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Full Length Research Paper

Sp1 and VEGF mRNA expression in the retina micrangium endothelial cells of streptozotocin-induced diabetic rats

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The aim of this study is to evaluate the Sp1 and VEGF mRNA expression in the retina micrangium endothelial cells of streptozotocin-induced diabetic rats. At the end of our study, we determined that SP1 positive expression rate and the Sp1, VEGF mRNA and protein expression in the retina micrangium endothelial cells in isolated rat eyes of diabetic rats were significantly increased compared to normal animals. The ERK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats was significantly decreased in high-surcose (Sp1 specific inhibitor Mithramycin A or ERK specific inhibitor PD98059) culture group in comparison to normal (vechile PBS) culture group. These findings suggest that ERK, Sp1 and VEGF protein expression is an important factor to affect the occurrence and development of vision diseases in diabetic patients.

Key words: Sp1, VEGF, PCR, Retina, Immunohistochemistry.

INTRODUCTION

Diabetic retinopathy (DR) is the leading cause of vision impairment and blindness throughout the world, including China (Resnikoff et al., 2004). There are a number of hypotheses for the potential causes of the microvascular, neural and glial cell changes that occur in the diabetic retina. A contribution of neural degeneration or dysfunction to the development of the retinal microvascular disease has been suggested (Antonetti et al., 2006; Barber et al., 1998; Gardner et al., 2002; Wolter, 1961), but not systematically investigated. Therefore, it is imperative to develop better approaches for the identification, evaluation, and prevention of diabetic retinopathy before the onset of vision damage (Antonetti et al., 2006). It appears that all of the major retinal cell types such as neurons, macroglias and microglias, as well as vascular cells, are involved in the pathogenesis of diabetic retinopathy (Gardner et al., 2002). In this study, we investigate the Sp1 and VEGF mRNA expression in the retina micrangium endothelial cells of diabetic rats. We also examine the RRK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats in high-surcose culture group and normal culture group.

METHODOLOGY

Animals

Male wistar rats were selected for the study. They were of the same age (2 months) and weight (190-230 g). The rats were housed in polycarbonated clean cages under a 12/12 h normal light/dark cycle. The animals were fed with standard diet and water ad libitum. After keeping in the laboratory condition for a week for acclimatization the experiment was initiated. A total of 20 male

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wistar rats were categorized into 2 groups, each consisting of ten rats.

Induction of diabetes

STZ-induced hyperglycemia has been described as a useful experimental model to study the activity of hypoglycemic agents (Junod et al., 1969; Ledoux et al., 1966). After overnight fasting (deprived of food for 16 hours had been allowed free access to water), diabetes was induced in rats by intraperitoneal injection of STZ (Sigma, St. Louis, Mo) dissolved in 0.1 M sodium citrate buffer pH 4.5 at a dose of 55 mg/kg body weight (Chattopadhyay et al., 1997). The control rats received the same amount of 0.1 M sodium citrate buffer. The animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycemia. After a week time for the development of diabetes, the rats with moderate diabetes having glycosuria and hyperglycemia (blood glucose range of above 250 mg/dl) were considered as diabetic rats and used for the further experiments. The change in the body weight was observed throughout the treatment period in the experimental animals. At the end of experiment, rats were killed and eyeballs were removed. Retina was taken from eyeballs for measuring Sp1 and VEGF mRNA and protein expression.

Cell culture

The retina micrangium endothelial cells were plated at a density of 10^6 cells/well in 12-well tissue culture plates overnight. The cells were pretreated with or without SAE (0–6 mg/ml), and incubated at 37°C in 5% CO_2 for 2 h before stimutation with Escherichia coli LPS (5 μg/ml). After 2-h stimulation at 37°C in 5% CO_2, the cells were harvested and stored in RNA stabilization reagent at −80°C until RNA extraction. Total cellular RNA was isolated by RNA isolation kit according to the manufacturer’s instructions and treated with DNase. RNA purity was confirmed with the spectrophotometric absorbance ratio at 260/280 and RNA quantity was determined by the absorbance at 260 nm.

Immunohistochemistry

Immunohistochemistry of Sp1was performed by the ABC method using the Vector ABC kit (Vector Laboratories, Burlingame, CA. Briefly, 1.5 mmsections were deparaffinized and incubated in methanol containing 0.3% H_2O_2 for 30 min and then with normal goat serum (1:20) to block nonspecific staining. Sections were intensely washed in PBS between the procedures. The sections were then incubated in antiserum against (P) RR (1:500) or non-immune rabbit serum (1:500) (the negative control) for 20 h at 4°C. Sections were incubated in biotinylated secondary antibody to rabbit IgG (1:400) for 30 min at room temperature and subsequently incubated with peroxidaseconjugated avidin for 30 min using the Vector ABC kit. These sections were visualized by immersion in 3, 30-diaminobenzidine solution (0.01 mol/l 3, 30-diaminobenzidine in 0.05 mol/l Tris–HCl buffer (pH 7.6) and 0.006% H_2O_2). The specificity of the Sp1 antiserum was examined by the absorption test. The diluted antiserum (1:500) was incubated with the antigen peptide at concentrations of 10 nmol peptide/ml of the diluted antiserum for 20 h at 4°C prior to use.

Evaluation of mRNA expression of Sp1 and VEGF

cDNA was synthesized from 240 ng of total RNA with a two step reverse transcription-polymerase chain reaction (RT-PCR) kit using a thermal cycler (GeneAmp® PCR system2400, Perkin–Elmer, Roche, NJ, USA). Specific oligonucleotides based on the published sequences (Won et al., 2006) and their annealing temperature (TA) were as follows: Sp I (forward): 5'-GGAGAAAACAGCCCAGGATGC-3', (backward): 5'-CTCATCCGAACGTGTGAAGC-3'; VEGF (forward): 5'-CTGTACCTCCACCATGCCAAG-3', (backward): 5'-ACAAGGCTCACAGTGAACGC-3' ; β-actin (forward): 5'-CATCCTGCGTCTGGACCT-3', (backward): 5'-TCAGGAGGAGCAATGATCTTG-3'. A dynamic test and cycle determination of PCR protocols were performed to determine the linear range of products and ensure an accurate semiquantitative analysis. There were 30–35 cycles of denaturation at 95°C for 2min, annealing at TA for 1 min and primer extension at 72°C for 1min (Sp I, VEGF, β-actin). The reactions were finally extended at 72°C for 10 min. A blank control (RT-PCR without RNA template) and RT(−) reactions (PCR without reverse transcription) were executed along with all RT-PCR reactions. The expected PCR products for Sp I, VEGF and β-actin were 453, 384 and 297 bp, respectively. The PCR products were analyzed by electrophoresis in 1.5% agarose gel and visualized by ethidium bromide staining.

Quantitative analysis was performed using the photographs and image-analysis software (Gel Documentation InGenius® L, Bio-Rod Lab, Hercules, CA, USA). The relative amount of COX-2, COX-1 and iNOS mRNA expression was calculated as its ratio to β-actin from the same template.

Western blot analysis

Tissues were homogenized for extract proteins in icecold protein extraction buffer (Wuhan Boster Biological Technology Company, China). The homogenates were centrifuged at 12,000g for 10 min to collect the supernatants. Bradford method was used to determine the concentration of proteins. About 20 mg of protein from each samplewas separated on SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane using an electroblotting apparatus (Bio-Rad). Membranes were blocked in 5% nonfat dry milk in PBS buffer for 1.5 h at room temperature. Blocked membranes were incubated in rabbit polyclonal antibodies specific for rat ERK, Sp1, VEGF (Santa Cruz, Santa Cruz, CA) or GAPDH (Wuhan Boster Biological Technology Company, China), in PBS (containing 1% nonfat dry milk) overnight at 4°C. Antirabbit secondary antibody (Wuhan Boster Biological Technology Company, China), at a concentration of 1:1000 in PBS (containing 1% nonfat dry milk), was added to membranes and incubated for 1.5 h at room temperature. The protein signal was developed using NBT/BCIP system. The results of Western blotting were quantified with Gene Snap software (Syngene, America).

Statistic analyses

Data were expressed as the mean ± standard deviation. One-way ANOVA was used to elucidate if there were significant differences between treatments groups and the control groups ($p < 0.05$ was considered as a statistically significant difference).

RESULTS

Positive expression rate of SP1 in rats’ retina micrangium endothelial cells were significantly increased ($p<0.01$) in diabetic model rats (78.17±8.51 vs. 51.23±6.03) when comparing with normal control animals (Table 1).

The Sp1 and VEGF mRNA expression in the retina micrangium endothelial cells of normal and diabetic rats

Table 1. Positive expression rate of SP1 in rats' retina micrangium endothelial cells.

Group	Positive expression rate (%)
Normal control group	51.23±6.03
Diabetic model group	78.17±8.51 [b]

[b] p<0.01, compared with Normal control group.

Table 2. The Sp1 and VEGF mRNA expression in the retina micrangium endothelial cells.

Group	Sp1 mRNA expression	VEGF mRNA expression
Normal control group	0.42±0.05	0.64±0.06
Diabetic model group	0.66±0.07 [b]	0.83±0.07 [b]

[b] p<0.01, compared with Normal control group.

Table 3. The Sp1 and VEGF protein expression in the retina micrangium endothelial cells.

Group	Sp1 protein expression	VEGF protein expression
Normal control group	58.04±4.29	62.69±6.93
Diabetic model group	79.13±6.32 [b]	86.21±6.38 [b]

[b] p<0.01, compared with Normal control group.

Table 4. The RRK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats in high-surcose (ERK specific inhibitor PD98059) culture group and normal (vechile PBS) culture group.

Group	ERK protein expression	Sp1 protein expression	VEGF protein expression
Normal (vechile PBS) culture group	68.25±5.39	77.47±3.82	88.35±5.39
High-surcose (PD98059) culture group	39.83±4.02 [b]	52.01±4.44 [b]	69.32±4.02 [b]

[b] p<0.01, compared with Normal control group.

are described in Table 2. In diabetic rats, Sp1 and VEGF mRNA expression were significantly increased (0.66±0.07 vs. 0.42±0.05; 0.83±0.07 vs. 0.64±0.06) in the retina micrangium endothelial cells (p<0.01) compared to normal control group.

The Sp1 and VEGF protein expression in the retina micrangium endothelial cells of normal and diabetic rats is described in Table 3. In diabetic rats, Sp1 and VEGF protein expression were significantly increased (79.13±6.32 vs. 58.04±4.29; 86.21±6.38 vs. 62.69±6.93) in the retina micrangium endothelial cells (p<0.01) compared to normal control group.

The ERK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats were evaluated in high-surcose (ERK specific inhibitor PD98059) culture group and normal (vechile PBS) culture group. After 24h of culture, the ERK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats in was significantly reduced (39.83±4.02 vs. 68.25±5.39; 52.01±4.44 vs. 77.47±3.82; 69.32±4.02 vs. 88.35±5.39) (p<0.01) in high-surcose (ERK specific inhibitor PD98059) culture group in comparison to normal (vechile PBS) culture group (Table 4).

The ERK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats were evaluated in high-surcose (Sp1 specific inhibitor Mithramycin A) culture group and normal (vechile PBS) culture group. After 24h of culture, the RRK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats in was significantly decreased (36.06±3.33 vs. 71.22±5.41; 37.59±3.29 vs. 59.11±4.17; 43.08±4.01 vs. 75.33±5.92) (p<0.01) in high-surcose (Sp1 specific inhibitor Mithramycin A) culture group in comparison to normal (vechile PBS) culture group (Table 5).

DISCUSSION

Retinal neovascular diseases, including age-related macular degeneration (AMD), diabetic retinopathy, and retinopathy of prematurity, are major causes of blindness Angiogenesis is known to be the most important

Table 5. The RRK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats in high-surcose (Sp1 specific inhibitor Mithramycin A) culture group and normal (vechile PBS) culture group.

Group	ERK protein expression	Sp1 protein expression	VEGF protein expression
Normal (vechile PBS) culture group	71.22±5.41	59.11±4.17	75.33±5.92
High-surcose (Mithramycin A) culture group	36.06±3.33 [b]	37.59±3.29 [b]	43.08±4.01 [b]

[b] p<0.01, compared with Normal control group

aggravating factor (Akiyama et al., 2002). VEGF is a potent angiogenic factor and has been postulated to play a key role in the process of retinal disease. VEGF is produced in many of the types of ocular cells of which the retina and choroid are composed (Lutty et al., 1996). The regulatory mechanisms of expression of the VEGF gene have been studied in many different cell types, including glioma cells, fibroblasts, endothelial cells, vascular smooth muscle cells, and cardiac myocytes.

Although Sp1 is generally considered a ubiquitous transcription factor, there is considerable evidence that Sp1 participates in cell type-specific gene expression (D'Angelo et al., 1996; Patterson et al., 1997; Noti et al., 1996; Baker et al., 1996; Chen et al., 1993), is developmentally (Saffer et al., 1991) and functionally regulated (Persengiev et al., 1996; Saffer et al., 1990), and is highly expressed during vasculogenesis (Saffer et al., 1991). In fact, the results of electrophoretic mobility shift assays (EMSAs) showed that Sp1 protein binds to this site and light stimulation increases the binding of nuclear factors to the Sp1 site. Previous studies support the idea that Sp1 plays a role in mediating the inducible expression of various genes, such as superoxide dismutase (Tanaka et al., 2000), glucose activation of the carboxylase (Daniel and Kim, 1996), plasminogen activator inhibitor-1 (Chen et al., 1998), and VEGF genes (Ryuto et al., 1996). In addition, a role of Sp1 has been described in PMA-induced expression of the WAF1/CIP1 (Biggs et al., 1996) gene and the platelet thromboxane receptor gene (D' Angelo et al., 1996).

This study has demonstrated altered positive expression rate of SP1 in rats' retina micrangium endothelial cells and the Sp1 and VEGF mRNA and protein expression in the retina micrangium endothelial cells in isolated rat eyes after the onset of STZ-induced diabetes. The results for control and diabetic eyes showed that SP1 positive expression rate and the Sp1, VEGF mRNA and protein expression in the retina micrangium endothelial cells in isolated rat eyes of diabetic rats were significantly increased compared to normal animals. This indicated that Sp1 and VEGF changes in retina micrangium endothelial cells are a feature of early diabetes. In addition, the ERK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats in was significantly decreased in high-surcose (Sp1 specific inhibitor Mithramycin A or ERK specific inhibitor PD98059) culture group in comparison to normal (vechile PBS) culture group. This indicated that Sp1 specific inhibitor Mithramycin A or ERK specific inhibitor PD98059 may decrease the ERK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats. This indicates that ERK, Sp1 and VEGF protein expression is an important factor to affect the occurrence and development of vision diseases in diabetic patients. This work can provide a useful molecular mechanism of the therapy of eye dieases.

Conclusion

We found that the VEGF gene expression was performed through Sp1-binding sites of the rat VEGF promoter. In addition, the ERK, Sp1 and VEGF protein expression in the retina micrangium endothelial cells of diabetic rats in was significantly decreased in high-surcose (Sp1 specific inhibitor Mithramycin A or ERK specific inhibitor PD98059) culture group in comparison to normal (vechile PBS) culture group. Therefore, our findings raise the possibility that pharmacological intervention that inhibits the Sp1, VEGF mRNA and protein expression may be effective in treating VEGF-mediated retinopathies.

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