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Inflammatory Cytokine-Specific Alterations in Retinal Endothelial Cell Function

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Abstract

Diabetic retinopathy (DR) is recognized as a chronic low-grade inflammatory disease. Retinal microvascular cell dysfunction and loss play an important role in the pathogenesis of DR. However, the basic mechanisms underlying the development and progression of DR are poorly understood. Many recent studies indicate that increased production of inflammatory factors either systemically and/or locally, is strongly associated with vascular dysfunction during diabetes. Here we sought to determine the specific impact of different inflammatory mediators on retinal endothelial cell (EC) function. Inflammatory mediators TNF- α and IL-1 β attenuated the migration and capillary morphogenesis of retinal EC. These dysfunctions were associated with increased production of reactive oxygen species, expression of inducible nitric oxide synthase, and production of total nitrate/nitrite. Incubation of retinal EC with TNF- α and IL-1 β altered VE-cadherin localization, as well as the expression of other junctional proteins. In addition, TNF- α and IL-1 β also altered the production of various ECM proteins including osteopontin, collagen IV, and tenascin-C. Mechanistically, these changes were concomitant with the activation of the mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) signaling pathways. In contrast, incubation of retinal EC with MCP-1 minimally affected their migratory, junctional, and ECM properties. Together our results indicate that the presence of inflammatory mediators in diabetes may have specific and significant impact on vascular cell function, and contribute to the pathogenesis of DR.

Keywords

Inflammation; Diabetic retinopathy; Cell migration; Endothelial cells; Extracellular matrix proteins; Cell-cell Junctions

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Competing Interests

The authors have no competing interests.

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Introduction

Diabetes predominantly affects the microvascular circulation of the retina and results in a range of microvascular structural changes that are unique to this tissue. The retinal microvessels are composed of endothelial cells (EC), which are resting on a basement membrane surrounded by pericytes (PC) (Darland and D'Amore, 2001; Fruttiger, 2007; Provis et al., 1997). Two of the earliest histopathological lesions, diffuse thickening of the vascular basement membrane and selective loss of PC, go undetected by routine fundus examinations. The earliest clinical manifestation of nonproliferative diabetic retinopathy is the occurrence of microaneurysms, punctate "dot and blot" hemorrhages, venous dilation/beading, hard exudates (extravasation of plasma proteins from leaky vessels), soft exudates (cotton-wool spots: nerve fiber swelling/damage as a consequence of ischemia) and intraretinal microvascular abnormalities (irregular dilation and tortuous varicosities of capillaries) are characteristic lesions of the nonproliferative stage. This stage is accompanied by focal capillary and arteriolar obstruction. The resultant retinal ischemia eventually promotes the production of vasoproliferative factors and subsequent new vessel formation, the hallmark of the proliferative or neovascular stage. The proliferative vascularization of the retina leads to vitreous hemorrhage and retinal detachment, and often if left untreated, destroys vision (Aiello et al., 1998; Chistiakov, 2012; Frank, 2004; Gardner and Antonetti, 2007; Hammes et al., 2011). Current therapies for this debilitating disease may slow disease progression, but they do not reverse visual loss in patients with DR (Brownlee, 2001; Glogorsky et al., 2012). Therefore, understanding the molecular mechanisms by which retinal neovascularization is dysregulated is vital to the development of new therapeutic strategies for DR.

Increased levels of proinflammatory cytokines, including TNF- α and IL-1 β , and chemokines, such as MCP-1, have been detected in the vitreous of patients with DR (Nehmé and Edelman, 2008). Moreover, incubation of human retinal EC with TNF- α and IL-1 β increases glucose consumption, mitochondrial superoxide production, and activation of MAPK and NF- κ B signaling pathways (Oshitari et al., 2008). Recent studies have demonstrated a role for TNF- α in the disruption of tight junction proteins, occludin and claudin-5, and increased vascular permeability through protein kinase C ζ (PKC ζ) activity in retinal EC (Aveleira et al., 2010). TNF- α can stimulate the expression of intracellular adhesion molecule-1 (ICAM-1) and disrupt tight junction protein complexes in EC resulting in increased permeability (Brownlee, 2001; Chen et al., 2012; El-Remessy et al., 2003; Tang and Kern, 2011; Zhang et al., 2011). In addition, inhibitors of the TNF- α receptor, as well as the blockade of NF- κ B, attenuate retinal EC apoptosis during diabetes (Joussen et al., 2009; Joussen et al., 2004).

In spite of the growing body of evidence that supports the involvement of proinflammatory cytokines in the development and progression of DR, the detailed mechanisms and cellular targets involved are not clearly defined. Furthermore, it is not known how inflammatory mediators produced, systemically and/or locally, during diabetes affect various cell types in the retinal vasculature. Thus, further investigation into the specific inflammatory mediators' affect on retinal vascular cells is needed. Here our study was designed to test the hypothesis that inflammatory mediators, including TNF- α , IL-1 β and MCP-1, differentially contribute to the dysfunction of retinal EC through increased oxidative stress and activation of MAPK and NF- κ B signaling pathways.

Material and methods

Experimental animals

All experiments were carried out in accordance to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health. Immortomice expressing a temperature-sensitive SV40 large T antigen were obtained from Charles River Laboratories (Wilmington, MA), and backcrossed into C57BL/6 mice in our laboratory as previously described (Huang and Sheibani, 2008; Su et al., 2003).

Reagents

Recombinant mouse cytokines, TNF- α and IL-1 β were purchased from Peprotech (Rocky Hills, NJ). Recombinant mouse MCP-1 was purchased from R&D Systems (Minneapolis, MN) or Peprotech.

Tissue preparation, isolation and culture of mouse retinal EC

Eyes from one litter (6 to 7 pups) of 4 week-old wild type immortomice were enucleated and hemisected. The retinas were dissected out aseptically under a dissecting microscope and kept in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Grand Island, NY) containing penicillin/streptomycin (Sigma, St. Louis, MO). Retinas (12 to 14 from one litter) were pooled together, rinsed with DMEM, minced into small pieces in a 60 mm tissue culture dish using sterilized razor blades, and digested in 5 ml of collagenase type I (1 mg/ml in serum free DMEM, Worthington, Lakewood, NJ) for 30–45 min at 37°C. Following digestion, DMEM with 10% FBS was added and cells were pelleted. The cellular digests then were filtered through a double layer of sterile 40 μ m nylon mesh (Sefar America Inc., Fisher Scientific, Hanover Park, IL), centrifuged at 400 $\times g$ for 10 min to pellet cells, and cells were washed twice with DMEM containing 10% FBS. The cells were suspended in 1.5 ml medium (DMEM with 10% FBS), and incubated with sheep anti-rat magnetic beads pre-coated with anti-PECAM-1 (MEC 13.3, BD Biosciences, Bedford, MA). After affinity binding, magnetic beads were washed six times with DMEM with 10% FBS and bound cells in endothelial cell growth medium were plated into a single well of a 24 well plate pre-coated with 2 μ g/ml of human fibronectin (BD Biosciences). Endothelial cells were grown in DMEM containing 10% FBS, 2 mM L-glutamine, 2 mM sodium pyruvate, 20 mM HEPES, 1% non-essential amino acids, 100 μ g/ml streptomycin, 100 U/ml penicillin, freshly added heparin at 55 U/ml (Sigma, St. Louis, MO), endothelial growth supplement 100 μ g/ml (Sigma) and murine recombinant interferon- γ (R & D, Minneapolis, MN) at 44 U/ml. Cells were maintained at 33°C with 5% CO₂. Cells were progressively passed to larger plates, maintained, and propagated in 1% gelatin-coated 60 mm dishes.

Flow Cytometry

Retinal EC were incubated with TNF- α , IL-1 β or MCP-1 (10 ng/ml) in EC Growth medium for 24 h on 60 mm culture plates. Plates were rinsed with phosphate buffered saline (PBS) containing 0.04% EDTA and incubated with 1.5 ml of cell dissociation solution (Tris-buffered saline [20 mM Tris-HCl and 150 mM NaCl; pH 7.6] TBS containing 2 mM EDTA and 0.05% BSA). Cells were rinsed from plates with DMEM containing 10% FBS, washed once with 5 ml TBS, and then blocked in TBS with 1% goat serum for 20 min on ice. Cells were centrifuged 5 min at 400 $\times g$, medium aspirated, resuspended in 0.5 ml TBS with 1% BSA containing an appropriate dilution of primary antibody (as recommended by the supplier), and incubated on ice for 30 min. Cells were incubated with rat anti-mouse VEGF receptor-1 (VEGFR-1) or control IgG (R&D Systems). Cells were washed twice with TBS

with 1% BSA and incubated with the appropriate FITC-conjugated secondary antibody for 30 min on ice. Cells were then washed twice with TBS with 1% BSA, resuspended in 0.5 ml TBS with 1% BSA, and analyzed using the FACScan Caliber flow cytometer (Becton-Dickinson, San Jose, CA). The isotype control was FITC-labeled isotype IgG as specifically stated above. Ten thousand cells were analyzed for each sample and at least three independent experiments were performed.

Apoptosis and cell viability

Apoptosis was determined by measuring caspase activation using Caspase-Glo 3/7-assay kit as recommended by the supplier (Promega, Madison, WI). The assay provides caspase-3/7 DEVD-aminoluciferin substrate and the caspase 3/7 activity is detected by luminescent signal. For the assay, retinal EC were plated at 8×10^3 cells per well of a 96 well plate and the next day incubated with 10 ng/ml TNF- α , IL-1 β or MCP-1 for 24 h. Caspase activity was detected using a luminescent microplate reader (Victor2 1420 Multilabel Counter, PerkinElmer, Waltham, MA). All samples were prepared in triplicate and repeated at least three times with similar results.

TUNEL staining was performed to confirm apoptosis. Retinal EC were plated at 1×10^5 in 4-well chamber slides (Lab-TEK, NUNC) coated with 2 μ g/ml fibronectin (BD Biosciences). Cells were incubated with TNF- α , IL-1 β , or MCP-1 (10 ng/ml) for 24 h or 48 h. The Click-iT TUNEL Alexa Fluor 594 kit (Invitrogen) utilizes a dUTP modified with an alkyne to detect apoptotic cells. Apoptotic cells were determined according to manufacturer's instructions. This assay was repeated at least three times with similar results.

Cellular viability of EC was determined using the CellTiter 96[®] Aqueous Non-Radioactive cell proliferation assay (MTS; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega). Retinal EC were plated at 8×10^3 cell per well of a 96 well plate and incubated with 10 ng/ml TNF- α , IL-1 β or MCP-1 for 24 h or 48 h in EC growth medium and incubated further with MTS solution according to manufacturer's instructions. The viability was determined by measuring absorbance at 490 nm using a microplate reader (Thermomax, Molecular Devices, Sunnyvale, CA), and determined as a percentage of control untreated cells. All samples were prepared in triplicate and repeated at least three times with similar results.

Scratch wound assays

Retinal EC were plated at 8×10^5 cells in 60 mm dishes and allowed to reach confluence (1–2 days). Cell monolayers were wounded with a 1 mL micropipette tip, rinsed with DMEM containing 10% FBS twice, and fed with EC growth medium with 10 ng/ml TNF- α , IL-1 β or MCP-1. Medium was refreshed every 24 h. Wound closure was monitored and photographed at 0, 24, 48 and 72h using a phase microscope in digital format. For quantitative assessment, the distances migrated as percent of total distance were determined.

Transwell migration assays

Prior to the assay, cells were incubated in serum-free EC growth medium for 24 h. Transwell filters (Corning, Acton, MA) were coated with 2 μ g/ml fibronectin in PBS and incubated overnight at 4°C. The bottom of the transwell was rinsed with PBS and blocked with 2% BSA in PBS for 1 h at room temperature. The transwell was rinsed with PBS and 500 μ l serum-free DMEM was added to the bottom of each well. Cells, suspended at 1×10^5 cells in 100 μ l of serum-free medium, were added to the top of the transwell membrane. Cytokines and chemokines were added to both top and bottom of the transwell. Following 4 hours in a 33°C tissue culture incubator, the cells and medium were aspirated and the upper side of the membrane was wiped with a cotton swab. The cells that had migrated through the

membrane were fixed with 4% paraformaldehyde, stained with hematoxylin-eosin, and mounted on a slide. Ten high power fields (x200) were counted for each condition and the average and standard error of the means were determined. All samples were prepared in duplicate and the experiment repeated at least three times with similar results.

Western blot analysis

Retinal endothelial cells were plated at 8×10^5 in 60 mm culture dishes and incubated in EC growth medium and 10 ng/ml TNF- α , IL-1 β , or MCP-1 for 24h. Cells were rinsed once in 0.2% EDTA in PBS and lysed in 100 μ l of lysis buffer (50mM HEPES pH 7.5, 100mM NaCl, 0.1M EDTA, 1mM CaCl₂, 1mM MgCl₂, 1% Triton X-100, 1% NP-40, 0.5% deoxycholate, and protease inhibitor cocktail (Roche Biochemicals, Mannheim, Germany), briefly sonicated, and centrifuged at 400 $\times g$ for 10 min at 4 °C. Protein concentrations were determined using the BCA protein assay kit (Pierce, Thermo Scientific, Rockford, IL). Samples were adjusted for protein content (50 μ g), mixed with appropriate volume 6x SDS-sample buffer, and analyzed by SDS-PAGE (4–20% Tris-glycine gels, Invitrogen). Proteins were transferred to nitrocellulose membrane and the membrane was blocked with blocking buffer (0.05% Tween-20 and 5% skim milk in TBS). Membranes were incubated with COX-2, VE-cadherin (Santa Cruz), iNOS, Caveolin-1, N-Cadherin, p120-Catenin (BD Biosciences), Occludin, ZO-1 (Zymed), Claudin-5 (Life Technologies), and β -actin (Sigma).

To analyze secreted proteins, retinal endothelial cells were plated at 7×10^5 in 60 mm culture dishes. After 24 h, cells were rinsed once with serum-free DMEM and incubated with either 10 ng/ml TNF- α , IL-1 β or MCP-1 for 48h in serum-free EC growth medium. Conditioned medium was collected and clarified by centrifugation. Cells were rinsed once in 0.2% EDTA in PBS and lysed in 100 μ l of lysis buffer (50mM HEPES pH 7.5, 100mM NaCl, 0.1M EDTA, 1mM CaCl₂, 1mM MgCl₂, 1% Triton X-100, 1% NP-40, 0.5% deoxycholate, and protease inhibitor cocktail (Roche Biochemicals), briefly sonicated and centrifuged at 400 $\times g$ for 10 min at 4C. Membranes were incubated with mouse anti-human TSP1 (A6.1 Neo Markers, Fremont, CA), rabbit anti-rat fibronectin (Life Technologies), rat anti-chicken tenascin-C, rabbit anti-mouse Collagen IV (Milipore), goat anti-mouse osteopontin (R&D Systems), and mouse monoclonal HB-EGF (Santa Cruz).

To assess Akt, ERK, JNK, p38, STAT3, RelB, and p65 activation, cells were plated at 7×10^5 cells on 60 mm tissue culture plates in EC growth medium. The next day, the cells were incubated with TNF- α , IL-1 β , or MCP-1 (10 ng/ml) for 24 h. Cells were rinsed twice with cold serum-free medium containing 1 mM Na₃O₄Va and subsequently rinsed with 1x PBS containing 1 mM Na₃O₄Va and 3 mM NaF. Cells were lysed, briefly sonicated and centrifuged and analyzed by SDS-PAGE as described above. Membranes were incubated with anti-JNK, anti-phospho-JNK (R&D Systems), rabbit anti-Akt, rabbit anti-phospho-Akt, rabbit anti-ERK1/2, mouse anti-phospho-Erk1/2, rabbit anti-p38, rabbit anti-phospho-p38 (Cell Signaling), rabbit anti-p65, rabbit anti-phospho-p65, rabbit anti-phospho RelB, rabbit anti-RelB, rabbit anti-STAT3, and mouse anti-phospho-STAT3 (Santa Cruz). Membranes were washed, incubated with horseradish-peroxidase-conjugated secondary antibody (1:5000, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature, and the protein was visualized according to the chemiluminescent procedure (Chemiluminescence reagent; GE Biosciences). The mean band intensities were measured densitometrically using Image J 1.46a (National Institutes of Health, Bethesda, MD).

Total Nitrate/Nitrite measurements

The amount of total endogenous nitrate/nitrite produced by retinal EC was determined using a Mouse Parameter Nitrate/Nitrite Immunoassay kit (R&D Systems). Cells were plated at 7×10^5 on 60 mm tissue culture dishes in EC growth medium. After 24 h, cells were rinsed

twice with serum-free DMEM and incubated with 10 ng/ml TNF- α , IL-1 β , or MCP-1 for 48 h in 2 ml of serum-free EC growth medium. The conditioned medium was collected, centrifuged for 5 min at 400 $\times g$ to remove cell debris, and the nitrate/nitrite was analyzed according to manufacturer's instructions. The amount of nitrate/nitrite was determined using a standard curve generated with known amounts of nitrate/nitrite in the same experiment.

Secreted VEGF measurements

The amount of secreted VEGF produced by retinal EC was determined using a Mouse VEGF Immunoassay kit (R&D systems). Cells were plated at 8×10^5 on 60 mm tissue culture dishes in EC growth medium. After 24 h, the cells were rinsed once with serum-free DMEM and incubated with 10 ng/ml TNF- α , IL-1 β or MCP-1 for 48 h in 2 ml of serum-free EC growth medium. The conditioned medium was collected, centrifuged for 5 min at 400 $\times g$ to remove cell debris, and the secreted VEGF was analyzed according to manufacturer's instructions. The amount of VEGF was determined using a standard curve generated with known amounts of VEGF in the same experiment. This assay was repeated three times with similar results.

Capillary morphogenesis assays

Tissue culture plates (35 mm) were coated with 0.5 ml Matrigel (10mg/ml, BD Bioscience, San Jose, CA) and allowed to harden by incubating at 37°C for at least 30 min. Prior to the assay, cells were incubated with 10ng/ml TNF- α , IL-1 β or MCP-1 for 24 h. The next day, cells were removed by trypsin EDTA and suspended at 1×10^5 cells/ml in serum free EC growth medium. Cells in a 2 ml volume were applied to the Matrigel-coated plates, incubated with 10 ng/ml TNF- α , IL-1 β or MCP-1 and photographed after 18 h using a Nikon microscope in a digital format. For quantitative assessment of the data, the mean number of branch points was determined by counting the number of branch points in five high-power fields ($\times 20$).

Determination of ROS levels

ROS levels were determined by staining cells with dihydroethidium (DHE; Life Technologies). DHE is oxidized to red fluorescent ethidium by $O_2^{\bullet -}$ in the cytosol and intercalates in the DNA. Retinal EC were plated at 5×10^4 cells in 4-well chamber slides (Lab-TEK, NUNC) coated with 2 μ g/ml fibronectin (BD Biosciences). Cells were incubated with 10 ng/ml TNF- α , IL-1 β , or MCP-1 for 24 h in EC growth medium. Cells were loaded with 10 μ M DHE for 20 min, washed with EC growth medium, and incubated with EC growth medium for two 30 min recovery periods. Fluorescent intensity was analyzed with a fluorescent microscope (Carl Zeiss Optical, Germany) and images were captured in digital format. Three independent experiments were performed. For quantitative assessment, the mean fluorescent intensities were determined using Image J 1.46a. Representative images are shown.

Indirect immunofluorescent

Cells were plated at 1×10^5 in 4-well chamber slides (Lab-TEK, NUNC) coated with 2 μ g/ml fibronectin. Cells were incubated with 10 ng/ml TNF- α , IL-1 β , or MCP-1 for 24 h in EC growth medium. Chambers were washed in PBS, fixed with 4% paraformaldehyde containing 0.1% TritonX-100 at room temperature, and blocked with 1% BSA in TBS at 37°C for 20 min. Slides were washed three times with TBS by dipping and incubated with goat anti-VE-cadherin (Santa Cruz), rabbit anti-vinculin (Sigma), and phalloidin/Alexa Fluor488 (Invitrogen) in TBS containing 1% BSA at 37°C for 40 min. After washing with TBS, cells were incubated with appropriate Cy3-conjugated secondary antibody in TBS containing 1% BSA at 37°C for 40 min. Cells were washed with TBS three times, mounted

with a 1:1 TBS:glycerol solution with DAPI, and analyzed with a fluorescent microscope (Carl Zeiss Optical, Germany). Images were captured in digital format. Three independent experiments were performed and representative images are shown.

Statistical analysis

Statistical differences between samples were evaluated with student's unpaired t-test (2-tailed) or two-way ANOVA with Bonferroni correction for multiple comparisons when appropriate. Data are represented as mean $\pm$ SEM. Each result is representative of at least three independent experiments. All statistical assessments were evaluated at the 0.05 level of significance. Statistical analyses were performed with GraphPad Prism statistical software (GraphPad Software, La Jolla, CA).

Results

TNF- α and IL-1 β did not alter EC viability but activated caspase 3/7

To exclude the possibility that the EC are undergoing apoptosis in response to TNF- α , IL-1 β , or MCP-1, we assessed cell viability after incubation for 24 and 48 h. We observed no change in cell viability in response to TNF- α , IL-1 β , or MCP-1 after 24 or 48 h. (Fig. 1A). We next addressed whether TNF- α , IL-1 β or MCP-1 induce apoptosis. Apoptotic cell death was determined by evaluation of the activation status of caspase-3/7. Both TNF- α and IL-1 β activated caspase-3/7 after 24 h. Further induction was observed after 48 h, with TNF- α activating caspase-3/7 more than IL-1 β (Fig. 1B). Caspase 7 has been documented to be activated during inflammatory processes (Oshitari et al., 2008), thus we evaluated late stage apoptosis using TUNEL staining to determine the contribution of Caspase 3/7 activation to late stage apoptosis. No increase in the rate of apoptosis was observed in the TNF- α , IL-1 β , or MCP-1 treated EC after 24 or 48 h by counting the number of TUNEL positive cells (Fig. 1C). Hoescht was used to stain the cell nuclei. Less than 1% cell death was observed in both control and cells incubated with inflammatory mediators. Thus, TNF- α and IL-1 β increased caspase-3/7 activation after short-term exposure. However, the 24 or 48 h exposure was not sufficient to increase the late-stage DNA fragmentation associated with cell death.

TNF- α and IL-1 β inhibited capillary morphogenesis and migration of retinal EC

Angiogenesis is fundamental in vascular development and remodeling during tissue injury. Vascular injury promotes production of inflammatory cytokines. We next determined whether capillary morphogenesis was affected by incubation of EC with TNF- α , IL-1 β or MCP-1. Fig. 2A shows that EC formed a well-branched capillary-like network. In contrast, both TNF- α and IL-1 β inhibited EC ability to organize into a capillary-like network. A longer incubation of the cells did not result in further branching morphogenesis. However, MCP-1, from two different sources, did not have any effect on retinal EC ability to form a capillary-like network. Quantitative assessment of the data is shown in Figure 2C (***P<0.0001, **P<0.001, n = 3).

To address whether TNF- α , IL-1 β or MCP-1 contribute to the inhibition of capillary morphogenesis through inhibition of cell migration, we utilized both scratch wound and transwell migration assays. Wound-healing assays were carried out using confluent retinal EC incubated with TNF- α , IL-1 β , or MCP-1. After 48 h, the wound inflicted on the control cultures was approximately 90% closed, whereas EC incubated with TNF- α and IL-1 β remained creviced (Fig. 2B). We observed an approximately 45% decrease in wound closure after exposure to TNF- α and 30% decrease with IL-1 β . Similar to untreated cells, cells incubated with MCP-1 fully closed the wound after 48 h, indicating an accelerated rate of wound closure. The quantitative assessments are shown in Fig. 2D.

To validate the wound assay results, retinal EC were seeded into the upper wells of transwell chambers with TNF- α , IL-1 β or MCP-1. We observed a decrease in the number of retinal EC that migrated through the membrane after TNF- α and IL-1 β , but not MCP-1 incubation (Fig. 2E). There was an approximately 40% decrease in cell migration after exposure to TNF- α and IL-1 β . These results supported our hypothesis that inhibition of cell migration negatively impact EC ability to undergo capillary morphogenesis. To further investigate the effects of inflammatory mediators on cell morphology and spreading, we stained cells using antibodies against vinculin and phalloidin which is used to label actin filaments. Vinculin is a membrane-cytoskeletal protein in focal adhesion plaques that is involved in linkage of integrins to the actin cytoskeleton. Loss of vinculin is associated with reduced cell adhesion and spreading, accompanied by reduced stress fiber formation and reduced migration. In cells exposed to TNF- α and IL-1 β , vinculin staining was reduced. The cells incubated with TNF- α or IL-1 β (Fig. 2Fb, c) also did not appear to spread as well as control cells (Fig. 2Fa) or cells incubated with MCP-1 (Fig. 2Fd) as indicated by their actin cytoskeleton organization.

Induction of iNOS by TNF- α and IL-1 β and increased oxidative stress in EC

Increasing evidence suggests that oxidative stress plays a causal role in the development of diabetic microvascular complications in the retina (Yamagishi and Matsui, 2011). Oxidative stress has been postulated to be a cause for the increase in retinal inflammation and vascular permeability observed in patients with DR (Giacco and Brownlee, 2010; Kim et al., 2013; Tan et al., 2011; Zheng and Kern, 2009). We next examined whether TNF- α , IL-1 β or MCP-1 induce oxidative stress in EC using dihydroethidium staining. Retinal EC exhibited increased oxidative stress in the presence of TNF- α or IL-1 β , but not MCP-1 (Fig. 3A). The mean fluorescent intensity was evaluated using Image J in Fig. 3B. Representative images are shown.

Since we demonstrated that the inflammatory cytokines induced oxidative stress, we next determined whether inducible nitric oxide synthase plays a role in generating oxidative stress. Nitric oxide synthases (NOSs) are a family of enzymes that catalyze the production of nitric oxide (NO) (Kibbe et al., 1999; Yamagishi and Matsui, 2011). Nitric oxide is a pleiotropic molecule that is key player in a number of physiological and pathological processes. High amounts of NO produced by inducible NO synthase (iNOS) and/or peroxynitrite (ONOO⁻), a reactive intermediate of NO with the superoxide anion, are involved in pro-inflammatory reactions and tissue damage (Kibbe et al., 1999). Incubation of retinal EC with TNF- α and IL-1 β significantly increased iNOS expression (Fig. 3C). We observed an approximately 20-fold increase with TNF- α and 50- to 80-fold increase with IL-1 β . Quantitative assessment of the Western blot data is shown in Fig. 3E. We further evaluated total NO levels after both 24 and 48 h of exposure to TNF- α , IL-1 β or MCP-1. Both TNF- α and IL-1 β increased total NO levels compared to control and MCP-1 after 24 h. A further increase in total NO was observed after 48 h of exposure (Fig. 3D). These results emphasize a link between inflammation, iNOS expression, and oxidative stress.

TNF- α and IL-1 β altered VE-Cadherin localization and production of junctional proteins in EC

A growing body of evidence indicates that proinflammatory cytokines contribute to vascular permeability in DR (Avelaira et al., 2010; Frey and Antonetti, 2011; Wolburg et al., 1994). Changes in retinal vascular permeability may result from alterations of tight and adherence junction proteins, including transmembrane protein occludin, the claudin family, and the junction adhesion molecule (JAM) family (Hartsock and Nelson, 2008). To determine whether TNF- α , IL-1 β or MCP-1 altered VE-cadherin localization the cellular localization was evaluated by immunofluorescence microscopy. In control EC, VE-cadherin appeared as

a near continuous staining at the cell border as indicated by arrow heads (Fig. 4A). In contrast, TNF- α and IL-1 β disrupted VE-cadherin localization to the junction in EC, leading to a fragmented staining pattern at the cell border, as indicated by arrows. Similar to control cells, cells incubated with MCP-1 exhibited continuous staining at the cell borders.

To determine whether these alterations were due to changes in protein expression, VE-cadherin was evaluated after 24 h of exposure to TNF- α or IL-1 β . No changes in VE-cadherin protein levels were observed via Western blot analysis (Fig. 4B). To further determine if TNF- α , IL-1 β , or MCP-1 alter expression of other junctional protein expression, lysates were collected after 24 h of exposure and subjected to Western blot analysis. TNF- α and IL-1 β significantly decreased occludin, zona-occluden-1 (ZO-1), and claudin-5 levels in retinal EC. In contrast, TNF- α and IL-1 β significantly increased N-cadherin and p120-catenin expression (Fig. 4B). The quantitative assessment of the data is shown in Fig. 4C.

VEGF production by EC increased after TNF- α and IL-1 β exposure

Vascular endothelial growth factor (VEGF) is an inflammatory mediator with a significant role in ocular inflammation, neovascularization, and vascular permeability (Amin et al., 1997; El-Remessy et al., 2003; Joussen et al., 2004; Lu et al., 2012; Murata et al., 1996; Tang and Kern, 2011). To determine whether incubation of retinal EC with TNF- α , IL-1 β or MCP-1 increases the secretion of VEGF, a VEGF immunoassay was employed. We observed a 13-fold increase in the level of VEGF produced by retinal EC exposed to TNF- α , and a 6-fold increase with IL-1 β , but no change with MCP-1 exposure. Quantitative assessment of VEGF levels is shown in Fig. 4D.

VEGFR-1 is a 180–185 kDa glycoprotein that is expressed on vascular cells throughout development and in the adult, and is activated in response to binding VEGF-A, VEGF-B, or PlGF (Koch et al., 2011). Deletion studies of the intracellular domain of VEGFR-1 revealed that VEGFR-1 may act as a VEGF decoy, to control VEGFR-2 signaling and angiogenesis. VEGFR-1 plays an important role in the inflammatory responses and pathological angiogenesis through the recruitment of monocytes (Koch et al., 2011). We next determined whether TNF- α alters VEGFR-1 expression using flow cytometry. Incubation of retinal EC with TNF- α for 24 h increased VEGFR-1 expression by 1.5-fold (Fig. 4E; * $P < 0.05$). The impact of IL-1 β and MCP-1 on VEGFR-1 expression was not significant (not shown).

Cyclooxygenase-2 (COX2) is a membrane-bound enzyme involved in the formation of prostaglandins (PGs) and thromboxanes from arachidonic acid. PGs, along with other soluble factors including bradykinin and leukotrienes, increase vascular permeability and contribute to fluid extravasation and edema (Kern, 2007). The impact of TNF- α , IL-1 β , and MCP-1 on COX2 expression was assessed. TNF- α and IL-1 β increased COX2 protein levels, but MCP-1 did not (Fig. 4F). Quantitative assessment of the Western data is depicted in Fig. 4G. We observed a 1.8-fold increase with TNF- α and 2.5-fold increase following IL-1 β exposure. Together, these data show that proinflammatory cytokines may significantly contribute to the increased vascular permeability observed during diabetes through multiple mechanisms.

TNF- α and IL-1 β increased ECM protein production in retinal EC

Increases in extracellular matrix proteins have been implicated in the development of DR (Huang and Sheibani, 2008; Joussen et al., 2004; Tang and Kern, 2011). Fibronectin, collagen IV, osteopontin, tenascin-C, and thrombospondin-1 (TSP1) are constituents of the ECM with significant roles in tissue remodeling, repair, cell migration, and vascular inflammation. We next investigated whether TNF- α , IL-1 β , and MCP-1 alter the expression

and production of various ECM proteins. In the presence of TNF- α or IL-1 β , EC increased expression and/or production of ECM proteins tenascin-C, collagen IV, and osteopontin (Fig. 5A, B). Changes in fibronectin were not statistically significant. We did not observe changes in EC TSP1 expression and secretion after exposure to inflammatory mediators (Fig. 5C, D).

Heparin binding EGF-like growth factor (HB-EGF) is a member of the EGF family that plays an important role in recruitment of PC to newly forming blood vessels (Stratman et al., 2010). The role of HB-EGF during the development and progression of DR is unclear. We next investigated whether TNF- α , IL-1 β or MCP-1 alter HB-EGF production in retinal EC by Western blot analysis. In the presence of TNF- α and IL-1 β , EC increased expression of HB-EGF, however release into the conditioned medium was undetectable in untreated and treated cells. MCP-1 did not alter the production of HB-EGF (Fig. 5A, D). The significance of increased HB-EGF level in EC incubated with inflammatory mediators is presently unknown.

The disruption of caveolae-mediated endocytosis partially prevents fibronectin degradation (Sottile and Chandler, 2005). Thus, caveolin-1 dependent signaling may be necessary for fibronectin internalization and degradation. In addition caveolae plays a critical role in various signaling pathways. Since we observed increased ECM protein deposition, we next assessed the expression of caveolin-1 in EC exposed to TNF- α , IL-1 β , and MCP-1 using Western blot analysis. Caveolin-1 expression was decreased after 24 h of exposure to TNF- α and IL-1 β but not MCP-1. Caveolin-1 expression was dramatically reduced after 48 h of exposure (Fig. 5E). Quantification of the Western data is provided in Fig. 5F. Further analysis of caveolin-1 by immunofluorescence in the presence of TNF- α revealed a significant reduction in caveolin-1 expression, as well as localization to the cell junction (Fig. 5G). Thus, reduced expression of caveolin-1 may have significant impact on formation of caveolae and turnover of the ECM and signaling proteins.

Activation of intracellular signaling pathways by TNF- α and IL-1 β in retinal EC

The mitogen activated protein kinases (MAPKs), including ERKs, p38 MAPK, and the c-Jun N-terminal Kinase (JNK), as well as nuclear factor- κ B (NF- κ B), Akt, and signal transducers and activators of transcription (STAT) pathways, specifically STAT3, play crucial roles in regulating cellular stress, inflammation, angiogenesis and survival, and are activated in DR (Aveleira et al., 2010; Chen and Han, 2008; Huang and Sheibani, 2008; Ibrahim et al., 2010; Kant et al., 2011; Tan et al., 2011; Zhang et al., 2011). To establish whether TNF- α , IL-1 β or MCP-1 impacted these signaling pathways and their phosphorylation status, Western blot assays were performed. As shown in Fig. 6A and B, stimulation with TNF- α and IL-1 β increased phosphorylation of ERK1/2, JNK, and p38 MAPK activity. No significant change in Akt phosphorylation or total protein level was observed. TNF- α and IL-1 β increased phosphorylation of p65 and RelB and also increased total RelB expression (Fig. 6C). MCP-1 exposure increased p65 phosphorylation, but did not affect any of the other pathways examined.

Mechanistically, STAT3 plays an important role during angiogenesis, in both physiological and pathological conditions affecting cell survival, proliferation, inflammation, and oncogenesis. STAT3 participates in angiogenesis, in part, through modulation of VEGF expression (Bartoli et al., 2003; Chen and Han, 2008). We also determined if TNF- α or IL-1 β impact STAT3 activation. We observed a 2.5-fold increase in the level of phosphorylated STAT3 in cells exposed to TNF- α and 4.5-fold increase with IL-1 β for 24 h (Fig. 6D). MCP-1 did not affect the level of phosphorylated STAT3. No significant change in total STAT3 protein from control was observed with any treatment. Increased STAT3

activation was consistent with the increased VEGF production observed after exposure to these inflammatory cytokines.

Discussion

Here we investigated the role of TNF- α , IL-1 β , and MCP-1 in inflammatory induced functional changes in retinal EC. Vitreous samples from patients with diabetic retinopathy exhibit higher levels of IL-6, IL-1 β , and TNF- α (Oh et al., 2010; Yuuki et al., 2001). Intravitreal administration of IL-1 β in rat retinas resulted in NF- κ B activation, increased vascular permeability, increased leukostasis, and formation of acellular capillaries (Kowluru and Odenbach, 2004). We demonstrated that TNF- α and IL-1 β attenuated retinal EC migration and capillary morphogenesis. These cells also exhibited increases in oxidative stress, iNOS expression, and total nitrate/nitrite production. Furthermore, these inflammatory mediators altered VE-cadherin junctional localization, as well as decreased expression of other junctional proteins including occludin and ZO-1. An increase in proinflammatory markers including VEGF, VEGFR-1, and COX-2 were observed in EC incubated with TNF- α and IL-1 β . In addition, we showed alterations in the production of various ECM proteins including collagen IV, tenascin-C, and osteopontin. These changes were concomitant with sustained activation of MAPK and NF- κ B signaling pathways.

Incubation with MCP-1 minimally impacted retinal EC function at the concentration used in these studies. Nawaz et al. demonstrated MCP-1 vitreal concentration to be approximately 2 ng/ml from proliferative DR patients. However, they were unable to demonstrate phosphorylation of ERK at concentrations lower than 30 ng/ml using human retinal microvascular endothelial cells (HMREC) (Nawaz et al., 2013). Another study showed that 1 ng/ml MCP-1 did promote angiogenesis in the chick CAM assay. However, maximal angiogenesis was observed using 10 ng/ml (Salcedo et al., 2000). In our studies, higher concentrations of MCP-1 (20 ng/ml and 50 ng/ml) promoted retinal EC migration using transwell assays (not shown). Thus, the effective concentration of MCP-1 may need to be higher to achieve a response using our culture model, similar to other studies described. We also confirmed the expression of MCP-1 receptor, CCR2, in our cells (not shown). CCR2 expression is also detected in human umbilical vein and dermal microvascular endothelial cells (Nawaz et al., 2013; Salcedo et al., 2000). Thus, inflammatory mediators may function in a cell specific manner contributing to vascular dysfunction during diabetes.

TNF- α and IL-1 β also increased bovine retinal EC permeability, while decreasing protein and mRNA content of tight junction proteins ZO-1 and claudin-5 (Aveleira et al., 2010). We observed alterations in VE-cadherin junctional localization further demonstrating the impact of inflammatory mediators on vascular permeability. TNF- α and IL-1 β also increased p120-catenin expression in retinal EC. The central function of p120-catenin is to regulate cadherin stability. This is mediated through the ability of p120 catenin to prevent clathrin-dependent endocytosis of VE-cadherin and stabilization of VE-cadherin at the plasma membrane (Xiao et al., 2005). Thus, the increase in p120 catenin expression in response to inflammatory mediators may be a feedback response to stabilize VE-cadherin at the cell membrane. In fact, the binding of p120 to the juxtamembrane domain of E-cadherin is recently shown to prevent its internalization and degradation (Hartsock and Nelson, 2012). In addition, increased N-cadherin expression may similarly compensate for the lack of VE-cadherin junctional localization (Luo and Radice, 2005). However, the significance of these inflammatory mediated changes in retinal EC junctional dysfunction and pathogenesis of diabetic retinopathy needs further investigation.

Remodeling of the extracellular matrix is a dynamic process that occurs during wound healing and pathological conditions such as diabetic retinopathy (Ljubimov et al., 1996).

Changes in ECM composition and increases in basement membrane thickness are early characteristic changes in diabetic retinopathy (Kern, 2007). We have previously demonstrated that the matricellular protein TSP1 is decreased in the vitreous of diabetic rats and patients with diabetic retinopathy (Sheibani et al., 2000; Wang et al., 2009). Osteopontin, also known as profibrotic adhesion molecule, is up regulated in kidneys of humans and mice with diabetes (Lund et al., 2009). Increases in tenascin-C mRNA have also been detected in diabetic retinas compared to normal retina (Mitamura et al., 2002). Tenascin-C is a large hexameric ECM glycoprotein that modulates cell growth, adhesion and angiogenesis, whose expression is affected by TGF- β 1 during diabetes (Castellon et al., 2002). Our studies demonstrated an important role for these inflammatory mediators in modulation of ECM composition of the endothelium. Further investigation is necessary to fully elucidate the contribution of these changes to vascular dysfunction and progression of the diabetic retinopathy.

Inducible nitric oxide synthase up-regulation has been found in retinas of diabetic rodents and patients with DR (Du et al., 2002; Kern, 2007; Yamagishi and Matsui, 2011). Diabetic mice, in which iNOS has been deleted or inhibited did not develop acellular capillaries or vascular permeability in the retina. Using an iNOS inhibitor, aminoguanidine, Du et al. observed decreases in NO production, iNOS expression and more importantly, Zhang et al. did not observe any development of microvascular lesions of DR in mice, rats, or dogs (Du et al., 2002; Zhang et al., 2011). We observed a significant increase in iNOS expression after TNF- α and IL-1 β exposure, as well as increases in total nitrate and nitrite production. During states of increased oxidative stress, NO interacts with increased levels of O₂⁻ to yield a potentially powerful oxidant, peroxynitrite, ONOO⁻. The protonated form of peroxynitrite decomposes readily to form free radical nitrogen dioxide (NO₂[•]) and hydroxyl radicals (OH[•]). Peroxynitrite can modify and thereby damage proteins through the oxidation of thiols, nitration of aromatic amino acids, and S-glutathiolation, which adds the small peptide glutathione to free SH groups of proteins. Imbalances in NO production can therefore have devastating effects in the cell and contribute further to tissue damage (Kibbe et al., 1999).

We also observed sustained activation of MAPK and NF- κ B signaling pathways, along with increased COX-2, NO, and VEGF/VEGFR-1 expression, and oxidative stress as previously reported (Tang and Kern, 2011). These changes were concomitant with changes in ECM proteins, junctional proteins, and decreased caveolin-1 expression. How inflammatory mediators alter biochemical pathways and intracellular signaling in other cell types of the eye including pericytes, astrocytes, müller glial cells, microglia, and RPE cells remain unexplored. The study of the cell specific impact of these inflammatory mediators is essential to how these and other individual inflammatory mediators contribute to retinal vascular dysfunction, cell death and retinopathy. Ultimately finding the most effective anti-inflammatory therapies is key to alleviating complications of DR. However, the best methods for delivery and the cell type and pathways to target which prove to be most effective requires rigorous testing.

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Abbreviations

EC	Endothelial Cells
VEGF	Vascular Endothelial Growth Factor
TNF-α	Tumor Necrosis-Factor-alpha
IL-1β	Interleukin1-beta
MCP-1	Monocyte Chemoattractant Protein-1
DR	Diabetic Retinopathy
ECM	Extracellular Matrix

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Highlights

- Inflammatory mediators contribute to vascular dysfunction during diabetes.
- TNF- α and IL-1 β , but not MCP-1, inhibited EC migration and capillary morphogenesis.
- The EC dysfunctions were associated with increased oxidative and nitrative stress.
- Sustained activation of MAPK and NF- κ B signaling pathways are involved.
- Alterations in EC junctional organization contribute to altered permeability.

Fig. 1.

TNF- α and IL-1 β do not alter cell viability, but activate caspase 3/7. (A) Cell viability after exposure to inflammatory mediators was determined using the MTS assay. (B) The rate of apoptosis was determined by measuring caspase activity with luminescent signal from caspase-3/7 DEVD-aminoluciferin substrate. TNF- α and IL-1 β induced caspase-3/7 activity compared to control (N=3, **P<0.001, ***P<0.0001). RLU, relative luminescent unit. (C) Apoptosis was also determined by DNA fragmentation. No change in TUNEL staining was observed (N=3, P>0.05).

Fig. 2.

TNF-α and IL-1β inhibit capillary morphogenesis and migration of retinal EC. (A) Capillary morphogenesis was assessed by culturing EC with TNF-α, IL-1β and MCP-1 in Matrigel for 18 h. Representative images are shown. TNF-α and IL-1β prevented capillary morphogenesis. Scale bar represents 500 μm. (B) Cell migration was determined by scratch wounding monolayers on gelatin-coated tissue culture plates. Wound closure was monitored by photography. Scale bar indicates 100 μm. (C) Mean number of branch points were counted for the capillary morphogenesis experiment (N=3, ***P<0.0001, **P<0.001). (D) Quantitative assessment of the data demonstrate a decrease in wound closure with TNF-α and IL-1β, and an increase with MCP-1 (N=3, ***P<0.0001, **P<0.001). (E) Transwell migration assays were performed to confirm the wound assay results (N=3, ***P<0.0001, **P<0.001). (F) Immunofluorescent staining of EC treated with TNF-α, IL-1β, or MCP-1 for 24 h. Phalloidin/Alex Fluor488 was used to stain the actin cytoskeleton, while rabbit anti-vinculin/Cy3 was used to stain focal adhesions. Scale bar represents 20 μm.

Fig. 3. Induction of iNOS by TNF- α and IL-1 β contributed to increased oxidative stress. (A) Oxidative stress was determined by dihydroethidium staining after 24 h incubation with control, 10 ng/ml TNF- α , IL-1 β , or MCP-1. Scale bar represents 20 μ m. (B) Quantitative assessment of the mean fluorescent intensity is shown (N=3, ***P<0.0001). (C) Production of total nitrate and nitrite was measure by Parameter Immunoassay kit after exposure to 10 ng/ml TNF- α , IL-1 β , and MCP-1 for 24 h and 48 h (N=2, *P<0.05, **P<0.001). (D) iNOS expression was analyzed by Western blot analysis of cell lysates after 24 h incubation with TNF- α , IL-1 β , and MCP-1. β -actin was used to assess loading. (E) Quantitative assessment of iNOS was determined by measuring band intensity using Image J (N=4, ***P<0.0001).

Fig. 4. TNF- α and IL-1 β disrupt tight and adherens junctions and increase VEGF production. (A) VE-cadherin localization was determined by immunofluorescent staining after exposure to TNF α , IL-1 β , and MCP-1 for 24h. Arrowheads indicate areas of continuous VE-cadherin junctional localization. Arrows indicate areas of discontinuous or punctate VE-cadherin junctional localization. Scale bar represents 20 μ m. (B) Expression of junctional proteins was determined by western blot analysis. The β -actin was used to assess loading. (C) Quantitative assessment was determined by measuring band intensity using Image J (N=3, *P<0.05, **P<0.01). (D) Production of VEGF was analyzed by a VEGF immunoassay. Analysis revealed a 13-fold increase in VEGF in the conditioned medium of cells incubated with TNF- α and a 6-fold increase with IL-1 β . (E) Expression of VEGF-R1 was determined by flow cytometry. Representative mean fluorescent intensities are indicated in bottom right corner of each panel. Shaded areas represent staining in the absence of primary antibody (N=4, *P<0.05). (F) COX2 expression was analyzed by Western blot analysis of cell lysates after 24 h incubation with TNF- α , IL-1 β , and MCP-1. The β -actin was used to assess loading. (G) Quantitative assessment of COX2 was determined by measuring band intensity using Image J (N=4, ***P<0.0001).

Fig. 5.

ECM and caveolin-1 protein expression is altered with TNF- α and IL-1 β . (A) Expression of various ECM proteins was determined by western blot analysis of cell lysates and conditioned medium after 48 h incubation with TNF α , IL-1 β , and MCP-1. The β -actin was used to assess loading. (B) Quantitative assessment was determined by measuring band intensity using Image J (N=3, *P<0.05, **P<0.001, ***P<0.0001). (C) TSP-1 expression was analyzed by western blot analysis of non-reduced cell lysates and conditioned medium after 48 h incubation with TNF- α , IL-1 β , and MCP-1. The β -actin was used to assess loading. (D) Quantitative assessment of TSP-1 was determined by measuring band intensity using Image J (N=3, P>0.05). (E) Caveolin-1 expression was analyzed by Western blot analysis of cell lysates after 24 h and 48 h incubation with TNF- α , IL-1 β , and MCP-1. The β -actin was used to assess loading. (F) Quantitative assessment of caveolin-1 was determined by measuring band intensity using Image J (N=3, ***P<0.0001). (G) Caveolin-1 intracellular expression and localization was determined by immunofluorescent staining. Scale bar represents 20 μ m.

Fig. 6. ERK 1/2, JNK, NF- κ B, p38 MAPK, and STAT3 pathways are activated by TNF- α and IL-1 β . (A–D) Expression of various signaling proteins was determined by Western blot analysis of cell lysates after 24 h incubation with TNF- α , IL-1 β , and MCP-1. The β -actin was used to assess loading. Quantitative analysis of each western blot is depicted next to its respective image. (N=3, *P<0.05, **P<0.001, ***P<0.0001).