affinity binding to EGFR (Schenk et al., 2003). These proteins participate in hemostasis, wound healing, and tissue remodeling.

Whether the EGF-like repeats of these proteins can, like TSP1, open the endothelial paracellular pathway is unknown.

EGFR Transactivation

EGFR not only responds to direct binding of EGF motif-containing ligands but can be transactivated by a wide range of diverse stimuli (Hackel et al., 1999; Carpenter, 1999). First, several heterologous receptors, including PDGFβR (Saito et al., 2001), G protein-coupled receptors (Luttrell et al., 1999), members of the cytokine receptor superfamily (Donato et al., 1989; Asano et al., 1997; Qiu and Kung, 1998), E-cadherin (Pece and Gutkind, 2000), and integrins (Miyamoto et al., 1996; Moro et al., 1998), associate with and/or transactivate EGFR. Environmental stressors, including hyperosmotic conditions (Carpenter, 1999), heat shock (Carpenter, 1999), UV and γ radiation (Carpenter, 1999, Huang et al., 1996; Bowers et al., 2001), oxidant stress with H_2O_2 (Carpenter, 1999), heavy metals (Carpenter, 1999), and alkylating agents (Carpenter, 1999), all can activate EGFR. The proposed mechanisms through which EGFR is transactivated are many. First, EGFR can cocluster and even heterodimerize with heterologous receptors (Saito et al., 2001). In other cases, agonists including thrombin, γ-interferon, and angiopoietin, each upregulate matrix metalloprotease (MMP)-mediated, proteolytic cleavage of transmembrane precursor(s), with release of EGFR ligands (Hackel et al., 1999; Carpenter, 1999; Luttrell et al., 1999; Prenzel et al., 1999). TSP1 reportedly upregulates MMP-2 and –9 in ECs (Donnini et al., 2004) and induces mesangial cell release of EGF (Marinides et al., 1994). We now know that in A431 cells, TSP1 upregulates MMP9, that liberates an as yet unidentified EGFR ligand which recognizes and binds to the ligand-binding portion of the EGFR ectodomain (Liu et al., 2007). Interestingly, several established mediators of increased vascular permeability and/or lung injury, including thrombin, TNFα, platelet-activating factor, bradykinin, angiopoietin, and H_2O_2, each transactivate EGFR (Hackel et al., 1999; Carpenter, 1999; Luttrell et al., 1999; Adomeit et al., 1999; Pan et al., 1995). Further, EGFR ligands, such as TGFα, are increased in both the lungs of experimental animals (Vivekananda et al., 1994) and the pulmonary edema fluids of patients (Chesnutt et al., 1997; Madtes et al., 1998) with acute lung injury. It is conceivable that EGFR is a pivotal signaling element in a final common pathway for the host response to a subset of injurious stimuli for acute lung injury.

SPARC/Osteonectin

Background

SPARC, also know as osteonectin and BM-40, is a highly secreted, 43 kDa multidomain glycoprotein expressed in multiple host tissues (Lane and Sage, 1994). Most relevant to the pulmonary vasculature, SPARC is expressed in ECs, vascular smooth muscle cells, macrophages, fibroblasts, and the alveolar epithelium (Lane and Sage, 1994; Madtes et al., 1998; Reed et al., 1993). SPARC is also present within the intravascular compartment, both freely circulating in the plasma (Stenner et al., 1986) and in monocytes and the α-granules of platelets (Lane and Sage, 1994; Reed et al., 1993; Stenner et al., 1986). In the adult, SPARC expression increases in response to tissue injury, including heat shock, heavy metal, and endotoxin (Sage et al., 1986; Sage et al., 1989a; Neri et al., 1992; Sauk et al., 1991), and during wound healing and angiogenesis (Reed et al., 1993; Iruella-Arispe et al., 1991a; Iruela-Arispe et al, 1991b). Most relevant to this discussion, SPARC permits EC attachment to underlying substrate but inhibits EC spreading (Lane and Sage, 1994; Sage et al., 1989b). It induces focal adhesion disassembly, actin reorganization, and EC shape changes. SPARC binds to Ca^{2+}, albumin, selected components of the ECM, and several growth factors (Sage et al., 1984; Raines et al., 1992; Hasselaar and Sage, 1992; Sage, 1992). SPARC also

influences expression of specific MMPs, ECM proteins, and TSP1. Whether one or more of these bioactivities are operative during SPARC-induced barrier disruption is unknown.

SPARC Opens Endothelial Paracellular Pathway through Protein Tyrosine Phosphorylation

In a bovine pulmonary artery EC system, SPARC increased transendothelial ^{14}C-BSA flux in a dose- and time-dependent manner (5). SPARC at ≥ 0.5 μg/ml increased albumin flux after a stimulus-to-response lag time of ~1h. These SPARC concentrations approximated the levels of SPARC detected in the plasma of healthy human subjects (Macko et al., 2002). SPARC exposure disrupted the F-actin network resulting with intercellular gap formation and F-actin redistribution to the subcortical compartment. Prior F-actin stabilization with phallicidin protected against SPARC-induced barrier disruption, suggesting that actin disassembly and reorganization is a prerequisite to opening of the paracellular pathway in response to the SPARC stimulus.

SPARC increased EC protein tyrosine phosphorylation in a dose- and time-dependent manner (Young et al., 1998). SPARC at ≥15 μg/ml increased phosphorylation which could be first detected at 15 min and maximal phosphorylation was seen at 60 min. Using phosphotyrosine fluorescence microscopy to immunolocalize phosphotyrosine-containing proteins, SPARC increased phosphotyrosine signal almost exclusively at the intercellular boundaries. One of the substrates for SPARC-induced tyrosine phosphorylation was identified as the zonula adherens protein, β-catenin. Finally, prior broad-spectrum PTK inhibition with either herbimycin or genistein profoundly diminished SPARC-induced barrier disruption, whereas prior PTP inhibition with either sodium orthovanadate or phenylarsine oxide, enhanced it. Taken together, SPARC increases tyrosine phosphorylation of proteins enriched to cell-cell contacts, reorganizes the actin cytoskeleton, and barrier disruption in response to SPARC is both F-actin- and PTK-dependent.

In a more recent study, immunoblockade of the vascular cellular adhesion molecule (VECAM)-1 or CD106 in human umbilical vein endothelia dramatically reduced SPARC-induced actin reorganization and intercellular gap formation (Kelly et al., 2007). SPARC bound to VCAM-1 in *in vitro* coimmunoprecipitation assays and the association appeared to be direct. Using both *in vitro* static migration assays and an *in vivo* thioglycollate-induced peritonitis model in wild-type and SPARC null mice, these intriguing studies focused on transendothelial leukocyte migration. Although VCAM-1 immunoblockade was not tested in an endothelial barrier assay, their findings are compatible with VCAM-1 serving as a receptor that couples the SPARC stimulus to opening of the endothelial paracellular pathway.

Conclusions

Two counteradhesive proteins, TSP1 and SPARC/osteonectin, open the paracellular pathway in pulmonary vascular endothelia in a tyrosine phosphorylation-dependent manner. In the case of TSP1, the receptor PTKs, EGFR and ErbB2, participate in barrier disruption. Whether another EGF repeat-bearing counteradhesive protein, tenascin, might also open the paracellular pathway through EGFR/ErbB2 activation is unknown.

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