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Inhibition of interleukin-6 trans-signaling prevents inflammation and endothelial barrier disruption in retinal endothelial cells.

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Abstract

Vascular inflammation plays a critical role in the pathogenesis of diabetic retinopathy. Recently, Interleukin-6 (IL-6) trans-signaling via soluble IL-6 receptor (sIL-6R) has emerged as a prominent regulator of inflammation in endothelial cells. This study was designed to test the hypothesis that selective inhibition of the IL-6 trans-signaling pathway will attenuate inflammation and subsequent barrier disruption in retinal endothelial cells. Human retinal endothelial cells (HRECs) were exposed to IL-6 and sIL-6R to induce IL-6 trans-signaling and the commercially available compound sgp130Fc (soluble gp-130 fused chimera) was used to selectively inhibit IL-6 trans-signaling. IL-6 trans-signaling activation caused a significant increase in STAT3 phosphorylation, expression of adhesion molecules, ROS production and apoptosis in HRECs whereas a significant decrease in mitochondrial membrane potential and NO production was observed in IL-6 trans-signaling activated cells. These changes were not observed in cells pre-treated with sgp130Fc. IL-6 trans-signaling activation was sufficient to cause barrier disruption in endothelial monolayers and pre-treatment of HRECs with sgp130Fc, maintained endothelial barrier function similar to that of untreated cells. Thus, in conclusion, these results indicate that IL-6 trans-signaling is an important mediator of inflammation, apoptosis and barrier disruptive effects in the retinal endothelial cells and inhibition of the IL-6 trans-signaling pathway using sgp130-Fc attenuates vascular inflammation and endothelial barrier disruption.

Keywords

Interleukin-6; trans-signaling; inflammation; endothelial cells; sgp130

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1. INTRODUCTION

Diabetic retinopathy (DR) is associated with several vascular abnormalities including vascular inflammation, occlusion of retinal capillaries and breakdown of the blood retinal barrier. The microvascular injury has been linked to up-regulation of several cytokines such as IL-6, TNF- α and VEGF and pathological over-expression of intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1) (Gustavsson et al., 2010; Soedamah-Muthu et al., 2006; Videm and Albriksen, 2008). Increasing evidence suggests that the IL-6 signaling pathway plays a prominent role in the pathogenesis of DR (Gabay, 2006; Rincon, 2012). This cytokine has an important role in endothelial cell dysfunction and vascular inflammation (Barnes et al., 2011; Chalaris et al., 2007; Hou et al., 2008; Rose-John, 2012; Rose-John et al., 2006).

IL-6 receptor (IL-6R) is present only on a few cell types in the body, such as hepatocytes, neutrophils, macrophages, and some lymphocytes (Bauer et al., 1989; Chalaris et al., 2011; Jones et al., 2011; Scheller et al., 2011). A soluble form of IL-6R (sIL-6R) comprising the extracellular portion of the receptor has been found in various body fluids. Classic IL-6 signaling is mediated by membrane-bound IL-6 receptor (mIL-6R) and membrane bound glycoprotein-130 (gp130) (Barnes et al., 2011; Rose-John, 2012; Rose-John et al., 2006; Rose-John et al., 2007). IL-6 trans-signaling operates via the soluble form of IL-6R forming a complex with IL-6 which associates with gp130 to initiate downstream signaling on the cells that lack the membrane-bound receptor (Chalaris et al., 2011; Jones et al., 2011; Rose-John, 2012; Scheller et al., 2011).

Currently, anti-IL6 therapeutic strategies globally block IL-6 signaling essentially targeting both pathways. It has been recognized that pro-inflammatory actions of this cytokine are mediated by trans-signaling whereas, many anti-inflammatory or regenerative activities of IL-6 are mediated by classic signaling. Recently, the role of IL-6 trans-signaling has emerged as a prominent regulator of inflammation and related diseases (Ebihara et al., 2011; Fisher et al., 2011; Rabe et al., 2008; Wei et al., 2013), and may be an important therapeutic target (Maes et al., 2014; Muangchant and Pope, 2013; Rose-John et al., 2007). The IL-6 trans-signaling pathway has been implicated in diseases such as rheumatoid arthritis, pancreatic cancer, chronic inflammatory bowel disease, pulmonary fibrosis, and diabetes (Barnes et al., 2011; Chalaris et al., 2011; Goumas et al., 2015; Jones et al., 2011; Le et al., 2014; Rose-John, 2012; Rose-John et al., 2006; Rose-John et al., 2007; Scheller et al., 2011).

The endogenous inhibitor of IL-6 trans-signaling is soluble gp130 (sgp130) (Goumas et al., 2015). The commercially available compound sgp130Fc (soluble gp-130 fused chimera) is an optimized fusion protein of the natural sgp130 and IgG1-Fc (Garbers et al., 2011; Tenhumberg et al., 2008). Sgp130Fc has been shown to possess 10–100x greater ability to inhibit IL-6 trans-signaling responses than natural sgp130 (Jostock et al., 2001). IL-6 and IL-6R individually do not have affinity for sgp130Fc (Rose-John, 2012; Rose-John et al., 2007). This allows for sgp130Fc to specifically inhibit IL-6 trans-signaling by binding to the IL-6/sIL-6R complex (Goumas et al., 2015; Jostock et al., 2001; Le et al., 2014). Cell types

that lack surface expression of IL-6R (such as endothelial cells) can be stimulated only by the IL-6/sIL-6R complex via the trans-signaling pathway and are insensitive to IL-6 alone (Ebihara et al., 2011; Hou et al., 2008; Rose-John et al., 2007; Sironi et al., 1989). In addition to the inflammatory response, IL-6 trans-signaling may regulate other critical endothelial functions. It has been hypothesized that IL-6 trans-signaling may be involved in the endothelial regulation of hemostasis, and the expression of anti-coagulant and anti-adhesion molecules (Hou et al., 2008; Rose-John et al., 2007; Verhamme and Hoylaerts, 2006). The use of Sgp130Fc to block IL-6 trans-signaling specifically has proven to be a valuable molecular tool for evaluating this process in various cells where IL-6 trans-signaling is implicated (Rose-John et al., 2006). Increased oxidative stress, reduced cellular function, adhesion molecule expression and 92 disrupted barrier function in retinal endothelial cells are important characteristics of DR pathogenesis (Coles et al., 2007; Hou et al., 2008; Lo et al., 2011). The goal of this study was to evaluate the effects of IL-6 trans-signaling in retinal endothelial cells. IL6 and sIL6R treatment was used to induce IL6 trans-signaling and sgp130Fc was used as a molecular tool to selectively inhibit IL-6 trans-signaling.

2. MATERIALS AND METHODS

2.1 Cell culture and treatments

Human retinal endothelial cells (Lonza, USA) were maintained on gelatin-coated culture dishes in M199 supplemented media with 10% fetal bovine serum (Hyclone Logan, UT, USA), 10% MCDB-131 (VecTech Rensselaer, NY, USA), and 1% L-glutamine/penicillin/streptomycin (Gemini West Sacramento, CA, USA) at 37°C in a humidified incubator containing 5% CO₂ with the media changed every alternate day. Physiological levels of IL-6 in human serum are in the range of 1–10 pg/ml. However, in case of inflammatory diseases, the IL-6 levels increase up to 10 ng/ml or more in severe cases (Baran et al., 2018; Gaillard et al., 1999; Garbers et al., 2011; Rose-John, 2017). The soluble-IL6R levels in humans are found in the range of 25–75 ng/ml and in case of inflammatory conditions, sIL6R reaches to a concentration of 100–200 ng/ml range (Baran et al., 2018; Rose-John, 2017). In this study, we have used 10 ng/ml of IL-6 and 150 ng/ml of sIL-6R to activate IL-6 trans-signaling mimicking the inflammatory conditions in humans. Previously, published studies have also used similar concentrations (Garbers et al., 2011; Rojas et al., 2010; Wolf et al., 2016) for their cell culture experiments. Cells were serum-starved for 4h prior to treatment and IL-6 trans-signaling was activated by exposing the cells to IL-6 (10ng/mL) and sIL-6R (150ng/mL; PeproTech, Rocky Hill, NJ, USA) overnight. To inhibit the IL-6 trans-signaling, cells were pre-treated for 1h with sgp130Fc (10µg/mL; R&D Systems, Minneapolis, MN, USA) before the addition of IL-6 and sIL6R.

2.2 Western blot analysis

HRECs were lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Sigma Aldrich, St. Louis, MO, USA). The cell lysates were subjected to Western blot analysis as described previously (Sharma et al., 2007b; Sharma et al., 2010; Sharma et al., 2008). Samples containing 20–40 µg protein were separated on a 4–20% SDS polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane using

the Trans-Blot® Turbo™ Transfer System (BioRad; Hercules, CA, USA). The membrane was then blocked in 5% bovine serum albumin (BSA) and incubated in primary antibody overnight at 4°C. Blots were then washed in TBST and incubated with the appropriate secondary antibody labeled with HRP. The bands were visualized with chemiluminescent substrate (Pierce Rockford, IL) and exposed on CL-Xposure™ X-ray film (Pierce Rockford, IL). Antibodies used were: phospho-STAT3 (1:500, Millipore Darmstadt, Germany), STAT3 (1:2000, Millipore Darmstadt, Germany), gp130 (1:200, Cell Signaling Danvers, MA, USA), IL6R (1:500, Santa Cruz Biotechnology, Dallas, Texas), ICAM-1 (1:500, Cell Signaling Danvers, MA, USA) and β -actin (1:5000, Cell Signaling Danvers, MA, USA). β -actin was used as loading control.

2.3 Measurement of Nitric oxide (NO) levels using DAF-2DA staining

Intracellular NO production was measured in HRECs using cell-permeable fluorescent NO indicator 4,5-diaminofluorescein diacetate (DAF-2DA) (Chemodex, Switzerland, Europe) (Leikert et al., 2001). HRECs were grown on an 8-well chamber slide and treated as described above. Following treatment, cells were washed and incubated in DAF-2DA (5 μ M, 40min) at room temperature in the dark followed by DAPI counter staining. A Zeiss LSM 510 Upright Confocal Microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) was used for imaging at 485nm excitation and 538nm emission wavelengths. The mean optical intensity (MOI) was quantified in 40 randomly selected fields per group using ImageJ software (U.S. NIH, Bethesda, Maryland, USA).

2.4 Hydrogen peroxide (H₂O₂) measurement by Amplex® Red Assay

H₂O₂ levels were determined using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Inc., Eugene, OR, USA) which uses the Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect H₂O₂. Briefly, HRECs were cultured and treated in a clear 96-well microplate. After treatment, 50 μ L of reaction solution containing 100 μ M Amplex® Red reagent and 0.2U/mL horseradish peroxidase was added to each well. The absorbance was measured at 560nm at multiple time points (0min – 6h) on Synergy HT microplate reader with KC4 3.3 Rev 10 from BioTek (Winooski, VT, USA). Concentrations were determined as previously described by using a H₂O₂ standard curve (Aggarwal et al., 2012).

2.5 Mitochondrial Reactive Oxygen Species (ROS) estimation by MitoSOX™

Mitochondrial ROS production was measured with fluorescence microscopy in live cells using MitoSOX™ Red mitochondrial superoxide indicator for live-cell imaging (Molecular Probes, Inc., Eugene, OR, USA). The HRECs were cultured and treated on a clear 8-well chamber slide. After treatment, 1–2 mL of 5 μ M MitoSOX™ reagent working solution was added to each well. Cells were incubated for 10 minutes at 37°C, protected from light. MitoSOX™ Red mitochondrial superoxide indicator has excitation/emission of 510/580 nm (Wiseman et al., 2007).

2.6 Mitochondrial membrane potential (ψ_m) detection by DePsipher

Quantification of mitochondrial membrane potential (ψ_m) was performed by fluorescent mitochondrial staining with DePsipher kit (Trevigen, Inc., Gaithersburg, MD, USA) as described previously (Cossarizza et al., 1993; Wiseman et al., 2007). HRECs were grown on an 8-well chamber slide and treated as described above. Following treatment, the cells were incubated in the dark with 5 μ g/ml DePsipher (5.5' .6.6'-tetrachloro-1.1' .3.3' -tetraethyl-benzimidazolyl car-bocyanin iodide) solution for 30 min at 37 °C, washed with reaction buffer with stabilizer, placed on a glass slide and covered with a glass coverslip. The stained cells were observed under an inverted fluorescence microscope using a fluorescein long-pass filter (fluorescein and rhodamine).

2.7 Immunofluorescence and Confocal Microscopy

Cells were grown to confluence and treated as described above on glass chamber slides. Following treatment, slides were washed with PBS and fixed and permeabilized with ice cold methanol: acetone (1:1). Slides were incubated in primary antibody ICAM-1 (1:1000, Abcam Cambridge, MA, USA) overnight followed by secondary antibody AlexaFluor-546 (1:300; Molecular Probes, Grand Island, NY, USA) for 1 hr. Slides were counterstained with DAPI for nuclear visualization. A Zeiss LSM 510 Upright Confocal Microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) was used to collect images with a 488nm excitation and 532nm wavelengths. The mean optical intensity (MOI) from 15–20 randomly selected fields per group were quantified using ImageJ software (U.S. NIH, Bethesda, Maryland, USA).

2.8 Caspase-Glo 3/7 Apoptosis Assay

Activity of caspase 3/7, was measured using a Caspase-Glo® 3/7 Assay kit (Promega Madison, WI, USA) as previously described (Aggarwal et al., 2012). This kit contains a proluminescent substrate (caspase-3/7 DEVD-aminoluciferin) which is cleaved by caspase 3/7 resulting in a luminescent signal. After treatment as described above, reaction mixture was added and luminescence read after 30 min incubation at room temperature. Luminescence was read on a Synergy H1 Hybrid Multi-Mode microplate reader equipped with Gen5 (BioTek, Winooski, VT, USA).

2.9 Detection of apoptosis using Annexin-V Assay

HRECs were grown to confluence and treated as described above on an 8-well glass chamber slide. Following treatment, cells were washed with binding buffer and stained for 15 min at room temperature with Annexin V-Alexa Fluor 488 (1:20) and propidium iodide (PI, 1 μ g/mL) (Molecular Probes, Grand Island, NY, USA). Annexin V binds to externalized phosphatidylserine indicating cells undergoing apoptosis while PI adheres to the nucleus of necrotic cells. Cells were then washed twice with binding buffer prior to imaging. A Zeiss LSM 510 Upright Confocal Microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) was used to collect images. The green MOI was quantified in 20 randomly selected fields per group using ImageJ software (U.S. NIH, Bethesda, Maryland, USA).

2.10 Measurement of Endothelial barrier integrity

Trans-endothelial electrical resistance (TER) was measured using an electric cell impedance sensing (ECIS) apparatus (Applied Biophysics, Troy, NY) as described previously (Müller et al., 2011; Sharma et al., 2010). An equal number of HREC_s were seeded on gold-coated electrode arrays (Applied Biophysics, Troy, NY), grown to confluence, then serum starved for 4 h. After resistances were equilibrated, treatment chemicals (sgp130Fc and IL-6/sIL-6R) were added directly to the wells. Impedance was measured for the duration of the experiment. The data was normalized from the initiation of treatment and plotted as normalized TER.

2.11 Statistical analyses

The mean $\pm$ SEM were calculated and comparisons were made by ANOVA followed by either Newman-Keuls or Bonferroni post-hoc testing (for 3 groups). The statistical significance of differences was set at $P < 0.05$. Statistical analysis was performed using GraphPad Prism version 6.05 for Windows (GraphPad Software, San Diego, CA). The ROUT method was utilized to identify and exclude outliers.

3. RESULTS

3.1 sgp130Fc treatment inhibits IL-6 trans-signaling mediated STAT3 activation in HREC_s

Previously published studies have shown that endothelial cells lack membrane bound IL-6 receptor and IL-6 alone is not sufficient to activate the IL-6 response in these cells (Chalaris et al., 2012; Lo et al., 2011; Modur et al., 1997; Romano et al., 1997; Su et al., 2017; Wei et al., 2013). Using western blot analyses, we also found that HREC_s express gp130 but lack IL-6R (Fig. 1A, B). Furthermore, our FACS analyses revealed that IL-6 alone did not elicit an IL-6 response, whereas IL-6/sIL-6R treatment caused a significant increase in the STAT3 phosphorylation in HREC_s (Fig. 1C, D). As an initial assessment of the ability of sgp130Fc to block IL-6 trans-signaling, we measured the phosphorylation of STAT3 since it is critical for its dimerization and downstream signaling. Western blot analysis of cell lysate showed that administration of IL-6/sIL-6R significantly increased (~4-fold) phosphorylation of STAT3 as compared to untreated cells (Fig. 2). Pre-treatment with sgp130Fc before IL-6/sIL-6R exposure significantly reduced the phosphorylation of STAT3 in comparison to the IL-6/sIL-6R treated group.

3.2 IL-6 trans-signaling mediated increase in H₂O₂ levels is prevented by sgp130Fc

Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) are an indicator of cellular stress and dysfunction. HREC_s were treated with IL-6/sIL-6R complex and H₂O₂ levels were measured dynamically (0–6 hours). We observed a significant increase in the H₂O₂ generation in the cells treated with IL-6/sIL-6R complex as compared to untreated cells (Fig. 3). However, pre-treatment with sgp130Fc significantly decreased the H₂O₂ production over 6 hours in the IL-6 trans-signaling activated cells.

3.3 sgp130Fc prevents IL-6 trans-signaling mediated mitochondrial dysfunction

Mitochondrial superoxide anion is the predominant ROS in mitochondria thus, its estimation is a marker of ROS production and oxidative stress within the cell. HRECs treated with IL6/IL6R complex revealed a significant increase in superoxide production compared to the untreated group (Fig. 4A). Administration of sgp130Fc preserved mitochondrial health, lowering the production of ROS. DePsipher kit was used to evaluate the effect of IL 6 trans-signaling on mitochondrial membrane potential (ψ_m). In healthy cells, the mitochondrial staining appears red whereas, in cells with disrupted potential the dye remains in its monomeric form and appears green. There was a significant decrease in the mitochondrial membrane potential of HRECs exposed to IL6/IL6R. In the cells pre-treated with sgp130Fc, exposed to IL6/IL6R, a decrease in mitochondrial membrane potential was not observed and it was almost similar to the untreated cells (Fig. 4B).

3.4 IL-6 trans-signaling decreases nitric oxide production in HRECs

Nitric oxide production in retinal endothelial cells was measured using the NO-specific fluorescent dye Diaminofluorescein –2 diacetate (DAF-2DA). The diacetate groups of DAF-2DA are hydrolyzed by cytosolic esterases releasing the non-fluorescent dye DAF-2. DAF-2 can be converted into its fluorescent triazole derivative, DAF-2T, by NO production. Exposure of HRECs to IL-6/sIL-6R resulted in a drastic decrease in NO production (Fig. 5). This decrease in NO levels was prevented if the cells pre-treated with sgp130Fc prior to IL-6/sIL-6R treatment and the NO levels in these cells were similar to that of untreated cells.

3.5 IL-6 trans-signaling increases ICAM-1 levels in endothelial cells

Intercellular adhesion molecular 1 (ICAM-1) is an endothelial cell-specific leukocyte adhesion molecule which plays an important role in leukostasis and immune response. When activated, leukocytes bind to endothelial cells via ICAM-1 leading to leukocyte-endothelial transmigration into the tissues. The ICAM-1 levels in endothelial cells were determined using immuno- fluorescent microscopy. ICAM-1 expression increased significantly with IL-6/sIL-6R treatment in comparison to the untreated cells. Pre-treatment with sgp130Fc prevented IL-6/sIL-6R mediated increase in ICAM-1 levels (Fig. 6A). These findings were further validated using western blot analyses to determine ICAM-1 levels (Fig. 6B)

3.6 sgp130Fc inhibits IL-6 trans-signaling induced apoptosis

To determine the effects of IL-6 trans-signaling on the apoptosis in retinal endothelial cells, Caspase 3/7 activity was measured in HRECs after IL-6/sIL-6R exposure with or without pre-treatment with sgp130Fc. IL-6/sIL-6R treated cells showed a significant increase in caspase 3/7 activity as compared to untreated cells. Additionally, sgp130Fc pre-treated cells showed a significant decrease in caspase 3/7 activity when compared to cells which did not receive pre- treatment (Fig. 7A). These results were further validated by fluorescent staining with Annexin V. The translocation of phosphatidylserine to the cellular membrane is an indication of apoptosis. Annexin V binds to exposed phosphatidylserine as a fluorescent indication of apoptotic cells. Untreated cells showed minimal Annexin V staining indicating a low level of apoptosis. Cells pre-treated with sgp130Fc prior to IL-6/sIL-6R treatment also

showed low levels of apoptosis (Fig. 7B). In contrast, the IL-6/sIL-6R treated cells which did not receive sgp130Fc pre-treatment showed a significant increase in apoptosis.

3.7 Endothelial barrier function is protected with sgp130Fc pre-treatment

To determine the effect of IL-6 trans-signaling on endothelial barrier function, we measured the trans-endothelial electrical resistance (TER) of a confluent endothelial monolayer treated overnight with IL-6/sIL-6R and either pre-treated or not with sgp130Fc. Intracellular gaps increased significantly with IL-6/sIL-6R treatment as shown by a substantial reduction in TER (Fig.8). Endothelial monolayers which were pre-treated with sgp130Fc maintained endothelial barrier function similar to that of untreated.

4. DISCUSSION

Retinal microvascular injury has been linked to up-regulation of several cytokines such as IL-6, TNF- α and VEGF and pathological over-expression of intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1) (Gustavsson et al., 2010; Soedamah-Muthu et al., 2006; Videm and Albrigtsen, 2008). Increasing evidence suggests that the IL-6 pathway plays a prominent role in DR pathogenesis (El-Asrar, 2012; Mocan et al., 2006; Shimizu et al., 2002) and interactions between IL-6 and endothelial cells regulate recruitment of leukocytes and expression of inflammatory proteins. A soluble form of the IL-6R is generated by limited proteolysis and alternative splicing. In cells lacking membrane-bound IL-6 receptor, the IL-6/soluble-IL6R complex associates with the ubiquitously expressed signal transducing protein gp130, initiating dimerization and intracellular signaling (IL-6 trans-signaling). There is abundant evidence demonstrating that IL-6 trans-signaling via the soluble IL-6R is the crucial step in the development and progression of several inflammatory diseases (Calabrese and Rose-John, 2014; Mitsuyama et al., 2006; Onogawa et al., 2013; Schaper and Rose-John, 2015; Schuett et al., 2012; Zhang et al., 2013). In this study, we found that IL-6 trans-signaling is sufficient to cause barrier disruption in endothelial cells and blockade of IL-6 trans-signaling using sgp130Fc, prevents inflammation and endothelial barrier disruption.

Successful activation of the IL-6 signaling is indicated by the dimerization of gp130. Since gp130 does not possess intrinsic kinase domain, the signaling is transduced via JAK1 and JAK2. JAKs are constitutively associated with gp130 and phosphorylate STAT3 in the cytoplasm. After the phosphorylation, STAT3 homo-dimerises and the pSTAT3 dimer translocate to the nucleus [38, 39]. In this study, we showed that the administration of IL-6/sIL-6R to HRECs was sufficient to increase the phosphorylation of STAT3 and sgp130Fc pre-treatment prevented this increase in STAT3 phosphorylation.

Reactive oxygen species are a side product of the oxidative phosphorylation, the key pathway for ATP synthesis. Superoxide anion ($\text{O}_2^{\cdot-}$) is the primary ROS generated in mitochondria through the monoelectronic reduction of O_2 (Daiber, 2010; Starkov, 2008). Dismutation of $\text{O}_2^{\cdot-}$ is mediated by superoxide dismutases (SODs) which convert the radical into a more stable molecule of hydrogen peroxide (H_2O_2). H_2O_2 can be detoxified by the action of glutathione peroxidase, catalase or thioredoxin peroxidase (Marchi et al., 2012; Nordberg and Arnér, 2001). Our study shows that the production of H_2O_2 significantly

increases after activation of IL-6 trans-signaling in endothelial cells and pre-treatment with sgp130Fc attenuates H₂O₂ production.

The maintenance of the mitochondrial membrane potential (MMP) is crucial for cellular bioenergetics such as ATP production and oxidative phosphorylation. It has been stated that an excess in ROS production is among the stimuli that target the protein responsible for the permeability transition pore formation (PTP) (Bernardi et al., 1992; Lemasters et al., 1997). ROS stimulation induces a high conductance state of the PTP which lead to an uncontrolled entrance of solute with a molecular mass less than 1.5kDa, and water. As a consequence, the permeability of the mitochondrial membrane dramatically changes thus dissipating MMP. This phenomenon is known as mitochondrial permeability transition (MPT). In this study, we showed that activation of IL-6 trans-signaling causes a loss of mitochondrial membrane potential in retinal endothelial cells. As a consequence of the ROS-induced disruption of the mitochondrial potential, cytochrome c is released through the PTP in the cytoplasm. Cytochrome c interacts with pro-caspase 9 and apaf1 leading to the formation of the apoptosome complex which triggers caspases activation (Acehan et al., 2002). Caspase 3 and 7, two executioner caspases that, once activated, promote the degradation of structural components of the nuclear matrix and fragmentation of nuclei. Activation of IL-6 trans-signaling significantly increased the caspase 3 and 7 protein levels in HRECs.

One of the major function of endothelial cells in the vasculature is NO production. As a gas, it diffuses to the vascular smooth muscle cells and activates guanylate cyclase, causing an increase in the production of intracellular cGMP and relaxation of the smooth muscle. NO levels are critical in maintaining vascular homeostasis and barrier function (Albrecht et al., 2003; Sharma et al., 2007a). Our results indicate that the ability of HRECs to generate NO is decreased when IL-6 trans-signaling is activated.

Previous studies have shown that endothelial cell activation via IL-6 signaling results in an increase in the expression of adhesion molecules (ICAM-1, VCAM-1), the release of chemokines (IL-8 and MCP), and the release of IL-6 itself (Barnes et al., 2011). The multiple inducers mentioned above promote interactions between endothelial cells and leukocytes, platelets or red blood cells, leading to further activation and damage of endothelium in an autocrine amplification pathway (Hou et al., 2008). We found a markedly increased endothelial expression of ICAM-1 in HRECs upon IL-6 trans-signaling activation that was prevented by sgp130Fc pre-treatment. In an earlier study, IL-6-induced upregulation of ICAM-1 was completely abolished by sgp130Fc in isolated endothelial cells (Clahsen and Schaper, 2008).

Retinal endothelial cells are among the components of the blood-retinal barrier (BRB) which plays a major role in the structural and functional integrity of the retina. The BRB regulates the flux of the molecules within the eye, protecting the retinal tissue from pathogens. The main components of the internal portion of the barrier are the endothelial cells of the intra-retinic capillaries, provided with tight junctions, together with pericytes, Muller cells and astrocytes (Cunha-Vaz et al., 2011) (Klaassen et al., 2013). The increase in BRB permeability is associated with changes in the expression of tight junctions in retinal vessels (Aveleira et al., 2010; Barber and Antonetti, 2003; Campbell and Humphries, 2011; Dejana

et al., 2008). The tight junctions not only strictly regulate the permeability of the BRB and contribute to its high level of trans-endothelial electrical resistance (TER) but they also regulate the paracellular flux of molecules across the barrier. An intact BRB is characterized by intact vessels, which demonstrate a high level of trans-epithelial resistance (TER). Intracellular gaps increased significantly with IL-6/sIL6R treatment as shown by a substantial reduction in TER. These data suggest that IL-6 trans-signaling activation is sufficient to cause barrier disruption. Endothelial monolayers, which when pre-treated with sgp130Fc, maintained endothelial barrier function similar to that of untreated cells suggesting that this compound prevented IL-6 trans-signaling mediated barrier disruption.

5. Conclusions

Our findings suggest that IL-6 trans-signaling causes ROS-induced damages, energy imbalance, inflammation, apoptosis and the BRB disruption in human retinal endothelial cells. Inhibition of IL-6 trans-signaling using sgp130Fc prevents inflammation and endothelial barrier disruption in retinal endothelial cells.

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HIGHLIGHTS

- IL-6 trans-signaling is an important mediator of inflammation, apoptosis and barrier disruptive effects in the retinal endothelial cells
- Selective inhibition of the IL-6 trans-signaling using a novel drug sgp130Fc attenuates vascular inflammation and endothelial barrier disruption.

Figure 1: IL-6 trans-signaling induces STAT3 phosphorylation in endothelial cells.

Western blot analysis was conducted in human retinal endothelial cells (HREC) to detect glycoprotein 130 (gp130) and IL6 receptor expression. (A) HREC express sgp130 (B) but lack the membrane bound IL6 receptor (IL6R). The positive control used were hepatocyte (HepG2) cells which expresses IL6R. β -actin served as a loading control. (C) HRECs were treated with and without sgp130Fc (10 μ g/mL) for 60 min followed by IL-6 (10 ng/ml) alone or combined with sIL-6R (150 ng/ml) for 10 min. Cells were collected and stained using monoclonal antibody anti-mouse p-STAT3 (pY705) (Millipore) and FITC-conjugated anti-mouse AlexaFluor 488 Conjugate (Cell Signaling) and analyzed using the FACS Calibur flow cytometer. (D) IL-6 alone did not show any response in HRECs but sgp130Fc pre-treatment, prevented STAT3 phosphorylation observed in IL-6+sIL-6R treated cells. Bar graphs represent fold untreated of peak means. Results are expressed as means $\pm$ SEM; n = 4 per group, * p < 0.05 versus IL-6+sIL-6R; † p < 0.05 versus IL-6+sIL-6R+sgp130 Fc.

Figure 2: sgp130Fc inhibits IL-6 trans-signaling mediated STAT3 phosphorylation in HRECs. Cells were pre-treated with and without sgp130Fc (10µg/mL, 60 min) and exposed to IL-6 (10ng/mL) and sIL-6R (150ng/mL) overnight. Western blot analysis of cell lysates was performed to evaluate phospho-STAT3 (Tyr705) and total STAT3 levels. Representative bands are shown in the figure. Bar graphs represent the quantification of band intensities. IL-6/sIL-6R treatment resulted in almost 4-fold increase in STAT3 phosphorylation, which was prevented by pre-treating with sgp130Fc. Results are expressed as means $\pm$ SEM; n=5 per group, *p < 0.05 vs untreated; † p < 0.05 vs IL-6/sIL-6R.

Figure 3: sgp130Fc partially prevents IL-6 trans-signaling mediated increase in H₂O₂ levels. Production of H₂O₂ was dynamically measured using the Amplex Red assay in HRECs exposed to IL-6 (10ng/mL, 60 min) and sIL-6R (150ng/mL) overnight, with and without sgp130Fc pre-treatment (10μg/mL). IL-6/sIL-6R exposure significantly increased H₂O₂ production compared to untreated cells which was partially prevented by sgp130Fc pre-treatment. Results are expressed as means ± SEM; n=7 per group, * p < 0.05 vs untreated; † p < 0.05 vs IL-6/sIL-6R.

Figure 4: IL-6 trans-signaling disrupts mitochondrial function in endothelial cells.

(A) Mitochondrial ROS production was measured in live cells using MitoSOX™ Red mitochondrial superoxide indicator. HRECs treated with IL6/sIL6R complex resulted in a significant increase in superoxide production as compared to the untreated group. This increase was prevented by inhibition of IL-6 trans-signaling using sgp130Fc. (B) Quantification of mitochondrial membrane potential (ψ_m) was performed by fluorescent mitochondrial staining using DePsipher in HRECs treated with and without sgp130Fc (10 μ g/mL, 60 min) and exposed to IL-6 (10ng/mL) and sIL-6R (150ng/mL) overnight. Graph represents the ratio of healthy (red):unhealthy (green) mitochondria quantified in images obtained via confocal microscopy. IL-6 trans-signaling activation significantly disrupted the ψ_m . This effect was prevented by pre-treating cells with sgp130Fc. Results are expressed as means $\pm$ SEM; n = 8 per group, scale bar = 50 μ m, * p < 0.05 vs untreated; † p < 0.05 vs IL-6+sIL-6R.

Figure 5: Anti-trans-signaling fusion protein exposure maintains nitric oxide (NO) production in endothelial cells.

DAF-2DA staining was used to detect NO levels. Before activating IL-6 trans-signaling with IL-6 (10ng/mL) and sIL-6R (150ng/mL), cells were treated with or without sgp130Fc (10μg/mL, 60 min). The treatment of cells with sgp130Fc resulted in maintenance of DAF-2 DA fluorescence which was reduced by IL-6+sIL-6R compared to untreated cells. Results are expressed as means $\pm$ SEM; n = 40 per group, scale bar = 25μm, * p < 0.05 vs untreated; † p < 0.05 vs IL-6+sIL-6R.

Figure 6: Inhibition of IL-6 trans-signaling decreases ICAM-1 protein levels in HRECs. HRECs were treated with and without sgp130Fc (10μg/mL, 60 min) and exposed to IL-6 (10ng/mL) and sIL-6R (150ng/mL) overnight. **(A)** ICAM-1 expression was measured after staining with anti-ICAM-1 antibody (red). Cells were counterstained by 4',6'-diamidino-2-phenylindole (DAPI, blue). All images were visualized by confocal microscopy. IL-6 trans-signaling activation resulted in a three-fold increase in the ICAM-1 expression. This effect was prevented by pre-treating cells with sgp130Fc. Results are expressed as means ± SEM; n = 8 per group, scale bar = 25μm, * p < 0.05 vs untreated; † p < 0.05 vs IL-6+sIL-6R. **(B)** Western blot analysis of cell lysates was performed to measure the ICAM-1 levels. Representative bands are shown in the figure. β-actin was used as a loading control. Bar graphs represent the quantification of band intensities. IL-6/sIL-6R treatment resulted in a significant increase in ICAM-1 levels, which was prevented by pre-treating with sgp130Fc.

Results are expressed as means $\pm$ SEM; n=4 per group, *p < 0.05 vs untreated; † p < 0.05 vs IL-6/sIL-6R.

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Figure 7: sgp130Fc protects against IL-6 trans-signaling induced endothelial cell apoptosis. (A) Cells were treated with and without sgp130Fc (10µg/mL, 60 min) and exposed to IL-6 (10ng/mL) and sIL-6R (150ng/mL) overnight. Following treatment, cells were analyzed with a Caspase-Glo 3/7 kit. The increase in caspase 3/7 activity seen in IL-6+sIL-6R treated cells was significantly decreased in presence of sgp130Fc. (B) Cell apoptosis was measured with Annexin V staining (green) using confocal microscopy. Cells were counterstained with propidium iodide (red) to show cell nuclei. Exposure to IL-6+sIL-6R significantly increased apoptosis which was prevented by specific inhibition with sgp130Fc. Results are expressed as means $\pm$ SEM; n = 10 per group, scale bar = 50µm, * p < 0.05 vs untreated; † p < 0.05 vs IL-6+sIL-6R.

Figure 8: sgp130Fc diminishes IL-6 trans-signaling induced disruption of endothelial monolayer barrier function.

HRECs were treated with and without sgp130Fc (10 μ g/mL, 60 min) and exposed to IL-6 (10ng/mL) and sIL-6R (150ng/mL) overnight. Trans-endothelial electrical resistance (TER) was measured using an electric cell impedance sensing (ECIS) apparatus. IL-6 trans-signaling significantly disrupted endothelial barrier function. Pre-treatment with sgp130Fc maintained the endothelial barrier function at near control levels. Results are expressed as means $\pm$ SEM; n = 3 per group, * p < 0.05 vs untreated; † p < 0.05 vs IL-6+sIL-6R.