Effect of Endothelin Dual Receptor Antagonist on VEGF Levels in Streptozotocin-Induced Diabetic Rat Retina

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Diabetic retinopathy (DR), one of the most serious causes of blindness, is often associated with the upregulation of vascular endothelial growth factor (VEGF) in retina. Recently, leukocyte adhesion (leukostasis) is blamed for the occlusion of retinal capillary vascularity, which ultimately contributes to the progression of diabetic retinopathy. In addition, intercellular adhesion molecule-1 (ICAM-1), a representative factor for leukostasis, is increased in the diabetic retina. Endothelin (ET)-1, a potent vasoconstrictor peptide, is deeply linked to the pathogenesis of diabetic retinopathy. Different therapeutic interventions concerning VEGF have already been proposed to prevent diabetic retinopathy. However, no study yet has reported whether ET-1 dual receptor antagonist could alter the upregulated VEGF and ICAM-1 levels in the diabetic retina. The present study investigated the effect of $ET_{A/B}$ dual receptor antagonist (SB209670; 1 mg/rat/day) on the expression of VEGF and ICAM-1 in the diabetic rat retina. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ; 65 mg/kg) in Sprague-Dawley rats, whereas control rats (non-DM control) received only citrate buffer. After 1 week, the STZ-administered rats were randomly divided into two groups: one group (DM+SB209670) received $ET_{A/B}$ dual receptor antagonist for 2 weeks, and a vehicle group (DM+vehicle) was treated only with saline. After the treatment period, the retinas were removed from the eyeballs. In DM+vehicle group, the VEGF expression of the retinas was significantly increased (32.8 pg/mg) in comparison to that in the non-DM control group (26.2 pg/mg); this upregulation of VEGF was reversed in the DM+SB209670 group (28.6 pg/mg). The expression of retinal ICAM-1 was increased in the DM+vehicle group (152.2 pg/mg) compared with the non-DM control group (121.6 pg/mg). However, SB209670 treatment did not alter the expression of retinal ICAM-1 level (154.8 pg/ml) in DM rats. Thus we conclude that an $ET_{A/B}$ dual receptor antagonist could reverse the expression level of VEGF in the diabetic retina while failing to normalize the upregulated ICAM-1 expression. Exp Biol Med 231:1090–1094, 2006

Key words: diabetic retina; VEGF; ICAM-1; endothelin antagonism; rat

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Introduction

Diabetic retinopathy (DR) is one of the most serious causes of visual impairment and blindness. Recent evidence showed that vascular endothelial growth factor (VEGF) is one of the major factors promoting the severity of DR (1–3). Therefore, a therapeutic option for controlling VEGF levels in the diabetic retina has been a target for both clinicians and basic researchers recently.

Leukostasis (leukocyte adhesion), which causes the stagnation of the blood flow in the capillary vessel of the retina, has gained attention recently and is an important contributing factor in the pathogenesis of DR (4–8). Intercellular adhesion molecule-1 (ICAM-1) is a representative molecular and biologic factor for leukostasis (6, 8), and its suppression has been proved effective for the improvement of leukostasis and retinopathy (7, 8).

DR progresses to regional retinal ischemia, large blood vessel tortuosity, and, when neovascularization develops, it becomes proliferative. Proliferative diabetic retinopathy (PDR) is a late or end-stage complication of DR. Thus the development of retinal ischemia, which is associated with capillary closure, is the first change in PDR. Therefore, it is urgent to clarify the cause of the retinal ischemia and to search for its prevention and therapeutic options. To date, enhanced VEGF expression and increased leukostasis are considered potential causes of retinal ischemia (2–4, 8, 9). Endothelin (ET)-1, which is a potent vasoconstrictive

Table 1. Characteristics of Nondiabetic Control and Diabetic Rats[a]

	Non-DM control	DM+vehicle	DM+SB209670
Body weight (g)	409.8 ± 41.8	368.6 ± 16.5*	378.7 ± 23.1*
Blood glucose (mg/dl)	119.9 ± 23.4	429.8 ± 111.8**	442.6 ± 102.9**

[a]Values are means ± SD. Significantly different from non-DM control: *$P < 0.01$ and **$P < 0.01$.

peptide, has been reported to be upregulated in the diabetic retina (10–15), and increased retinal ET-1 levels have been shown as one of the causes of decreased retinal blood flow in early DR (11). In addition, the improvement of retinal blood flow in DR by ET antagonist has been reported (11, 16, 17). The change in retinal blood flow is considered to be closely related to retinal ischemia. Therefore, we hypothesized that ET-1 antagonism may be useful in the prevention of the progression of DR by reversing the altered expression levels of VEGF and ICAM-1 in the diabetic retina.

In the present study, we used streptozotocin (STZ)-induced diabetic rats to examine the expression levels of VEGF and ICAM-1 in the diabetic retina. An $ET_{A/B}$ dual receptor antagonist (SB209670) was administered for 2 weeks to diabetic rats and its effects on retinal VEGF and ICAM-1 were evaluated. The present study tries to investigate whether there is any treatment option that would reverse the alterations in key molecules responsible for the development and progression of DR.

Materials and Methods

Animals and Drug Treatment. Male, 8-week-old Sprague-Dawley rats were obtained from Charles River Japan, Inc. (Yokohama, Japan) and cared for according to the *Guiding Principles for the Care and Use of Animals* based on the Helsinki Declaration of 1964. The rats were made diabetic by means of a single 65 mg/kg ip injection of streptozotocin (STZ; Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in 0.1 m*M* citrate buffer (pH 4.5). Control nondiabetic animals were administered citrate buffer only (non-DM control). Animals with blood glucose levels more than 250 mg/dl 48 hrs after the STZ injection were considered to be diabetic. One week after the STZ injection, the diabetic animals were randomly divided into two groups. One group received $ET_{A/B}$ dual receptor antagonist (SB209670; SmithKline Beecham Pharmaceuticals, King of Prussia, PA) at a dose of 1 mg/rat/day for 2 weeks total by osmotic mini pump (model 2002; Durect Corporation, Cupertino, CA) (DM+SB209670), whereas the vehicle group was treated with physiologic saline only (DM+vehicle). Before the start of the drug treatment, blood glucose was determined almost every day, but after the treatment started the diabetic status was assessed every week. The rats were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12:12-hr light:dark cycle until sacrificed. After 2 weeks of treatment, rats were sacrificed under anesthesia and the retinas were harvested. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Enzyme-Linked Immunosorbent Assay for VEGF and ICAM-1. Enzyme-linked immunosorbent assay (ELISA) was performed for the determination of retinal VEGF and ICAM-1 protein levels. The retina was carefully dissected, placed in cold phosphate-buffered saline and homogenized. Then the protein concentration was measured using the bicinchoninic acid kit (Bio-Rad, Hercules, CA). The levels of VEGF and ICAM-1 in retinal tissue were measured using commercially available kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Histopathology Examination. For histopathologic analysis, the retinal tissues from the nondiabetic and diabetic rats were fixed in 10% buffered formalin solution, dehydrated, embedded in paraffin, and then sliced into 5-μm–thick sections. After being deparaffinized, hematoxylin and eosin–stained slides were prepared by using the standard method.

Statistical Analysis. All results are expressed as mean ± SD. Data were analyzed using the StatView version 5.0 program (SAS Institute, Cary, NC). Comparisons among groups were made by Mann-Whitney *U* test, with post-hoc comparisons using the Scheffé procedure. Differences were considered statistically significant when the *P* values were < 0.05.

Results

Characteristics of Experimental Animals. Table 1 shows the body weight and blood glucose data of each group of experimental animals. The blood glucose level was significantly higher in the diabetic group than in the age-matched non-DM control group. Treatment of diabetic rats with $ET_{A/B}$ dual receptor antagonist for 2 weeks did not alter blood glucose levels.

Expression of VEGF. VEGF protein expression from ELISA in the retinas of three experimental groups was shown in Figure 1. A 25% increase in VEGF protein level was observed in the DM+vehicle retinas compared with that of those in non-DM control animals ($P < 0.05$). Two weeks treatment with an $ET_{A/B}$ dual receptor antagonist in diabetic rats could significantly reverse this upregulation ($P < 0.05$).

Expression of ICAM-1. Similar to VEGF, ICAM-1 protein level by ELISA was also increased in the retinas of the

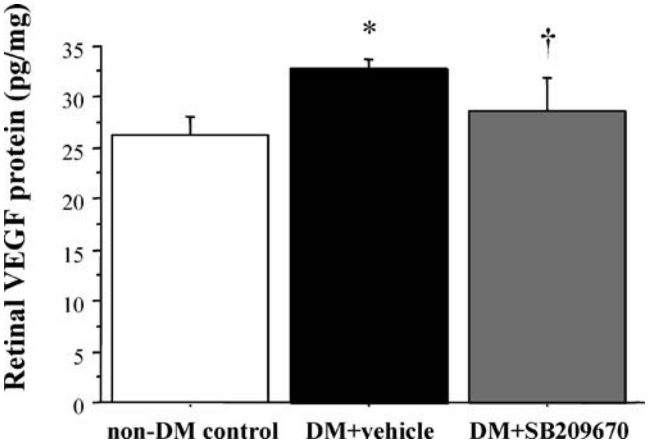

Figure 1. VEGF protein levels were quantitatively measured by ELISA (each group, $n = 5$) in the retina of non-DM control, DM+vehicle, and DM+SB209670 rats. $^*P < 0.05$ compared with non-DM control rats; $^\dagger P < 0.05$ compared with DM+vehicle rats.

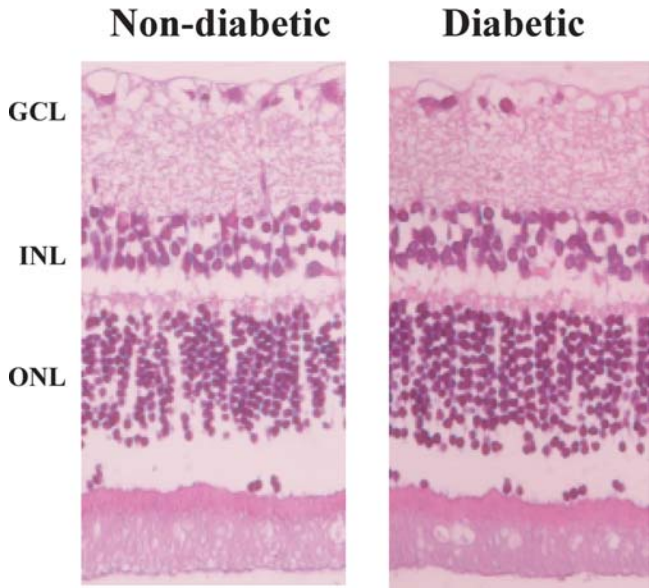

Figure 3. Representative photographs obtained from the sections of retina stained by hematoxylin and eosin in nondiabetic and diabetic rats. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Magnification: ×400.

DM+vehicle rats (25.1%) compared with that in the non-DM control group ($P < 0.05$; Fig. 2). However, SB209670 treatment did not alter the expression of retinal ICAM-1 level.

Histologic Analysis. Hematoxylin and eosin staining showed no evident morphologic abnormality in the retinas of nondiabetic or diabetic rats (Fig. 3).

Discussion

In the present study, we investigated whether there is any treatment option that would reverse the alterations in key molecules responsible for the development and progression of DR. The present study demonstrated that the expressions of VEGF and ICAM-1 were upregulated in the diabetic retina and revealed that an $ET_{A/B}$ dual receptor antagonist could reverse VEGF, but failed to alter the ICAM-1 level.

In the present study, we showed a 25% upregulation of VEGF protein level in the diabetic retina. Indeed, a significant number of studies have already reported the upregulation of VEGF in the retinas of experimental diabetic animals such as diabetic rats (18–24) and in the vitreous fluid of diabetic patients—especially those with PDR (25–27). Because of the potential role of VEGF in the development and progression of DR, therefore, VEGF has been considered as an important target for the treatment of DR although many of the mechanisms remain unrevealed. Hypoxia is thought to be a strong stimulator of increased VEGF expression in the retinas of diabetic subjects (28). On the other hand, the decrease of the retinal blood flow would contribute to the development of tissue hypoxia in the diabetic retina. Thus the present examination focused on the concept that the reversal of hypoxia-induced VEGF upregulation could subsequently normalize the alteration in retinal blood flow in diabetic subjects. Retinal vasculature lacks direct nerve innervation. Thus retinal blood flow is regulated primarily via local factors (29). Among them, two important vasoregulators are ET-1 and nitric oxide. ET-1 causes the contraction of the blood vessel through ET_A and ET_{B2} receptor (30), which is primarily located in vascular smooth muscle cells. Moreover, the expression of ET_B receptor has been shown to be augmented in the STZ-induced diabetic retina without the upregulation of ET_A receptor (31). These observations have led us to use a dual receptor blocker in the current experimental setting. It should be noted that in our preliminary experiment, hematoxylin and eosin staining showed no obvious morphologic abnormality in retinas of diabetic or non-diabetic rats (Fig. 3). However, in the diabetic retina, the histopathologic changes often followed the molecular biologic alterations. Thus it may be important to arrest the

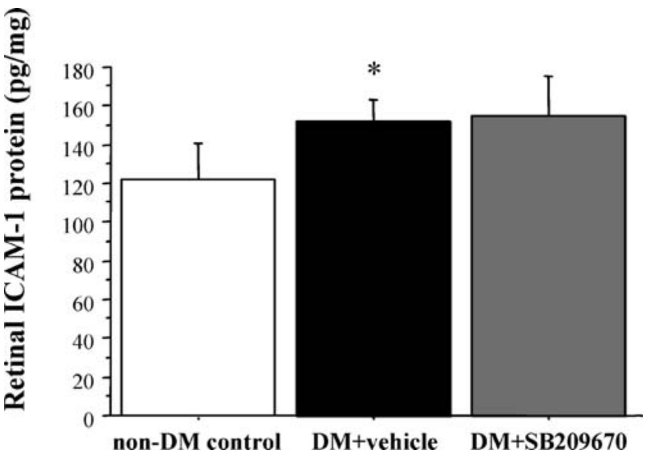

Figure 2. ICAM-1 protein levels were quantitatively measured by ELISA (each group, $n = 5$) in the retina of non-DM control, DM+vehicle and DM+SB209670 rats. $^*P < 0.05$ compared with non-DM control rats.

progression of VEGF-mediated alterations in the diabetic retina at a stage of diabetes when the retina is still morphologically intact.

As with VEGF, leukocyte adhesion to the retinal vasculature has also gained interest as a therapeutic target for the prevention of retinopathy (6–8). A growing body of evidence demonstrates leukocyte adhesion as one of the central factors for DR, which is predominantly involved in capillary nonperfusion (4–8). ICAM-1 is a representative molecular and biologic factor for leukostasis. Thus, in the present study, we investigated whether the treatment by an $ET_{A/B}$ dual receptor antagonist also could be effective for the reversal of the upregulated retinal ICAM-1 levels. But, SB209670 treatment could not alter the expression of upregulated retinal ICAM-1 levels. As far as we know, there is no report that has investigated the effect of a dual endothelin antagonist on the expression of ICAM-1 in the retina. But there are some reports stating the decrease of the expression of ICAM-1 in other different tissues by the ET antagonist. Hayasaki and colleagues reported that ET-1–induced ICAM-1 expression in cardiac myocytes has been inhibited by a selective ET_A receptor antagonist (S-0139), but not by a selective ET_B receptor antagonist (BQ788; Ref. 32). Pu and colleagues reported that the increased expression level of ICAM-1 in the vessel wall of aldosterone-infused rats has been reduced by the treatment of a selective ET_A receptor antagonist (BMS 182874; Ref. 33). Thus a specific ET_A receptor antagonist is shown to be effective in reversing the ICAM-1 level in different studies. In the present study, we used the $ET_{A/B}$ dual receptor antagonist. It would be hard to rule out the mechanism of the unchanged ICAM-1 levels in the ET dual receptor antagonist-treated retinas in DM rats from the current investigation; however, we speculate that blockade of ET_B receptor in the present study may cause a counteraction or effect on the reversal process of expression of upregulated ICAM-1 in the retina of DM animals. Before reaching a concrete conclusion in this issue, one should follow the same experimental design of the present study by using the selective ET_A and selective ET_B receptor antagonist separately.

The present study demonstrated that the expression levels of VEGF and ICAM-1 in the retina of STZ-induced diabetes were significantly upregulated compared with those in nondiabetic control animals in early diabetic stage (diabetes of 3 weeks). An $ET_{A/B}$ dual receptor antagonist could reverse the increased expression level of VEGF in the diabetic retina while failing to normalize the upregulated ICAM-1 expression.

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