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IGFBP-3 inhibits TNF-α production and TNFR-2 signaling to protect against Retinal Endothelial Cell Apoptosis

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ABSTRACT

In models of diabetic retinopathy, insulin-like growth factor binding protein-3 (IGFBP-3) protects against tumor necrosis factors-alpha (TNF-α)-mediated apoptosis of retinal microvascular endothelial cells (REC), but the underlying mechanisms are unclear. Our current findings suggest that at least two discrete but complimentary pathways contribute to the protective effects of IGFBP-3; 1) IGFBP-3 directly activates the c-Jun kinase/tissue inhibitor of metalloproteinase-3/TNF-α converting enzyme (c-Jun/TIMP-3/TACE), pathway, which in turn inhibits TNF-α production; 2) IGFBP-3 acts through the IGFBP-3 receptor, low-density lipoprotein receptor-related protein 1 (LRP1), to inhibit signaling of TNF-α receptor 2 (TNFR2). Combined, these two IGFBP-3 pathways substantially reduce REC apoptosis and offer potential targets for the treatment of diabetic retinopathy.

Introduction

Diabetic retinopathy is the leading cause of blindness in the world (Cheung et al., 2010; Leon et al., 2013). Retinal microvascular endothelial cell (REC) apoptosis is a key step in the initiation of diabetic-related pathologies (Jiang et al., 2012; Steinle, 2012). Progressive microvascular alterations, including leukocyte adhesion, endothelial cell death and formation of degenerated capillaries, are hallmarks of the disease (Ishida et al., 2003; Joussen et al., 2001). Various mediators, such as tumor necrosis factor alpha (TNF-α), the insulin-like growth factor 1(IGF-1)/insulin like growth factor binding protein (IGFBP) system and vascular endothelial growth factor (VEGF) are known to regulate cell apoptotic pathways in various cell types including REC (Ben-Mahmud et al., 2004; Kielczewski et al., 2011; Titchenell and Antonetti, 2013); however, the specific pathways involved are unclear. We have previously reported that IGFBP-3 inhibits TNF-α production, leading to an inhibition of REC apoptosis (Zhang et al., 2013a,b). Building on this important finding, our goal in the current study is to uncover the mechanistic pathways that underlie IGFBP-3 regulation of TNF-α and REC apoptosis, and specifically to determine if binding of IGFBP-3 to its receptor, LRP1, is involved in its protective effects.

IGFBP-3 is one of seven proteins that constitute the IGFBP family. In addition to being the most abundant circulating IGF-1 binding protein, IGFBP-3 binds to IGF-1 with high affinity and specificity (Baxter, 2000; Mohan and Baylink, 2002), and appears to have multiple functions (Ning et al., 2006). In many cases, the high affinity binding of IGFBP-3 to free IGF-1 acts to control IGF-1 distribution in interstitial fluids (Clemmons, 2001; Lovett-Racke et al., 1998). For example, following tissue injury, IGFBP-3 helps concentrate IGF-1 at wound sites and reduces its rate of clearance. IGFBP-3 binds directly to fibrinogen/fibrin clots and in turn IGF-1 binds the immobilized IGFBP-3/fibrinogen and IGFBP-3/fibrin complexes. The lower IGF-1 affinity for fibrin-bound IGFBP-3 allows IGF-1 release to higher affinity type I IGF receptors of stromal cells migrating into the fibrin clot. Of particular relevance to our studies, IGFBP-3 also has separate, IGF-1 independent actions that have been shown to support cell survival (Jarajapu et al., 2012; Lofqvist et al., 2007), and as such IGFBP-3 had been shown to reduce angiogenesis and mediate protective effects on blood retinal barrier integrity in diabetic retinopathy, independent of its actions on IGF-1. By using a mutant form of IGFBP-3 that cannot bind IGF-1, it is possible to observe IGF-1 independent actions of IGFBP-3. Under these conditions (in our case, transfection of REC with mutant non-binding, IGFBP-3 NB plasmid), we found that overexpression of IGFBP-3 NB led to a reduction in TNF-α levels and a subsequent reduction in REC apoptosis (Zhang et al., 2013a,b). The studies described here also use IGFBP-3 NB plasmid and thus, all the findings contained in this report are limited to actions of IGFBP-3 that are independent of IGF-1 binding.

Since c-Jun N-terminal kinases act as mediators of angiogenesis in other systems, we hypothesize that c-Jun might play a role in vascular changes associated with diabetic retinopathy and be one of the targets of IGFBP-3. It has been established in other tissues that c-Jun can control TNF-α activation by direct transcriptional activation of tissue inhibitor of metalloproteinase-3 (TIMP-3), an inhibitor of the TNF-α-converting enzyme (TACE) (Guinea-Viniegra et al., 2009). Thus, we wished to

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determine if c-Jun acts as an essential physiological regulator of TNF-α by controlling the TIMP3/TACE pathway in retinal vascular cells and whether IGFBP-3 regulates c-Jun.

TNF-α was first identified as an inducer of cell death (Takahashi et al., 1998; Tracey and Cerami, 1993). It has also been shown to be an inflammatory biomarker associated with the development of vascular leakage and apoptosis in diabetic retinopathy (Zhang et al., 2011). TNF-α interacts with two membrane-bound receptors, tumor necrosis factor receptor 1 (TNFR1; CD120a; p55) and tumor necrosis factor receptor 2 (TNFR2; CD120b; p75) (Riches et al., 1998). While TNFR1 is expressed ubiquitously, basal TNFR2 expression has only been detected in vascular endothelial cells, cardiac myocytes and some neuronal cells (Carpentier et al., 2004; Vandenabeele et al., 1995). TNFR2 expression can also be induced by ischemia in vascular endothelium (Luo et al., 2006). On binding of TNF-α, TNFR recruits the adaptor protein, TNFR associated death domain (TRADD), directly signaling to the Caspase 8 cytoplasmic death complex (Cabal-Hierro and Lazo, 2012).

Changes in retinal microvascular structure, due in part to REC cell death, represent hallmark features of early diabetic retinopathy (Cheung et al., 2010). Because of the potential role of REC in the initiation of this disorder, we have utilized cultured primary human REC grown in normal versus high glucose conditions as a model in which to examine functional relationships between TNF-α and IGFBP-3. Results confirm that cultured REC respond to high glucose, IGFBP-3, and TNF-α in a manner similar to that observed in whole retina. Furthermore, culture conditions permit highly controlled genetic and chemical manipulation of key elements so that mechanistic pathways within REC can be clearly identified. Our findings suggest that functional pathways linking IGFBP-3 to c-Jun N-terminal kinase and to the TNF-α receptor, TNFR2, are responsible for the observed decrease in REC apoptosis.

Methods

Reagents

Caspase 8, TRADD and TIMP3 antibodies were purchased from Cell Signaling (Danvers, MA). LRP1 and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human IGFBP-3 immunoassay ELISA kit was purchased from R & D (Minneapolis, MN). InnoZyme™ TACE activity kit was bought from Millipore (San Diego, CA). Human LRP1 siRNA and Non-Targeting siRNA #1 were purchased from Dharmacon RNAi Technologies (Chicago, IL). RNAimax was purchased from Invitrogen (Carlsbad, CA). SuperFect transfection reagent was bought from Qiagen (Valencia, CA). Horseradish peroxidase (HRP) conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI). Enhanced chemiluminescence for immunoblot development and signal detection was purchased from Amersham Biosciences (Piscataway, NJ). C-Jun peptide was purchased from Tocris Bioscience (Bristol, UK). IGFBP-3 NB plasmid DNA was a gift from Dr. Maria B. Grant (University of Florida).

Cell culture

Primary human REC were acquired from Cell System Corporation (CSC, Kirkland, Washington). Cells were grown in M131 medium containing microvascular growth supplements (Invitrogen) (MVGS), 10 μg/Πl gentamycin and 0.25 Πg/Πl amphotericin B. In the high glucose condition, cells were transferred to high glucose (25 ΠM) (Cell Systems) medium, supplemented with MVGS and antibiotics for 3 days. Only primary cells within passages 6 were used. Cells were quiesced by incubating in high or normal glucose medium without MVGS for 24 h and used to perform the experiments. For experiments to test inhibition of c-Jun, we treated REC with 400ΠM c-Jun peptide to block c-Jun activity as previously reported (Holzberg et al., 2003), followed by transfection with IGFBP-3 NB.

Transfection of siRNA and plasmid

DNA-ON-TARGETplus SMARTpool, human LRP1 siRNA (LRP1 siRNA) were purchased from Dharmacon, Inc. We used 4 sets of siRNA, with target sequences of GCGAAGGCAUUGUGUGUUC, GCACCAUUCUCAAG AGUAU, GCGCAUCGAUCUUCACAAA and GAACAAACACACUGGCUAA. siCONTROL Non-targeting siRNA #1 (Dharmacon) was used as a non-specific control. REC were transfected with siRNA at a final concentration of 20 nM using RNAiMAX transfection reagent according to the manufacturer's instructions. The cells were used for experiments 24 h after transfection. For the cells in high glucose condition, cells were transfected on Day 2 in high glucose medium and were also kept in high glucose medium during transfection. The cells were also transfected with IGFBP-3 NB plasmid DNA at 1 μg/ml using SuperFect transfect reagent, according to the manufacturer's instructions. The cells were used for experiment 24 h after transfection.

ELISA analysis

An ELISA for IGFBP-3 and TNF-α levels was performed using the ELISA assay kits according to the manufacturer's instructions to evaluate the IGFBP-3 and TNF-α levels after the treatment. Equal protein was loaded into all wells for both assays.

TACE analysis

TACE activity was measured using a solution-based assay containing a fluorescently labeled TACE substrate. Equal proteins were added to the TACE-coated 96-well plate. The activity of the captured TACE was measured using an internally quenched fluorescent substrate, MAC-KPLGL-Dpa-AR-NH2. Fluorescence of the cleaved product, MAC-KPLG, was measured at an excitation wavelength of 320 nm and emission wavelength of 405 nm. The specific enzymatic activity of TACE was calculated as relative fluorescence units (RFU).

Western blot analysis

After appropriate treatments and rinsing with cold phosphate-buffered saline, REC were scraped into lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein from the cell extracts were separated on the pre-cast tris-glycine gel and blotted onto a nitrocellulose membrane. After blocking in TBST (10 mM Tris–HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 5% (w/v) BSA, the membrane was treated with anti-phospho-c-Jun, c-Jun, TIMP3, TRADD and caspase 8 antibodies followed by incubation with HRP conjugated secondary antibodies. The antigen–antibody complexes were detected using chemiluminescence reagent kit (Thermo Scientific).

Statistics

All the experiments were repeated at least three times, and the data are presented as mean ± SEM. Data was analyzed by Kruskal–Wallis test, followed by Dunn's testing with p values < 0.05 were considered statistically significant. In the case of Western blotting, one representative blot is shown. Normal glucose was normalized to 1, with all treatment compared to normal glucose followed by normalization to actin levels.

Results

When grown in the presence of 25 mM glucose (diabetic-like conditions) and transfected with IGFBP-3NB plasmid, REC expressed increased levels of IGFBP-3, concomitant with increased levels of phosphorylated c-Jun and TIMP3, as well as decreased levels of TACE. Given our previous observation that IGFBP-3 decreases TNF-α levels and thus protects against REC apoptosis, we wished to determine