·Basic Research·

Increased expression of Ets-1 in the diabetic retina of streptozotocin-induced rat model

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Abstract

• AIM: To determine the involvement of Ets-1 in the pathological progress of the experimental diabetic retina.

• METHODS: Diabetes was induced by intraperitoneal injection of STZ. Total RNA and total proteins were isolated from retinas of experimental and control eyes at 4 weeks after STZ-injection and were assessed by Northern blot analysis and Western blot analysis, respectively.

• RESULTS: Expression of both Ets-1 mRNA and Ets-1 protein was significantly increased in the experimental diabetic rat's retina after STZ-injection compared with the control group (P<0.001).

• CONCLUSION: Our results indicate that Ets-1 is involved in the pathological progress of experimental diabetic retina. Further studies should be conducted to focus on the relationship between Ets-1 and VEGF in the diabetic retina.

• KEYWORDS: Ets-1; VEGF; diabetic retina; angiogenesis

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INTRODUCTION

Ets-1 (E26 transformation-specific-1) is the prototype of the ETS family of transcription factors, which are characterized by a conserved ETS domain that is capable of binding to DNA sequences containing a core GGAA/T motif [1,2]. It plays an important role in cell proliferation, differentiation, development, transformation, angiogenesis, and apoptosis [3,4]. Ets-1 has been reported to regulate angiogenesis in vascular endothelial cells. Increased Ets-1 expression is observed in cultured endothelial cells and in endothelial cells of new vessels during tumor angiogenesis in the adult [5, 6]. Numerous angiogenesis-related genes have been demonstrated to be targets of Ets-1 [6-9]. Moreover, VEGF-induced angiogenesis can be inhibited through inhibition of Ets-1 expression in cultured human aortic endothelial cells and in a rabbit corneal neovascularization model[10].

Although the association of Ets-1 with the development of angiogenesis is known, the role of Ets-1 in the diabetic retina has been seldom studied. The purpose of this study is to determine the involvement of Ets-1 in the pathological progress of the experimental diabetic retina.

MATERIALS AND METHODS

Rabbit polyclonal anti-Ets-1 antibody and murine Ets-1 cDNA were kindly provided by Doctor Jian (the Fourth Military Mediccal University). STZ was purchased from Sigma (St. Louis, MO). Anti-actin polyclonal antibody was obtained from NeoMarkers, Inc. (Fremont, CA). The BCA Protein Assay Kit was obtained from Beyotime (Beijing, China). All of the other reagents were purchased from Sigma unless otherwise specified.

Animals All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Care and Use of Laboratory Animals and were authorized by the Fourth Military Medical University Bioethics Committee. Age-matched female Sprague-Dawley rats (obtained from animal centre of the Fourth Military Medical University) were used in all experiments. Rats were housed under a

12-hour light/dark cycle with free access to a standard rat food and water. Diabetes was induced by intraperitoneal injection of STZ (65mg/kg)[11, 12] dissolved in sodium citrate buffer, pH 4.5, and control rats received equivalent volumes of buffer alone. Food was withdrawn 16 hours before the experiments. Rats were anesthetized with 100mg/kg of sodium pentobarbital injected intraperitoneally. STZ-injected rats were considered diabetic when exhibiting blood glucose levels >13.9mmol/L (250mg/dL) within 3 days after diabetes induction. Each week, rats were weighed and blood glucose (nondiabetic, 4 to 8mmol/L; diabetic, 13.9 to 27mmol/L) estimated using an Accutrend α glucometer (Mannheim Boehringer, NSW, Australia). Ten rats of each group were killed at 4 weeks after STZ injection, and total RNA and total proteins were isolated from retinas to detect Ets-1 expression.

Northern Blot Analysis Total RNA was isolated from retinas using guanidinium thiocyanate at the indicated time point. Northern blot analysis was performed on 20mg total RNA after 10g/L agarose/2mol/L formaldehyde gel electrophoresis and subsequent capillary transfer to Biodyne nylon membranes (Pall BioSupport) and ultraviolet cross-linking using a FUNA-UV-LINKER (FS-1500, Funakoshi Inc). Radioactive probes were generated using Amersham Megaprime labeling kits and [^{32}P]dATP (Amersham). Blots were prehybridized, hybridized, and washed in 0.53 SSC and 50g/L SDS at 65℃ with four changes over 1 hour in a rotating hybridization oven (TAITEC). All signals were analyzed using a densitometer (BAS-2000II, Fuji Photo Film), and lane loading differences were normalized using a 36B4 cDNA probe, which hybridizes to acidic ribosomal phosphoprotein PO. Murine Ets-1 cDNA was used as a probe.

Western Blot Analysis Total proteins from retinas were extracted with cold RIPA containing 20g/L protease inhibitors and supernatant fluid was collected after centrifuging at 10 000g for 10 minutes. The protein concentration of the supernatant was determined using the BCA Protein Assay Kit. Total proteins (50μg) were subjected to 100g/L polyacrylamide gel electrophoresis, and were transferred to nitrocellulose membrane (Schleicher & Schuell; Dassel, Germany). The blots were incubated overnight at 4℃ with Ets-1 primary antibody followed by incubation for 2 hours with horseradish peroxidase-conjugated secondary antibody (1:2 000 dilution)(Amersham International, Buckinghamshire, UK). Primary antibody specific for Ets-1 was used at 1:500 dilution. Visualization was performed by enhanced chemiluminescence detection system (Amersham International).

Statistical Analysis Determinations were performed in triplicate, and experiments were repeated at least three times. Results are expressed as the mean ±SD. The paired Student's *t*-test was used to evaluate the differences of Ets-1 expression between control and experimental groups.

RESULTS

Rat Characteristics All diabetic rats gained significantly less weight than their age-matched control groups. The blood glucose values of the diabetic rats were significantly higher than control values for the entire length of the experiment (P <0.01). These data were similar with other previous reports[11-13].

Expression of Ets-1 mRNA in the Diabetic Retina of STZ-induced Rat Model Expression of Ets-1 mRNA was studied by Northern blot analysis. Total mRNA was isolated from retinas of rats at 4 weeks (10 retinas from five rats of each group). Ets-1 mRNA level was increased significantly, reaching a 4.5-fold increase of control at the indicated time point (P <0.001, Figure 1A).

Expression of Ets-1 Protein in the Diabetic Retina of STZ-induced Rat Model Western blot analysis was used to investigate Ets-1 protein expression (Figure 1B). Total proteins were isolated from retinas of rats at 4 weeks (10 retinas from five rats of each group). Ets-1 protein level in the experimental eyes were much higher than that in the control eyes, reaching a 4-fold increase of control at the indicated time point (P <0.001).

DISCUSSION

Diabetic retinopathy remains a leading cause of blindness in the western countries[14], and is characterized by an increased retinal neovascularization due to the action of the angiogenic factor, VEGF [15,16]. The promoter region of VEGF gene contains Ets binding sites, and previous reports implicated that Ets-1 played an important role in angiogenesis by induction of VEGF[17]. In addition, VEGF increased the level of Ets-1 mRNA in cultured BRECs in a time-and dose-dependent manner [18], and Ets-1 up-regulated VEGF expression and increased angiogenesis in oral cancer [17]. Thus, the aim of the present study is to investigate the possibility of Ets-1 being involved in the pathological progress of the diabetic retina.

Our results showed that expression of both Ets-1 mRNA and

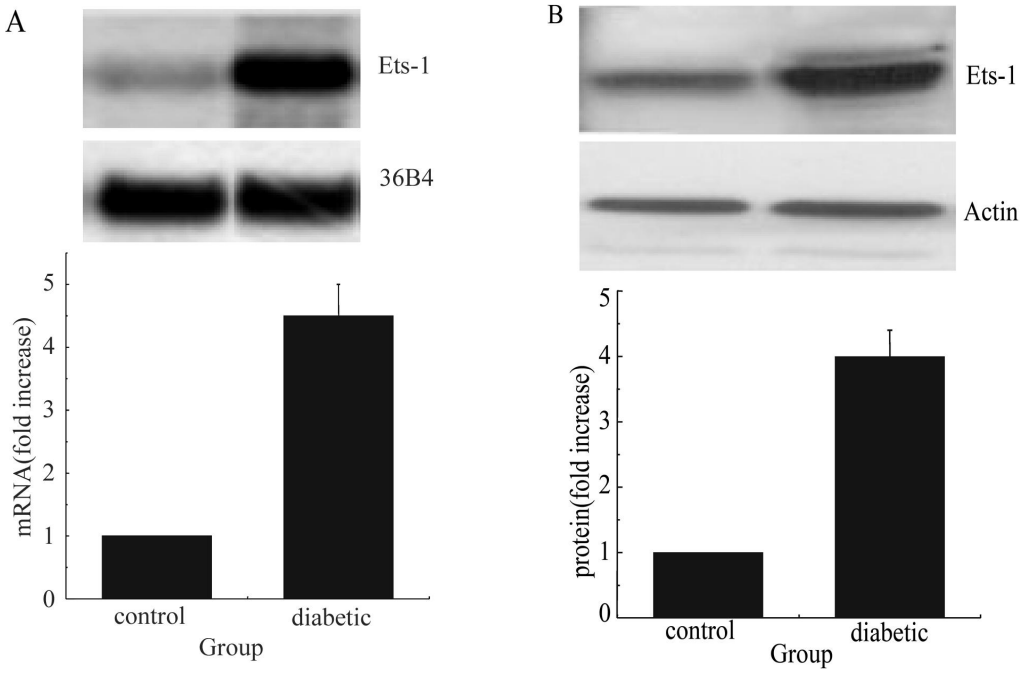

Figure 1 Up-regulation of Ets-1 mRNA and protein levels in the diabetic retina. Total RNA and total proteins were isolated from rat´s retinas at 4 weeks after STZ-injection A: Ets-1 mRNA expression in retinas. Total RNA was assessed by Northern blots analysis. Representative Northern blots and control 36B4 (top) and quantitation (bottom); B: Ets-1 protein expression in retinas. Total proteins were assessed by Western blot analysis. Actin was used as a loading control. Representative Western blots (top) and quantitation (bottom). Results are shown as fold increase of control. Mean±SD of three independent experiments (each in triplicate). $P<0.001$

Ets-1 protein was significantly increased in the experimental diabetic rat's retina after STZ-injection. It was thought that angiogenesis was regulated by a balance of angiogenic factors and angiogenesis inhibitors[19]. Ets-1 was one of these angiogenic factors and functioned as a principle transcription factor converting endothelial cells (ECs) to the angiogenic phenotype [20]. Furthermore, there was a significant correlation between microvessel counts (MVCs) and Ets-1 mRNA levels in ovarian cancers, and Ets-1 was indicated to be an angiogenic mediator in uterine endometrium linked to VEGF [21,22] Therefore, our results together with these previous reports suggested that Ets-1 was involved in the pathological progress of diabetic retina and might be one of initial angiogenic factors in the diabetic retinal neovascularization.

Other results of our experiments (unpublished) demonstrate that the time courses of VEGF and Ets-1 expression are identical to each other. Ets-1 is indicated to regulate angiogenesis through the induction of VEGF, and at the same time, VEGF is also proved to induce the expression of Ets-1 mRNA in several endothelial cells [23-25] Thus, up-regulation of Ets-1 by VEGF activated its own gene expression. This auto-loop of up-regulation of Ets-1 may have a role in the regulation of diabetic retinal neovascularization.

In summary, the present study demonstrates that Ets-1 is involved in the pathological progress of experimental diabetic retina. Further studies should be conducted to focus on the relationship between Ets-1 and VEGF in the diabetic retina.

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