Effects of Inhibiting High Glucose-Induced Uptregulation of ECM on Connexin 43 Expression in Retinal Endothelial Cells

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Abstract

Introduction: Diabetic retinopathy (DR) is the leading cause of blindness in the working age population. Early stage DR is characterized by apoptotic vascular cell death in the retina. However, it is unclear how hyperglycemia triggers apoptosis in these cells. In this study we investigated if high glucose-induced overexpression of fibronectin (FN) and collagen type IV (Coll IV), two basement membrane (BM) components upregulated in diabetes, promote apoptosis by reducing connexin 43 (Cx43) expression and gap junction intercellular communication (GJIC).

Methods: To determine if correction of the abnormally high FN and Coll IV expression improved GJIC and subsequently rescued cells from apoptosis, retinal endothelial cells grown in high glucose (HG) were subjected to FN- or Coll IV siRNA transfection. Protein and mRNA levels from these cells was analyzed by Western blot for FN, Coll IV, and Cx43 levels. Parallel assays were conducted to determine GJIC and apoptosis. Cells were immunostained for FN, Coll IV, and Cx43.

Results: Western blot analysis indicated significantly increased FN and Coll IV and reduced Cx43 in HG cells. When cells grown in HG were transfected with FN siRNA or Coll IV siRNA, a significant reduction in their respective protein levels was observed; scrambled siRNA had no effect. Interestingly in cells grown in HG and transfected with FN siRNA or Coll IV siRNA, both Cx43 expression and GJIC was significantly upregulated. This finding was supported by immunofluorescence studies in which Cx43 expression was upregulated in HG cells transfected with FN siRNA or Coll IV siRNA. TUNEL assays indicated that the transfected cells grown in HG exhibited reduced number of apoptotic cells.

Conclusion: Our findings indicate that HG-induced upregulation of FN and Coll IV component expression reduces Cx43 and GJIC and thus, promote apoptosis. The siRNA strategy may be useful in preventing retinal vascular cell death in DR.

Introduction

Increased synthesis of basement membrane components, fibronectin and collagen type IV, is closely associated with the development of diabetic retinopathy (1, 2, 3). Cx43 gap junction channelals allow the exchange of small molecules between adjacent cells and provide retinal vascular homeostasis. In diabetes, overexpression of FN and Coll IV expression and subsequent downregulation of Cx43 has been reported to be reduced (4). Both of these abnormalities play an important role in the pathogenesis of early stage diabetic retinopathy. However, an association between these two abnormalities has not been identified.

Excess synthesis of BM components contributes to endothelial cell loss, pericyte loss, and vascular permeability in diabetic retinopathy (5). Downregulation of Cx43 expression and subsequent reduction of GJIC have been shown to induce apoptosis in retinal endothelial cells and pericytes (6). A common feature is that both these abnormalities contribute to apoptosis in the retinal vascular cells. However, it is unknown whether one abnormality influences the other or whether they occur independently.

In this study, we have investigated if specific reduction of FN and Coll IV could impact Cx43 expression and GJIC. Results from this study would provide novel information related to regulation of abnormal overexpression of basement membrane components on Cx43 expression/GJIC in the pathogenesis of early stage diabetic retinopathy.

Methods

Cell Culture

Retinal endothelial cells (RECs) were grown in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum and antibiotics and antimycotics in normal (5nm) or high (30nm) glucose medium for 8 days.

Transfection

RECs plated in high glucose medium were grown to subconfluency and transfected with FN siRNA, Coll IV siRNA, or scramble siRNA. Cells were transfected in reduced serum medium Optimum with 5mL Lipofectamine reagent and 2mL FN siRNA, 2mL Coll IV siRNA, or 4mL scrambled siRNA. The cells were then analyzed 48 hours later.

Cell Electrophoresis and Western Blotting

The protein samples from the five groups of cells were analyzed for FN, Coll IV, and Cx43 expression by Western blot analysis. Briefly after electrophoresis and transfer, the membrane was blocked and exposed to rabbit FN antibody, or rabbit Coll IV antibody, or rabbit Cx43 antibody. The membranes were then incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase and subjected to chemiluminescent assay. The membrane was then exposed to x-ray films and densitometry analysis of the Western blot image was performed using image analysis software (NIH Image J).

Scrape Load Dye Transfer (SLDT)

SLDT was used to evaluate GJIC activity. RECs were grown to confluence on coverslips and exposed to Lucifer Yellow and simultaneously random cuts were made on the confluent monolayers. The cells were then fixed with 10% formalin and imaged under fluorescence microscopy. The number of dye couple cells layers was counted on each slide and from at least two random fields.

TUNEL Assay

TUNEL assay was performed using the ApopTag in situ Apoptosis Detection kit according to manufacturer’s instruction. Following TUNEL, assay cells were mounted together with DAPI. DAPI was used to identify the cell nuclei. Images of 10 random fields were digitally captured for each cover slip.

Double Immunofluorescence Staining

Cells were grown on cover slips, fixed, and exposed to primary antibody (rabbit FN antibody, or goat Coll IV antibody, or mouse Cx43 antibody). The cells were incubated in a moist chamber at 4 °C overnight, washed with PBS and exposed to secondary antibody (FITC-conjugated anti-rabbit IgG or rhodamine conjugated anti-mouse IgG). After exposure to secondary antibody, the cells were washed in PBS, mounted, and examined under fluorescence microscope. Images were digitally recorded and analyzed for FN, Coll IV, and Cx43 immunostaining using image analysis software (NIH Image J).

Results

The expression and immunostaining of FN and Coll IV was significantly increased whereas those of Cx43 was significantly reduced in RECs exposed to high glucose condition. FN and Coll IV cell expression induced increased Cx43 expression. Cells transfected with FN siRNA exhibited increased Cx43 expression. Cells transfected with Coll IV siRNA exhibited increased Cx43 expression.

Summary

The expression and immunostaining of FN and Coll IV was significantly increased whereas those of Cx43 was significantly reduced in RECs exposed to high glucose condition.

Cells grown in high glucose condition exhibited reduced GJIC activity.

Downregulation of FN or Coll type IV expression using siRNA strategy resulting in upregulation of Cx43 expression and increased GJIC, which in turn enhanced cell communication, and rescued cells from apoptosis.

Conclusion

This finding indicates that high glucose induced overexpression of ECM components may play a role in regulating cell-cell communication and mediate apoptosis.

References


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