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Blockade of VEGF-induced GSK/ β -catenin signaling, uPAR expression and increased permeability by dominant negative p38 α

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Abstract

The goal of this study was to define the role of p38 α MAP kinase in VEGF-induced vascular permeability increase. Activation of p38 is correlated with increased permeability in endothelial cells treated with VEGF or high glucose and in retinas of diabetic animal models. We have shown previously that p38 inhibitors preserve endothelial barrier function and block VEGF-induced GSK/ β -catenin signaling. Here, we present data demonstrating that adenoviral vector delivery of a dominant negative p38 α mutant blocks this signaling pathway and preserves barrier function. This p38 α mutant was altered on its ATP-binding site, which eliminates its kinase activity. Bovine retinal endothelial (BRE) cells were transduced with recombinant adenovirus containing the p38 α mutants or empty vector. Successful transduction was confirmed by expression of GFP and p38 increase. Blockade of p38 activity by p38 α mutant was demonstrated by inhibition of VEGF-induced phosphorylation of a p38 target, MAP kinase activated protein kinase 2 (MK-2). The mutant also prevented VEGF-induced GSK phosphorylation and β -catenin cytosolic accumulation and nuclear translocation as shown by cell fractionation and Western blotting. Quantitative real-time PCR demonstrated that this mutant inhibited VEGF-induced uPAR gene expression. Importantly, this same mutant also strongly abrogated VEGF-induced endothelial barrier breakdown as determined by measuring transcellular electrical resistance and tracer flux through endothelial cell monolayer. This study indicates a critical role of p38 α in VEGF-induced permeability and offers a new strategy for developing potent and specific therapies for treatment of retinal diseases associated with vascular barrier dysfunction.

Keywords

VEGF; endothelial permeability; p38 MAP kinase; β -catenin; uPAR

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1. Introduction

Vascular endothelial growth factor (VEGF) is a potent vascular permeability factor. Blood-retinal barrier (BRB) dysfunction, one of the hallmarks of retinal diseases including diabetic retinopathy has been correlated with increased levels of VEGF (Funatsu et al., 2002; Viores et al., 1997). Therapies that inhibit VEGF have shown a beneficial effect on diabetic macular edema as well as macular edema caused by retinal vein occlusion (Kreutzer et al., 2008; Kriechbaum et al., 2008; Nguyen et al., 2006; Scott et al., 2007). Although the role of VEGF as a critical vascular permeability factor is well established, the signaling pathways that lead to VEGF-induced vascular permeability increase are still not clear. Meanwhile, VEGF is responsible for other functions of vascular endothelial cells, including proliferation, survival and migration (El-Remessy et al., 2005; Storkebaum and Carmeliet, 2004), as well as physiological functions of other cell types in the retina. The multiple functions of VEGF underline the importance of defining more precisely the mechanisms by which VEGF increases endothelial cell permeability and identifying specific steps in the signaling pathway as potential therapeutic targets.

Disruption of the tight junction and adherens junction protein complex causes endothelial permeability increase (Antonetti et al., 1998; Davidson et al., 2000; Gardner, 1995; Navaratna et al., 2007). Beta-catenin, one component of adherens junctions, acts as a transcription factor once it is translocated into the nucleus. Under normal physiological conditions in endothelial cells, cytosolic beta-catenin will bind to a protein complex including adenomatosis polyposis coli (APC), axin and a serine/threonine kinase, glycogen synthase kinase 3 beta (GSK3-beta) (Aberle et al., 1997). GSK3 is constitutively active and GSK3-dependent phosphorylation of beta-catenin leads to beta-catenin ubiquitination and degradation. When GSK3 is phosphorylated it loses its activity, thus, beta-catenin escapes ubiquitination, accumulates in the cytosol and translocates into the nucleus to induce gene transcription.

Previous studies have shown that one of the targets for beta-catenin transcriptional activation is urokinase plasminogen activator receptor (uPAR) (Mann et al., 1999). uPAR is a cell surface glycoprotein tethered to the cell membrane with a glycosylphosphatidylinositol anchor, and binding of pro-urokinase plasminogen activator (pro-uPA) to uPAR leads to uPA activation (Blasi et al., 1986). Activated uPA in turn activates plasminogen into plasmin, and converts pro-MMPs (matrix metalloproteinase) into active MMPs. This cascade of proteolytic activations results in extracellular matrix (ECM) degradation and disruption of cell-cell and cell-matrix attachments, which leads to permeability increase and facilitates the subsequent angiogenic processes (Dano et al., 1985). Moreover, uPAR expression has been correlated with p38 activation in cancer cell lines (Huang et al., 2000; Lee et al., 2004). We have shown previously that the VEGF-induced paracellular permeability increases in endothelial cells involve GSK3beta deactivation, beta-catenin nuclear translocation and uPAR expression which results in activation of uPA and matrix metalloproteinase MMP-9 (Behzadian et al., 2003, 2001). However, the role of p38 in this GSK/beta-catenin signaling pathway, which leads to uPAR expression and proteolytic events in endothelial cells, is still not clear.

Members of the p38 mitogen-activated protein (MAP) kinase family can be activated by various cytokines, growth factors and stresses (Han et al., 1994; Zarubin and Han, 2005). Four splice variants of p38 have been identified: p38 alpha, beta, gamma and delta. Among those p38 alpha, beta and gamma are expressed in endothelial cells (Hale et al., 1999; Yu et al., 2004). Phosphorylation of threonine and tyrosine on the Thr-Gly-Tyr dual phosphorylation motif is a shared activation mechanism of all four p38 isoforms (Raingeaud et al., 1995). Activated p38 in turn transfers phosphate from bound-ATP to its substrates and

phosphorylates them, which leads to activation of transcription factors or protein kinase cascades (Zarubin and Han, 2005). In endothelial cells, the VEGF-induced activation of p38 has been shown to initiate a protein kinase cascades that result in actin reorganization and endothelial cell migration (Rousseau et al., 1997).

Our previous study has shown that blockade of p38 by pharmacological inhibitors abrogates VEGF effects on the GSK/beta-catenin signaling pathway, uPAR expression and endothelial barrier function (Yang et al., 2010). Those commercially available pharmacological inhibitors are pyridinyl imidazole compounds, which compete with ATP for the same binding site on p38 kinase. Due to the structural similarity between p38 alpha and beta isoforms these inhibitors inhibit both. Although pharmacological inhibitors are very useful tools to study the role of MAP kinases, they are non-specific when the two closely related kinases are studied. To demonstrate direct evidence for the specific role of p38alpha in this signaling cascade, mutants of p38alpha were introduced into retinal endothelial cells.

Primary endothelial cells are resistant to taking up foreign genes and are also highly sensitive to cytotoxicity effects of transfection reagents. Therefore recombinant viral delivery of mutant genes is a very helpful tool to study signaling mechanisms in endothelial cells. Of the different types of recombinant virus, adenovirus, with its high transduction efficiency and capability of expressing recombinant proteins in non-dividing cells, is the optimal choice for studies of barrier function in retinal endothelial cells. In this study adenovirus-mediated delivery of p38alpha mutants was used to test the role of p38alpha in VEGF-induced activation of GSK/beta-catenin signaling pathway, uPAR expression and permeability increase in vascular endothelial cells.

2. Materials and methods

2.1. Cloning p38alpha mutants into adenoviral vectors

The recombinant adenoviral vector expressing p38alpha mutants (designated clone 703 and clone 1102) or the empty vector (designated Empty) were constructed using AdEasy™ XL adenoviral vector system from Stratagene (La Jolla, CA). The retroviral vectors BabeHygo-mp38KM and BabeHygo-mp38AF were kind gifts from Dr. J. Han (Scripps Research Institute, CA). Briefly, those mutants of p38alpha were isolated by PCR using primers in which 5' *SpeI* and 3' *PvuI* restriction sites were added at the 5' and 3' ends of the p38 encoding sequence, respectively. The fragments of p38alpha mutants and the shuttle vector (pShuttle-IRES-hrGFP-1, supplied by Stratagene) were digested with *SpeI* and *PvuI* and the p38alpha mutant fragments were cloned into the *SpeI*, *PvuI* orientation of the shuttle vector. The resulting plasmids were characterized by restriction enzyme analysis. These recombinant plasmids were linearized by *PmeI* and transformed into the *Escherichia coli* strain BJ-5183-AD-1 (supplied by Stratagene) by electroporation. The *PmeI*-linearized empty shuttle vector was separately transformed into the same strain of *E. Coli* to be used as empty adenoviral vector control. For each mutant the resulting recombinant plasmids were characterized by restriction enzyme analysis and by sequencing of the p38alpha inserts. The recombinant plasmids were linearized by *PacI* and transfected into the adenoviral packaging 293A cell line (supplied by Invitrogen) to produce viable, replication-deficient adenovirus expressing p38alpha mutants and green fluorescent protein (GFP).

Recombinant adenoviruses were then isolated and purified by CsCl gradient. The titer of the purified recombinant adenovirus clone used for this experiment was determined by plaque assay using agarose overlay and at least 10^{11} PFU/ml of vector particles were obtained. The purified recombinant adenovirus was dialyzed and stored at -80°C . The recombinant adenovirus carrying p38alpha mutant with altered ATP-binding site (K57 to M) was designated as clone 703. This mutant can be phosphorylated by upstream signal (from GSK)

but lacks kinase activity. The recombinant adenovirus carrying p38alpha with altered phosphorylation sites (TGY180–182 to AGF), was designated clone 1102. This mutant cannot be phosphorylated by upstream signals and lacks kinase activity as well. The recombinant adenovirus clone carrying empty shuttle vector was designated as Empty.

2.2. Endothelial cell culture and adenovirus transduction

Primary cultures of bovine retinal microvascular endothelial cells (BRE cells) were isolated according to methods established in our laboratory (Yang et al., 2010; Behzadian et al., 1995). For this study, BRE cells were maintained in M199 medium supplemented with 10% fetal bovine serum (Gemini), 10% CSC complete medium (Cell Systems), penicillin (100 U/ml), and streptomycin (100 µg/ml) under an atmosphere of 95% air and 5% CO₂ in a humidified 37 °C incubator and were used at passage 5–9. For all experiments, unless otherwise designated, culture medium was switched to a serum free endothelial basal medium (EBM, Lonza Walkersville, Inc. Walkersville, MD) including 0.1% BSA upon 90% confluence. Cultures were incubated for an additional 20–24 h before the indicated treatments were carried out. The NCI Preclinical Repository (Frederick, MD) kindly provided all VEGF used for this study. BRE cells were transduced with various multiplicity of infection (MOIs) of recombinant adenoviruses to determine a titer that gave maximal expression without significant cell death. In subsequent experiments, cells were transduced for 12–16 h at an optimal MOI (~20) followed by overnight serum starvation before VEGF treatment (30 ng/ml).

2.3. Protein extraction and Western blot analysis

Endothelial cells were grown in 100 mm diameter dishes and treated as indicated. At the end of the treatment time, cells were collected and extracted as described before (Behzadian et al., 2003; Yang et al., 2010). Equal amounts of sample (10 µg) were resolved on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. The proteins were then transferred into a nitrocellulose membrane (BioRad laboratories, Hercules, CA). After blocking with 5% non-fat milk, the membranes were probed with antibodies against beta-catenin (BD Transduction Laboratories), phosphorylated and total GSK3-beta, p38 MAP kinase and MK-2 (Cell Signaling Technology Inc, Danvers, MA) followed by horseradish-peroxidase (HRP)–conjugated secondary antibody (GE Healthcare). Protein bands were then visualized by chemiluminescence (ECL, Amersham), exposed to X-ray films, scanned for densitometry and normalized to appropriate internal markers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Ambion Inc.) served as cytosolic protein internal marker and histone H1 (Millipore) served as nuclear protein marker.

2.4. Real-time quantitative PCR

The method for quantifying specific gene expression by polymerase chain reaction (PCR) was as previously described with some modification (Behzadian et al., 2003; Yang et al., 2010). In brief, after the indicated treatments, cells were removed from the dish by Teflon coated razor blade, RNA was extracted using RNeasy min kit (QIAGEN Inc., Valencia, CA) and stored in ethanol-precipitated form at –70 °C. Aliquots of 2.0 µg total RNA were reconstituted and reverse transcription (RT) was carried out using random primers (Omniscript RT Kit, QIAGEN Inc.). Quantitative PCR was performed using fluorescence-labeled probes (TaqMan) and the Step-One-Plus system (Applied Biosystems, Foster City, CA). The TaqMan probes and primers for uPAR and the internal marker HPRT (Hypoxanthine-guanine phosphoribosyl-transferase) were designed based on the bovine DNA sequences (bovine uPAR accession number: AF-144762 and HPRT accession number: BC103248). The PCR products were also examined by agarose gel electrophoresis.

2.5. Endothelial cell permeability assays

BRE cell barrier function was assessed by measuring changes in trans-endothelial electrical resistance (TER) using ECIS (electrical cell-substrate impedance sensing, Applied Biophysics Inc., Troy New York) and by measuring FITC-dextran flux across the BRE cell monolayers grown on porous membranes (Transwell; Corning Costar, Acton, MA). Decreased TER and increased dextran flux across the cell monolayer are indications of increased paracellular permeability.

In ECIS, cells are grown in fibronectin-coated chambers of 8-well electrode arrays and the electric currents passing through the monolayers are measured independently in each chamber (Tiruppathi et al., 1992). The ECIS system is superior to the previously used manual methods in that, instead of measuring the endpoint TER, the electrical resistance is monitored uninterruptedly before and during the treatments. In this study, BRE cells were plated at a density of 5×10^4 cells/chamber (8W10E) to obtain a confluent cell monolayer within two days, upon which, cells were transduced with recombinant adenovirus and treated according to the experimental design. Data from the electrical resistance measurements (ohms) were recorded over the experimental time course. Integrity of the endothelial monolayer was confirmed at the end of each experiment microscopically and also by a final TER measurement. Resistance values for each chamber were normalized as the ratio of measured resistance to baseline resistance and plotted as a function of time.

For the tracer flux assay, BRE cells were grown to confluence on fibronectin-coated membranes with 0.4 μ M pores (Transwell; Corning Costar, Acton, MA). After transduction of recombinant adenovirus and serum starvation, cultures were treated with VEGF (30 ng/ml, 2 h). Then, FITC-dextran (10 μ M) was added to the upper chamber of the inserts. After 5 h, aliquots were taken from the lower and upper chambers, placed in a 96-well plate (black with clear bottoms, polystyrene; Corning Costar) and fluorescence was quantified using a plate reader. The FITC-dextran that passed across the endothelial cell monolayer was normalized to the fluorescence reading from the upper chamber and permeability was calculated as relative fluorescence units.

2.6. Statistical analysis

The comparisons between multiple groups and the overall differences were analyzed by ANOVA with post-hoc comparison (two-tailed student's *t*-test). *P* values < 0.05 were taken as significant.

3. Results

3.1. Evidence for successful transduction of BRE cells with adenovirus carrying p38alpha MAP kinase gene

BRE cells were incubated with empty adenovirus or adenovirus carrying p38 mutants (48 h, MOI~20). Transduction efficiency was monitored by fluorescence microscopy and Western blotting. As shown in Fig. 1A, ~80% of the endothelial cells express GFP and the GFP-positive cells exhibit normal cobblestone morphology. The transduction was further confirmed by Western blot analysis of whole cell lysate from parallel cultures. The p38alpha mutant clone 703 has a single amino acid substitution in the ATP-binding site (K57 to M) and clone 1102 has two altered phosphorylation sites (TGY180–182 to AGF) thus the p38 antibody can still recognize the mutated protein. This analysis demonstrated a 20-fold increase in p38 in cells transduced with the p38alpha mutant as compared with the empty adenovirus transduced cells (Fig. 1B).

3.2. Suppression of VEGF-induced p38 activation by p38alpha MAP kinase mutants

Over expression of the p38alpha mutant should extensively dilute, and thus reduce the effects of wild type p38alpha in the cell. This was confirmed by Western blot analysis of the phosphorylation status of a p38 substrate, MAP kinases-activated protein kinase 2 (MK-2). BRE cells were transduced with either empty adenovirus or adenovirus carrying p38alpha mutant, serum-starved and treated with VEGF (30 ng/ml, 10 min). As shown in Fig. 2, there is a significant reduction of MK-2 phosphorylation after VEGF treatment by both p38 mutant clone 1102 and 703. The results clearly show that the transduction of BRE cells with the p38alpha mutants blocks VEGF-induced p38 activation compared to the cells transduced with the empty adenovirus vector.

3.3. Blockade of VEGF-induced permeability by p38alpha mutant

We next examined the specific involvement of p38alpha in VEGF-induced permeability increase by measuring TER in BRE cell monolayers transduced with empty adenovirus or the p38alpha mutants. As shown in Fig. 3A&B, VEGF reduced TER in cultures transduced with empty adenovirus. Interestingly, the mutant carrying the altered ATP-binding site (clone 703) blocked VEGF-induced TER reduction (Fig. 3A), while the clone containing mutated phosphorylation sites (clone 1102) failed to preserve the barrier function upon VEGF treatment (Fig. 3B). This observation was further verified by analysis of FITC-dextran (70 kD) passage across the endothelial monolayer (Fig. 4). After VEGF treatment there is a 50% increase in tracer flux in cultures transduced with empty adenovirus. This VEGF-induced permeability increase was remarkably reduced in cultures transduced with p38alpha mutant clone 703, whereas clone 1102 has no effect.

3.4. Blockade of VEGF-induced GSK phosphorylation by p38alpha mutant

The above experiments demonstrate that the p38alpha mutant clone 703 carrying an altered ATP-binding site blocked VEGF-induced retinal endothelial cell permeability increase. We have previously established that VEGF-induced expression of uPAR in endothelial cells, a key mediator of barrier breakdown, involves GSK3 deactivation and beta-catenin redistribution (Behzadian et al., 2003). Therefore, we wanted to see if this p38alpha mutant also affects GSK phosphorylation. BRE cells transfected with the p38alpha mutants or empty vector were treated with VEGF and the phosphorylation state of GSK3beta was analyzed by Western blotting (Fig. 5). BRE cells transduced with the empty adenovirus showed increased GSK3beta phosphorylation within 10 min after VEGF treatment. VEGF-induced GSK3beta phosphorylation was markedly reduced in the cells transduced with the p38alpha mutant clone 703. The blocking was not significant in cells transduced with clone 1102.

3.5. Blockade of VEGF-induced beta-catenin cytosolic accumulation and nuclear translocation by p38alpha mutant

The kinase activity of GSK3 is abolished upon its phosphorylation, allowing beta-catenin to escape ubiquitination and accumulate in the cytosol and the nucleus. To evaluate the downstream effects of the p38alpha mutant clone 703, beta-catenin distribution was determined by cell fractionation and Western blotting. One hour after VEGF treatment cytosolic and nuclear fractions were separated and beta-catenin was quantified. There was a significant increase in the amount of beta-catenin in the cytosol as well as in the nucleus in control cells transduced with empty adenovirus and treated with VEGF (Fig. 6). However, in cells transduced with adenovirus carrying p38 mutant clone 703, the VEGF-induced redistribution of beta-catenin was prevented. Together with the results shown in Fig. 5, these data indicate that blockade of p38 activity by clone 703 restores GSK3beta activity and prevents beta-catenin cytosolic accumulation and nuclear translocation.

3.6. Blockade of VEGF-induced uPAR expression by p38alpha mutant

The above results strongly support the role of p38alpha in the VEGF/GSK/beta-catenin signaling pathway. In this experiment, the expression of uPAR as an outcome of VEGF/GSK/beta-catenin signaling was measured. BRE cells transduced with either empty adenovirus or adenovirus carrying dominant negative p38alpha mutant were treated with VEGF and mRNA was harvested 2 h later. Reverse transcription and quantitative PCR were performed following RNA extraction. As shown in Fig. 7, VEGF-induced elevation of uPAR mRNA was abrogated by transducing BRE cells with adenovirus carrying the p38alpha mutant clone 703 but not by p38alpha mutant clone 1102.

4. Discussion

To determine the specific role of p38alpha MAP kinase activity in VEGF-induced endothelial cell permeability increases, this study employed an adenoviral vector to deliver mutants of p38alpha into cultured endothelial cells. Our results show that p38alpha mutant (clone 703) prevents VEGF-induced barrier breakdown, blocks the GSK/beta-catenin signaling pathway and prevents uPAR expression. Consistent with our previous study using pharmacological inhibitors of p38 MAP kinase, these experiments strongly suggest that p38alpha is responsible for VEGF-induced signaling pathway leading to permeability increase. Our findings shed more light on the mechanism by which VEGF increases vascular permeability and introduce a potential therapeutic target for retinal diseases characterized by blood/retinal barrier breakdown.

Breakdown of the blood/retinal barrier (BRB) is a prominent characteristic of many retinal diseases including diabetic retinopathy, retinal vein occlusion and age-related macular degeneration. It leads to macular edema, pathological neovascularization and in later stages, retinal detachment. A number of stimuli have been implicated in BRB dysfunction, including growth factors, inflammatory cytokines and oxidative stress, all of which have been shown to activate p38 (Claudio et al., 1994; Doly et al., 1992; Poulaki et al., 2002). Several molecular mechanisms have been implicated in p38 regulation of barrier function. However, the role of p38alpha in regulating extracellular proteolytic activity and vascular permeability in response to VEGF has not been reported to date. This study links a signaling molecule (p38 alpha), an adherens junction associated protein (beta-catenin) with the proteolytic process (activation of uPA/uPAR system) in endothelial cell permeability increase.

In our previous study, transcriptional activation of beta-catenin and uPAR expression were associated with permeability increase (Behzadian et al., 2003). This study identifies p38alpha as an upstream regulator of GSK phosphorylation/inactivation, beta-catenin transcriptional activation and uPAR expression.

Increased proteolytic activity at the endothelial cell surface and the consequent degradation of the extracellular matrix is a critical feature of vascular permeability increase. Activation of the uPA/uPAR system has been very well studied in tumor metastasis and angiogenesis. Studies have shown that uPAR is expressed at cell-cell and cell-matrix contacts, activating an extracellular proteolytic cascade involving uPA, plasminogen and MMPs. In addition to promoting degradation of the extracellular matrix, increased proteolytic activity contributes to vascular permeability by promoting degradation of VE-cadherin (Navaratna et al., 2007) or release of VEGF and other cytokines.

There are four p38 isoforms in endothelial cells: p38 alpha, beta, gamma and delta. The commonly used p38 inhibitor reagents (SB202190 or SB203580) are pyridinyl imidazole compounds that compete with ATP for the same binding site on p38 kinase and thus inhibit

both p38 alpha and beta subtypes. Previous studies indicate that p38 alpha and beta differentially regulate cellular functions (Silva et al., 2006). In order to determine the specific role of p38alpha in VEGF-induced activation of the GSK/beta-catenin pathway, we used two p38alpha mutants. One with altered ATP-binding site (K57 to M) designated here as clone 703. This mutant can be phosphorylated by upstream signals but lacks kinase activity on downstream substrates. The second mutant (clone 1102) carries altered phosphorylation sites (TGY180–182 to AGF). This mutant cannot be phosphorylated by upstream signals and doesn't have any kinase activity as well.

The fact that the phosphorylation mutant (clone 1102) has limited ability to prevent VEGF-induced GSK/beta-catenin signaling, uPAR expression and endothelial barrier breakdown underscores the complexity of p38 signaling. This may be due to cross talk between p38 feedback signaling and alternative kinase pathways upstream of GSK. These putative pathways may remain active under conditions where unphosphorylated p38alpha (clone 1102) is accumulated in the cell. Whereas accumulation of phosphorylated p38 (703) may block, by negative feedback, most of the other alternative pathways. Probably, it is for the same reason that in the absence of VEGF, the 703 mutant increases TER (Fig. 3A). On the other hand, it appears that the protective effects of the 703 mutant on TER (in the presence of VEGF) are not complete (the same figure). This is probably due to the fact that over expression of p38 mutant (at the MOI used here) considerably dilutes, but does not totally abrogate the activity of the intrinsic p38 kinase normally expressed in the cell.

Regulation of barrier function in endothelial cells is an intricate multi factorial process involving a number of signaling pathways, many of which may converge at the p38 kinase activation level. In addition to p38alpha, there are other modulators of the GSK/beta-catenin signaling pathway. For example, GSK3beta is a well-known substrate for ERK and PI3 kinase/Akt signaling. However, we found that the phosphorylation of ERK and Akt was not blocked by transduction of endothelial cells with the p38alpha mutants used in this study (data not shown). Further investigations are needed to identify the alternative pathways.

Besides mediating vascular permeability, this signaling pathway may also contribute to neovascularization which follows permeability increase in various pathological conditions. It has been shown that constitutive activation of p38alpha is required for uPA/uPAR expression and matrix invasion by breast cancer cells (Huang et al., 2000). Inhibition of the uPA/uPAR system by uPAR gene deletion, pharmacological inhibitors or adenoviral delivery of the noncatalytic amino-terminal fragment of uPA reduces neovascularization in a mouse model of retinopathy of prematurity (Le Gat et al., 2003; McGuire et al., 2003). A recent study also demonstrated the critical role of p38 in regulating chronic inflammation-mediated angiogenesis through MMPs, the downstream target of uPA/uPAR system (Rajashekhar et al., 2011). Moreover, as part of the *Wnt* signaling, the GSK/beta-catenin pathway has been implicated in vascular injury and pathological angiogenesis (Bikkavilli et al., 2008; Chen et al., 2009; Zhou et al., 2010). Thus, the p38alpha-mediated GSK/beta-catenin/uPAR signaling pathway may contribute to abnormal vascular growth as well as vascular hyperpermeability.

5. Conclusion

This study demonstrated the critical role of p38alpha in a VEGF-induced signaling pathway (GSK3 inactivation, beta-catenin nuclear translocation and uPAR expression) that leads to the pathogenesis of endothelial barrier breakdown. The different effects of the kinase mutant and phosphorylation site mutant of p38alpha on VEGF-induced endothelial barrier dysfunction imply the complexity of intracellular p38 signaling and indicate multiple pathways upstream of GSK/beta-catenin. In all, using adenovirus-mediated delivery of

p38alpha mutants, validates the important role of p38 kinase in diseases characterized by increased vascular permeability but also provides useful information for identifying specific signaling pathway as potential therapeutic targets for blood-retinal barrier breakdown.

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Fig. 1.

Transduction of BRE cells with adenovirus carrying p38alpha MAP kinase mutants. Cell morphology and efficiency of transduction were demonstrated by phase contrast and fluorescence microscopy (A). The recombinant adenovirus carries the GFP gene. Thus expression of GFP indicates successful transduction. BRE cells were incubated with empty adenoviral vector or the recombinant vector carrying the kinase mutant of p38alpha (designated clone 703) or the phosphorylation mutant of p38alpha (designated clone 1102) for 12–16 h at an optimal MOI (~20) and then serum-starved for 20 h. Western blott analysis of the cell extracts show a 20-fold increase in p38 protein levels in the cells expressing either p38alpha mutant as compared with the cells carrying the empty adenoviral vector (B). $n = 3$, $\#P < 0.001$ vs. Empty adenovirus group.

Fig. 2. Blockade of p38 activity by p38alpha MAP kinase mutants. Function of the dominant negative p38alpha mutants was evaluated by the phosphorylation of its substrate, MAP kinase activated protein kinase 2 (MK-2). BRE cells transduced with either empty vector or the recombinant adenovirus clone 703 or the recombinant adenovirus clone 1102 were serum-starved and treated with VEGF (30 ng/ml, 10 min). Whole cell lysate was collected and subjected to SDS-PAGE and Western blotting for phosphorylated MK-2. $n = 3$, # $P < 0.05$ vs. Empty adenovirus control, * $P < 0.05$ vs. Empty adenovirus VEGF-treated group.

Fig. 3.

Preservation of endothelial barrier function by p38alpha MAP kinase mutant. Confluent BRE cells grown on fibronectin-coated electrode arrays were incubated with either empty vector or the recombinant adenovirus clone 703 (Fig. 3A) or the recombinant adenovirus clone 1102 (Fig. 3B) for 12–16 h and then serum-starved overnight. The effects of VEGF (30 ng/ml) treatment on TER of the monolayer were demonstrated using the ECIS system. Decreased TER indicates increased permeability.

Fig. 4.

Blockade of VEGF-induced endothelial permeability increase by p38alpha MAP kinase mutant. Effects of VEGF on permeability were assayed using BRE cells plated on porous membrane and incubated with either empty vector or the recombinant adenovirus clone 703 or the recombinant adenovirus clone 1102. Paracellular permeability was quantified by measuring flux of FITC-dextran (70kD). Two hours after VEGF (30 ng/ml) treatment FITC-dextran (10 μ M) was added to the upper chamber. Five hours later, samples (100 μ l in triplicate) were taken from the lower and upper chambers and the relative flux of FITC-dextran across the monolayer was assessed by fluorescence reading. $n = 3$, # $P < 0.05$ vs. Empty-control, * $P < 0.05$ vs. Empty-VEGF treated group.

Fig. 5. Blockade of VEGF-induced GSK3 β phosphorylation by p38 α MAP kinase mutant. BRE cells were incubated with either empty vector or the recombinant adenovirus clone 703 or the recombinant adenovirus clone 1102 for 12–16 h, serum-starved overnight and then treated with VEGF (30 ng/ml, 10 min). To monitor GSK3- β activity status, cell lysates were subjected to SDS-PAGE and Western blotting for analysis of phosphorylated GSK3- β . $n = 4$, # $P < 0.01$ vs. Empty adenovirus control, * $P < 0.01$ vs. Empty adenovirus VEGF-treated group.

Fig. 6.

Blockade of VEGF-induced beta-catenin cytosolic accumulation and nuclei translocation by mutant of p38alpha MAP kinase. BRE cells were incubated with either empty vector or the recombinant adenovirus clone 703 for 12–16 h and treated with VEGF (30 ng/ml, 1 h). Cell lysates were fractionated and membrane-free cytosolic (A) and nuclear (B) preparations were subjected to Western blotting for beta-catenin protein content. GAPDH and Histone H1 served as cytosolic and nuclear markers, respectively. ($n = 3$), [#] $P < 0.05$ vs. Empty adenovirus control * $P < 0.05$ vs. Empty adenovirus VEGF-treated group.

Fig. 7. Inhibition of uPAR expression by a mutant of p38alpha MAP kinase. BRE cells were incubated with either empty vector or the recombinant adenovirus clone 703 or the recombinant adenovirus clone 1102 for 12–16 h and treated with VEGF (30 ng/ml, 2 h). Total cellular RNA was extracted and used for reverse transcription and quantitative real-time PCR analysis. $n = 3$, $\#P < 0.01$ vs. Empty adenovirus VEGF-treated group.