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Linoleic Acid is a Diabetes-relevant Stimulator of Retinal Inflammation in Human Retinal Muller Cells and Microvascular Endothelial Cells

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Abstract

Objective: To determine the effect of oleic acid and linoleic acid on the production and secretion of specific diabetic retinopathy- (DR-) related cytokines: vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and interleukin-8 (IL-8) by human retinal glial cells, retinal endothelial cells, and retinal pigment epithelial cells. These expression profiles will be compared to those obtained by treatment of the same cell types with elevated D-glucose, a diabetes-relevant stimulus often used in retinal cell culture experiments.

Methods: Primary cultures of human retinal Müller cells, astrocytes, and microvascular endothelial cells (RMEC) and a human retinal pigment epithelial cell line (ARPE-19) were treated with oleic acid, linoleic acid, elevated D-glucose, or L-glucose as an osmotic control. VEGF, IL-6, and IL-8 concentrations in conditioned media were determined by colorimetric ELISA and normalized to total cellular protein.

Results: In the conditioned medium of human Müller cells, linoleic and oleic acid increased VEGF production by 6.4-fold and 9.9-fold, respectively. Linoleic acid also significantly increased IL-6 by 2.9-fold and IL-8 by 5.7-fold. L-glucose and D-glucose both increased VEGF by 3.1-fold in Müller cell conditioned medium. Linoleic acid increased IL-8 concentrations by 56% in human RMEC conditioned medium. Human retinal astrocytes and ARPE-19 were unaffected by all stimuli.

Conclusions: Linoleic and oleic acid induce inflammatory mediators believed to be involved in the pathogenesis of diabetic retinopathy (DR). In culture, the free fatty acid insults, particularly linoleic acid, significantly increased cytokine production by Müller cells. In summary, these data identified Müller cells as the primary producer of these inflammatory mediators when treated with unsaturated fatty acids. This study also demonstrates that elevated glucose is an inadequate stimulus for assessing the production of inflammatory mediators. Therefore this study provides a novel in vitro model system of the dyslipidemia-induced inflammation occurring in DR.

Keywords: Diabetic retinopathy; Vascular endothelial growth factor; Interleukin-6; Interleukin-8; Linoleic acid; Oleic acid

Introduction

Inflammation is an early component of diabetic retinopathy (DR) that can lead to vision-threatening pathology. A number of soluble pro-inflammatory growth factors, cytokines and chemokines, including vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and interleukin-8 (IL-8), are elevated in the vitreous of diabetic patients [1]. These factors are expressed and secreted by several retinal cell types, and studies have shown that they elicit retinal inflammatory responses such as disruption of retinal tight junctions and induction of cell adhesion proteins, among others.

Although hyperglycemia is often considered to be the dominant insult in diabetes-related retinal inflammation, other stimuli may be of critical importance. Recent studies have demonstrated a strong association between dyslipidemia and DR. Interestingly, in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) and The Action to Control Cardiovascular Risk in Diabetes (ACCORD) studies, the lipid lowering drug, fenofibrate, delayed retinopathy progression, independent of glycemic control [2]. In humans and animal models, diabetes increases fatty acid concentrations in systemic circulation and tissues, leading to inflammation, insulin resistance, and disease progression [3,4]. Increased retinal levels of oleic and linoleic acids have been observed in an animal model of diabetes [5] and in vitro studies have shown that linoleic acid induces inflammatory adhesion molecule production in retinal endothelial cells [6].

These data suggest that elevated fatty acids may be causally linked to retinal inflammation occurring early in the pathogenesis of DR. Therefore, we sought to characterize the effect of oleic and linoleic acids on the expression and secretion of VEGF, IL-6, and IL-8 by human retinal glial cells (Müller cells and astrocytes) and human blood-retina-barrier cells [retinal microvascular endothelial cells (RMEC) and retinal pigment epithelium (RPE)]. We compared these results to those obtained by treating the same cells with elevated glucose, to assess the relative effects of each stimulus on these retinal cell cultures.

Methods

Retinal cell culture

Primary human retinal Müller Cells were isolated from human donor tissue (NDRI; Philadelphia, PA) within 24 hrs post-mortem, using a modified protocol developed by Hicks and Courtois [7]. The retina was dissected from the eye cup and dissociated in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies; Grand Island, NY, USA) containing trypsin and collagenase. Following incubation in dissociation medium, cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1X antibiotic/antimycotic solution.

Primary human RMEC (Cell Systems; Kirkland, WA, USA) were cultured on attachment factor (Cell Signaling; Danvers, MA) in phenol red-free endothelial basal medium (EBM: Lonza; Walkersville, MD, USA) containing 10% FBS and SingleQuots (Lonza).

The ARPE-19 cell line (American Type Culture Collection; Manassas, VA, USA) was cultured in DMEM/F12 (Life Technologies) containing 10% FBS and 1X antibiotic/antimycotic solution.

Primary human retinal astrocytes (Sciencell; Carlsbad, CA, USA) were cultured on poly-L-lysine (Sciencell) in Astrocyte Growth Medium containing 2% FBS, astrocyte growth supplement, and a penicillin streptomycin solution (Sciencell).

Cell treatment and enzyme-linked immunosorbent assays

Growth medium was replaced with 2% FBS-containing medium for 12 hrs prior to treatment. All cell types were matched for confluency at the time of treatment. Cells were treated with bovine serum albumin (BSA; 100 mg/ml), BSA-bound oleic acid (OA; 60 μM), or BSA-bound linoleic acid (LA; 60 μM). These doses were chosen for their physiological relevance, because total circulating levels of free fatty acids can be as high as 600 μM [8,9]. Furthermore, these concentrations are within ranges used in studies of other retinal cell behaviors [6]. Other cells were treated with 2% FBS medium, or 2% FBS medium plus L-glucose (osmotic control; 25 mM) or D-glucose (25 mM). After 36 hrs of treatment, cell medium was collected for secreted protein analysis by ELISA. Cell lysates were collected and total cellular protein was determined by BCA assay (Pierce; Rockford, IL, USA). The cytokine levels were normalized to total cellular protein. Human VEGF, IL-6 and IL-8 ELISAs (R&D Systems; Minneapolis, MN, USA) were performed according to the manufacturer's protocol.

Statistical Analysis

Data were analyzed with commercial software (JMP; SAS Institute; Cary, NC, USA) using ANOVA with Student's post hoc analysis. $p<0.05$ was considered statistically significant.

Results

The Effect of Oleic Acid, Linoleic Acid, and Elevated Glucose on VEGF, IL-6 and IL-8 Production by Human Retinal Cells.

As shown in Figure 1, human retinal Müller cells responded robustly to treatment with LA. LA induced a 9.9-fold increase of VEGF ($p<0.0001$), a 2.9-fold increase of IL-6 ($p=0.0002$) and a 5.7-fold increase of IL-8 ($p=0.0004$). OA increased VEGF production 6.4-fold ($p=0.0007$). L-glucose and D-glucose both significantly induced VEGF production in Muller cells, suggesting the osmotic stress may be responsible. LA significantly up-regulated IL-8 (56%; $p=0.0153$) in human RMEC; however, RMEC did not respond to any other stimulus. None of the experimental stimuli induced ARPE-19 or human retinal astrocytes to produce any of the inflammatory mediators assayed in this study. These response patterns were also observed at both earlier (24 hr) and later (48 hr) time points (data not shown).

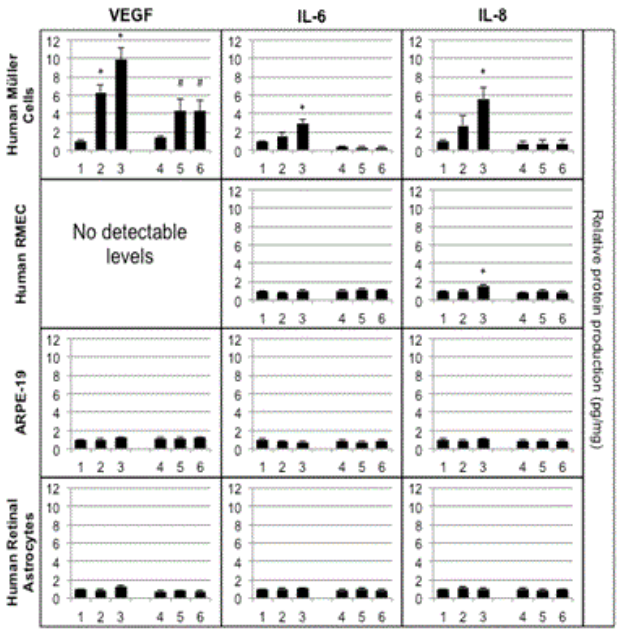

Figure 1: The effect of oleic acid, linoleic acid, and elevated glucose on VEGF, IL-6, and IL-8 production. Human retinal Müller cells, RMEC, ARPE-19 and retinal astrocytes were treated for 36 hours with the following: 1: BSA (100 mg/ml), 2: Oleic Acid (BSA-bound; 60 μM), 3: Linoleic Acid (BSA-bound; 60 μM), 4: Normal glucose (5.5 mM), 5: L-glucose (25 mM), 6: D-glucose (25 mM). Data is presented as the average ± SEM; *$p<0.05$ compared to BSA control, and #$p<0.05$ compared to normal glucose control; n=9.

Discussion

The initial causes of early inflammation in the diabetic retina are still undefined. A prevalent theory is based on the notion that elevated blood glucose induces inflammatory mediators in the diabetic retina, leading to low-grade chronic inflammation and subsequent vision-threatening pathology. Accordingly, elevated D-glucose is often used to mimic diabetic inflammatory conditions in cell culture. In this study, D-glucose did not induce the expression and secretion of VEGF, IL-6, or IL-8 by primary cell types involved in retinal inflammation (glial cells and blood-retina-barrier cells). However, we did find that linoleic acid was an effective inducer of these cytokines by Müller cells and RMEC, suggesting its relevance to diabetes-induced retinal inflammation.

Müller glia are found exclusively in the retina, and are the only retinal cells that span almost the entire retinal thickness. Diabetic conditions activate Müller glia, causing them to become a significant source of cytokines that signal monocyte/macrophage recruitment and activation. Our results suggest that, based on their responsiveness to

fatty acids, Müller cells may be the most significant retinal cell type to effect cytokine induction brought about by diabetes-related dyslipidemia. Notably, this was the only cell type tested in which linoleic acid stimulated VEGF, IL-6, and IL-8, all of which are up-regulated in vitreous of diabetics. These inflammatory mediators may act by both autocrine and paracrine mechanisms to promote inflammatory responses in retinal cells. Therefore, cytokine-induced cytokine production by Müller cells might present an important therapeutic target to prevent the progression of DR from early to late stages.

We found that linoleic acid induced human RMEC to express and secrete IL-8. IL-8 is a neutrophil chemotactic factor that facilitates monocyte homing to activated endothelium. IL-8 is an activator of NF-κB, and therefore elevated levels of IL-8 can induce the NF-kB target genes, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Both adhesion proteins are leukocyte adhesion molecules that are localized to the surface of the retinal endothelium and are up-regulated in DR. Their expression precedes leukocyte adhesion to the retinal endothelium, leukostasis, and vascular regression, all hallmarks of DR pathology. In a previous study, linoleic acid induced the expression of VCAM-1 and ICAM-1 in human RMEC [6] and this might be through an IL-8-dependent mechanism. Taken together the results suggest that linoleic acid can trigger diabetes-related pro-inflammatory events in RMEC that may be directly related to those brought about by dyslipidemia in DR.

Future studies will seek to determine a mechanism whereby linoleic acid induces Müller cells and RMEC to produce inflammatory mediators. The lipoxygenase (LOX) pathway may warrant exploration in this context. For example, linoleic acid is a LOX substrate, LOX activity induces pro-inflammatory mediators following linoleic acid treatment [6] and 12-LOX activity is increased in experimental diabetes [10,11]. Peroxisome proliferator-activated receptors (PPARs) are nuclear transcription factors that regulate inflammatory signaling events. Linoleic acid directly binds PPARs [12], and the impact of these binding events on DR pathology may point to another plausible mechanism of significant interest. It is important to note, however, that there is no evidence to support that dyslipidemia alone induces DR-like pathology. Thus, combined use of D-glucose and fatty acids may potentiate the production of inflammatory cytokines. Future studies will aim to characterize the interdependence of these stimuli in order to elucidate the mechanisms by which dyslipidemia interacts with the diabetic condition to generate retinal pathology.

In summary, linoleic acid significantly induced the expression and secretion of pro-inflammatory cytokines by human retinal Müller cells and RMEC; in this study, LA was a more effective stimulus than elevated glucose in culture. Based on these data, we propose that linoleic acid challenge of retinal cells is relevant to DR-induced inflammatory changes and may be preferable to high glucose challenge in cell-based experiments. These findings support the significance of dyslipidemia in the induction of DR pathology.

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