Dexamethasone Inhibits Leukocyte Accumulation and Vascular Permeability in Retina of Streptozotocin-Induced Diabetic Rats via Reducing Vascular Endothelial Growth Factor and Intercellular Adhesion Molecule-1 Expression

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Intravitreal injection of corticosteroid has been used to treat diabetic macular edema, however, the exact mechanism remains unknown. In the present experiment, four weeks after streptozotocin administration, intravitreal injection of dexamethasone (50 μg/10 μl) was performed. After 2 d injection, we investigated the effect of dexamethasone on leukocyte accumulation, vascular permeability and the expression of vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1) in streptozotocin-diabetic rats. Accumulated leukocytes were counted in vivo by acridine orange leukocyte fluorography, the retinal vascular permeability was measured by the Evans blue assay. The mRNA and protein level of VEGF and ICAM-1 were analyzed with real-time quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) respectively. Dexamethasone downregulated VEGF and ICAM-1 expression in diabetic rats which correlated with its effect on leukocytes accumulation and retinal vascular permeability. The present data revealed that dexamethasone may inhibit retinal accumulation and leukostasis accumulation and vascular permeability through its blockage on VEGF and ICAM-1 expression.

Key words dexamethasone; vascular endothelial growth factor; intercellular adhesion molecule-1; diabetes

Retinopathy, a major complication of diabetes, often leads to diabetic macular edema which causes adult blindness in many countries. The acetazolamide and topical application of nonsteroidal antiinflammatory drugs (NSAIDs) are helpful in treating macular edema, but their efficacy as therapeutic agent remains to be proved. Glucocorticoids were used to treat the edema caused by brain tumors. Intravitreal injection of steroids may be used in treating macular edema when oral administration of acetazolamide and topical application of NSAIDs or steroids are not effective. Some studies have reported the efficacy and complications of intravitreal triamcinolone acetonide injection and shown the positive results of intravitreal corticosteroid injection. However, the exact mechanism of such treatments was not demonstrated and a large-scale, randomized clinical trial was needed to prove their effects.

Several lines of evidence suggest that the pathogenesis of diabetic retinopathy is mediated by inflammatory processes, including leukocyte adhesion and the cytokine network. Retinal vasculature in diabetes is accompanied by inflammatory cell adhesion, which triggers vascular hyperpermeability and pathologic retinal neovascularization. Vascular endothelial growth factor (VEGF) was thought to mediate leukocytes bind to the vasculature, inducing retinal vascular permeability and leukostasis partially through intercellular adhesion molecule-1 (ICAM-1). So, VEGF and ICAM-1 may be two critical factors for diabetes-induced leukostasis and blood-retinal barrier breakdown.

The streptozotocin-induced diabetic rat shows many of the early changes in retinal structure and function that are associated with human diabetic retinopathy. Therefore, in the present experiment, we investigated the effect of dexamethasone on VEGF and ICAM-1 in streptozotocin-diabetic rats. Accumulated leukocytes were counted in vivo by acridine orange leukocyte fluorography, the retinal vascular permeability was measured by the Evans blue assay. The mRNA and protein level of VEGF and ICAM-1 were analyzed with real-time quantitative polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) respectively. We found that intravitreal dexamethasone injection inhibited VEGF and ICAM-1 expression and reduced leukocyte accumulation, vascular permeability in streptozotocin-induced diabetic rats, which may be the underlying mechanisms of its effect.

MATERIALS AND METHODS

Materials The experiments were carried out in compliance with the guidelines for animal care and use of China and the experimental protocols were approved by the animal ethics committee of Beijing Capital Medical University. Male pigmented Brown-Norway rats (180 g, n = 72) were purchased from Vitalriver Laboratory Animals Co., Ltd. (Beijing, China). After an overnight fast, some rats received intraperitoneal streptozotocin injection (60 mg/kg; Sigma Chemical, St. Louis, MO, U.S.A.) in 10 mmol/l citrate buffer (pH 4.5) for diabetes induction. Control nondiabetic animals received 10 mmol/l citrate buffer alone. After that, rat’s abdominal aorta blood was drawn and treated with the anticoagulant EDTA then analyzed with a hematology analyzer (XT-2100i, Sysmex).

After diabetes induction, those rats with blood glucose levels higher than 250 mg/dl at all time points tested in 24 h were considered as successful diabetic models. All rats were kept in an air-conditioned room with a 12 h light and 12 h dark cycle and given free access to water and food until they were used for the experiments. All experiments were performed 4 weeks after the induction of diabetes.

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Intravitreal Injection of Dexamethasone Rats were anesthetized with a 1:1 mixture of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg), and their pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. For additional topical anesthetia, 0.5% alcaine (Alcon Co.) was used. Then, 0.5% of levofloxacin ophthalmic solution (Santen Co.) was applied to the ocular surface to prevent infection. A 27-gauge needle was used to make a punch incision 1 mm posterior to the rat’s temporal limbus, then the microinjector (n=6) was inserted through the incision, approximately 1.5 mm deep, toward the optic nerve and into right vitreous. A single dose of 50 μg dexamethasone (phosphate, 10 μl) was injected. Those rats with injection-damaged lenses or retinas were excluded from our further experiment. Ten microliters physiological saline was injected into the right eye of other rats as control (n=6). All rats were observed 2 d after intravitreal injection which was at the dexamethasone effect peak.11)

Evaluation of Leukocyte Accumulation in Rat Retina Six different rats were tested in each group. Leukocyte acculumation in retinal microcirculation was evaluated with acridine orange (AO) digital fluorography, a technique using a scanning laser ophthalmoscope (Heidelberg Engineering, Heidelberg, Germany) to get high-resolution images of fundus stained by AO (Sigma Chemical, St. Louis, MO, U.S.A.). That technique has been reported,12) and was modified slightly in our study. AO, a metachromatic fluorochrome, is a widely used probe in biochemical and cytochemical studies. The dye emits a green fluorescence when it interacts with DNA. Because the spectral properties of leukocytes stained with AO are similar to those of sodium fluorescein, so the argon blue laser was used as illumination source and with a regular emission filter for fluorescein angiography. Before AO digital fluorography, rats were anesthetized and their pupils were dilated and body temperatures were maintained between 37 °C and 39 °C during experiment. AO (0.1% solution in saline) was injected continuously through the jugular vein at a rate of 1 ml/min. Thirty minutes later, the fundus were observed with the scanning laser ophthalmoscope. The real time video images (640×480 pixels; intensity resolution 256) were captured with a frame-grabber for later digital processing. An observation area around the optic disc in 5 diameter was determined by drawing a polygon surrounded by the adjacent major retinal vessels, which was used for retinal leukostasis evaluation. The leukocytes area was measured in pixels, the trapped leukocytes were recognized as fluorescent dots and the average density of which were calculated generally in eight peripapillary observation areas.

Measurement of Blood–Retinal Barrier Breakdown Rats with 4-week-duration diabetes were anesthetized, and Evans blue dye (45 mg/kg; Sigma Chemical, St. Louis, MO, U.S.A.) was injected through the jugular vein.13) After 120 min, the chest cavity was opened and rats were perfused for 2 min at a physiological pressure via the left ventricle with 0.05 M, pH 3.5, citrate-buffered paraformaldehyde (1% w/v; Sigma). A pH of 3.5 was used to optimize binding of Evans blue to albumin, and the perfusion solution was warmed to 37 °C to prevent vasoconstriction. Immediately following perfusion, both eyes were enucleated and bisected at the equator. The retinas were carefully dissected and thoroughly dried in a Speed-Vac for 5 h. The dry weight was used to normalize the quantitation of Evans blue leakage. Evans blue was extracted by incubating each retina in 120 μl formamide (Sigma) for 18 h at 70 °C. The supernatant was filtered through Ultrafree-MC tubes (30000 NMWL UFC3LTK00, Millipore, Bedford, MA, U.S.A.) at 2500 g for 2 h, and 60 μl of the filtrate were used for triplicate spectrophotometric measurements. Each measurement occurred over a 5-s interval, and all sets of measurements were preceded by evaluation of known standards. The background-subtracted absorbance was determined by measuring each sample at 620 nm (the absorbance maximum for Evans blue in formamide) and 740 nm (the absorbance minimum). The concentration of dye in the extracts was calculated from a standard curve of Evans blue in formamide. Blood-retinal barrier breakdown was calculated using the following equation, with results being expressed in μl plasma×g retina dry weight⁻¹×h⁻¹.

Measurement of ICAM-1 and VEGF Protein Levels The retina-vitreous-lens capsule complexs of 48 h intravitreal injected rats were carefully separated and placed in 150 μl lysis buffer (20% glycerine, 10 mmol/l KCl, 1 mmol/l MgCl₂, 0.1% Triton, 300 mmol/l NaCl, 0.5 mmol/l dithiothreitol (DTT), 0.1 mmol/l phenylmethanesulfonyl fluoride (PMSF), 20 mmol/l HEPES, pH 7.9) with protease inhibitor cocktail and were sonicated. The lysate was centrifuged at 12000 g for 15 min at 4 °C in a tabletop microfuge and supernatant was collected. The VEGF and ICAM-1 protein levels were calculated by ELISA based on kit instructions and were normalized with a protein assay kit.

Measurement of ICAM-1 and VEGF mRNA Levels Six rat eyes in the following four respective group: nondiabetic control, DM without intravitreal injection, vehicle-treated, and dexamethasone-treated were enucleated for quantification of ICAM-1 and VEGF gene expression, VEGF and ICAM-1 mRNA was measured by real-time reverse transcription PCR (RT-PCR) as previously described.14) The total

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligo</th>
<th>Primer sequence</th>
<th>Predicted size (bp)</th>
<th>Genebank accession</th>
</tr>
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<tr>
<td>ICAM-1</td>
<td>Forward</td>
<td>5′-CTGTCAAACGGGAGATGAATGGT-3′</td>
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<tr>
<td></td>
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<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′-CTGACCGTACTGGACGCTATGCT-3′</td>
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RNA from different experimental conditions was obtained by TRIzol method. The concentration of RNA was determined by an absorbance at 260 nm and RNA was reverse transcribed to cDNA using the Taqman® Reverse Transcription Reagents (Applied Biosystems). Reverse transcription was performed at 20 °C 5 min, 42 °C 60 min, 70 °C 5 min (PerkinElmer GeneAmp 9600, Foster City, CA, U.S.A.). cDNA was analyzed immediately or stored at −20 °C. The oligonucleotide primers sequences, predicted product lengths and gene bank accession by amplification in real-time PCR are listed in Table 1. Real-time PCR assay was carried out with ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems) to observe mRNA level. The basic protocol for real-time PCR was an initial incubation at 93 °C for 3 min, followed by 40 cycles of 93 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and finally cooling to 40 °C. All samples were run in triplicate, the relative expression values were normalized with β-actin value.

Plasmids containing cDNA was used as standard in quantifying the PCR results. The interest cDNA was amplified by RT-PCR using the same premiers as for real-time RT-PCR. The PCR products were cloned into pGEM-T easy vector (Invitrogen) and confirmed by sequencing. The purified recombinant plasmid DNA was quantified by UV spectrophotometer and then serially diluted in double-distilled water as standard for numerical quantification. The standard curve was prepared for each target cytokines and β-actin was used as housekeeping gene. The PCR products were sequenced to verify the analytical specificity. Melting curve was analyzed after PCR amplification.

**Statistical Analysis** Experiments were repeated two to four times and the data were pooled. All data were expressed as mean ± S.D. The results were analyzed by Kruskal–Wallis test and Dunn’s multiple comparisons test. p value < 0.01 was considered statistically significant.

**RESULTS**

**Physiologic Data** Changes in physiologic variables of each group were shown in Table 2. There were no significant differences in any physiological variable among groups except blood glucose levels, which was significantly lower in control group than in all three other groups. However, no significant blood glucose difference was found among those groups.

**Leukocyte Accumulation** The changes of leukocytes accumulation in the dexamethasone-treated groups’ retinal microcirculation were shown in Figs. 1 and 2. Few leukocytes were found in the control group. In the DM and vehicle-treated DM groups, leukocytes significantly increased 2.7 folds as many as that in control group (both p<0.01), while with no significant difference interclass. After 48 h dexamethasone treatment, leukocytes reduced to 44.1% compared with vehicle-treated DM group (p<0.01).

**Quantitation of Diabetes-Induced, Blood-Retinal Barrier Breakdown** To determine the relation between leukocytes accumulation and blood-retinal barrier breakdown, the Evans blue assay was performed (Fig. 3). The retinal Evans blue mean leakage (μl plasma × g retina dry weight^{-1} × h^{-1}) was 12.26 and 11.69 respectively in DM and vehicle-treated DM group, which were approximately 2.1-fold and 2.0-fold higher than that of the control group (both p<0.01). Intravitreal injection of dexamethasone decreased blood-retinal barrier breakdown in the diabetic retinas by 47.5% compared with vehicle-treated diabetic (p<0.01) or diabetic groups (p<0.01).

**Table 2. Physiological Variables of Each Group**

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC (×10^6/μl)</th>
<th>Blood glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.71±1.53</td>
<td>127.17±10.61</td>
</tr>
<tr>
<td>Untreated DM</td>
<td>7.32±1.84</td>
<td>447.83±25.65*</td>
</tr>
<tr>
<td>Vehicle-treated</td>
<td>5.93±0.95</td>
<td>403.83±26.17*</td>
</tr>
<tr>
<td>Dexamethasone-treated</td>
<td>6.13±1.16</td>
<td>418.51±19.36*</td>
</tr>
</tbody>
</table>

Data shows physiological variables of four groups 2 d after intravitreal injection of 50 μg dexamethasone. Data are expressed as mean ± S.D. (n=6). * (p<0.01) indicates a significant difference compared with the control group. WBC, peripheral leukocyte count.

![Fig. 1. Effect of Dexamethasone on Leukostasis 2 d after Intravitreal Injection of 50 μg Dexamethasone](image.png)

![Fig. 2. Effect of Dexamethasone on Leukostasis 2 d after Intravitreal Injection of 50 μg Dexamethasone](image.png)

The number of leukocytes accumulating in the retina was inhibited significantly via intravitreal administration of dexamethasone. Each column represents the mean ± S.D. (n=6). * (p<0.01) indicates a significant difference compared with the control group. △ (p<0.01) indicates a significant difference compared with the vehicle-treated diabetic retinas group.
ICAM-1 and VEGF Gene Expressions

Real-time quantitative PCR and ELISA were used to reflect the effect of dexamethasone on ICAM-1 and VEGF expression in retina. Real-time RT-PCR was performed to amplify VEGF and ICAM-1, and the standard curve was drawn for each target gene (for example, VEGF in Figs. 4A and B). Melting curve analysis confirmed that there was no primer dimer in the PCR products (Fig. 4C). For each primer set, non-specific amplification was seen after agarose gel electrophoresis and ethidium bromide staining (Fig. 4D).

ICAM-1 and VEGF Protein Levels

The ICAM-1 and VEGF protein levels in untreated DM group and the vehicle-treated group were 76.74 pg/mg (26.26 pg/mg) and 74.32 pg/mg (27.17 pg/mg) respectively, higher than these in the control group ($p<0.01$). Dexamethasone reduced ICAM-1 protein level significantly compared with the DM group and vehicle-treated group ($p<0.01$). These findings were correlated with the ELISA results (Figs. 6A, B).

DISCUSSION

Our study investigated the mechanism of clinical steroid in treating streptozotocin-induced diabetic rats. The results demonstrated that intravitreal injection of dexamethasone suppressed the leukostasis and blood-retinal barrier breakdown in diabetic rats. And such dexamethasone-induced changes were mediated by VEGF and ICAM-1 suppression in retinal vasculature in vivo, which was similar to that of...
eosinophils, lymphocytes, and platelets.\textsuperscript{18,19} Leukocytes, well known to increase vascular permeability. VEGF has been found in neutrophils, monocytes, and platelets.\textsuperscript{20} The expression of VEGF was increased in the diabetic retina of humans and rodents.\textsuperscript{20,21} It has been confirmed that VEGF induced retinal vascular permeability and leukostasis, partially through ICAM-1.\textsuperscript{22}

Retinal leukostasis is a very early event in diabetic retinopathy. Indeed, activated leukocytes increased in diabetes. Capillaries are occluded from downstream static leukocytes. Increased leukocyte adhesion correlate spatially and temporally with endothelial cell injury and death in the diabetic rat retina, and cause blood-retinal barrier breakdown finally.\textsuperscript{23,24} Based on the present studies, we believe that there is a strong relationship between dexamethasone induced leukostasis or blood-retinal barrier breakdown and ICAM-1 or VEGF expression in diabetic retina.

Dexamethasone may decrease diabetes-induced permeability changes either by reducing ICAM-1 and VEGF expression or by suppressing their interactions, decreasing leukocyte adhesion to vascular endothelium and leukocytes accumulation within the retina. Several \textit{in vitro} studies proved that corticosteroids inhibited the metabolic pathway of VEGF,\textsuperscript{25,26} and dexamethasone binded to glucocorticoid receptor to inhibit VEGF induction as confirmed in glucocorticoid receptor antagonist reverse effect.\textsuperscript{27} Another report indicated that corticosteroids could suppress VEGF responses \textit{in vivo}.\textsuperscript{15} A serial of previous studies have also proved that dexamethasone (and other glucocorticoids) inhibited leukocytes recruitment by affecting adhesion molecule expression on both endothelial cells and leukocytes. These studies have also provided insights into the underlying molecular mechanisms. One result demonstrated that PMA-induced expression of ICAM-1 on ECV304 cells was significantly reduced by triamcinolone acetonide suggesting that triamcinolone acetonide had the potential to downregulate ICAM-1 expression \textit{in vitro}.\textsuperscript{28} Dexamethasone may interfere with ICAM-1 expression, suppressing leukocyte accumulation, reducing ICAM-1 expression on rat circulating unstimulated monocytes and peritoneal macrophages \textit{in vivo}. A recent \textit{in vivo} study found that intravitreal corticosteroid attenuated leukostasis and blood-retinal barrier breakdown in diabetic retinal edema, which indicated that intravitreal corticosteroid might improve blood-retinal barrier breakdown and then diabetic retinal edema by inhibiting leukostasis.\textsuperscript{11} Additionally, glucocorticoids directly induce the formation of endothelial tight junctions, thus providing a provascular effect. These activities may be an effective means of reducing vascular permeability in diabetic retinopathy.\textsuperscript{30} Our study suggested that diabetes-induced leukostasis and permeability changes caused by dexamethasone correlated with mRNA and protein levels of both VEGF and ICAM-1. In other words, intravitreal corticosteroid was beneficial to diabetic retinopathy prevention by suppressing VEGF and ICAM-1-mediated leukocyte adhere to vessel walls.

Clinically, triamcinolone acetonide has been used for treatment of diabetic macular edema and the typical dosage is 1 mg/ml (4 mg triamcinolone in 4 ml human vitreous volume). As intravitreal injection of triamcinolone may affect observing the leukocyte accumulation, so dexamethasone was used in this study. A single dose of 0.18 mg/ml dexamethasone is equivalent to 1 mg/ml triamcinolone in terms of equal corticosteroid efficacy.\textsuperscript{31} Since rat vitreous volume is

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure6.png}
\caption{Effect of Dexamethasone on Protein Expression of ICAM-1 and VEGF in the Retina 2 d after Intravitreal Injection of 50 \textmu g Dexamethasone}
\end{figure}

Tamura \textit{et al.}\textsuperscript{11} Tamura \textit{et al.} stated that considerable evidence did not support glucocorticoids activity on VEGF expression. We used real-time RT-PCR to detect mRNA expression, which offered more powerful evidence compared with the result of Tamura \textit{et al.}\textsuperscript{11} On the contrary, Edelman \textit{et al.} proved the efficacy of glucocorticoids in reducing VEGF activity on vascular leakage in the eye.\textsuperscript{15} Their job was based on a VEGF-induced retinal blood-retinal barrier breakdown in rabbits, while we investigated in streptozotocin-induced diabetic rats model. In our study, we also tested both pathways in rats. The results suggested that dexamethasone-induced changes were mediated \textit{via} depressing both VEGF and ICAM-1 expression in the retinal vasculature \textit{in vivo}.

VEGF and ICAM-1 seem to be two critical factors during the process of diabetes-induced leukostasis and blood-retinal barrier breakdown. VEGF is thought to increase retinal blood vessels permeability by at least two mechanisms. Previous reports have shown that leukocyte-endothelial interactions can trigger endothelial cell adherens, tight junction disorganization, and increase vascular permeability.\textsuperscript{16,17} Others have demonstrated that VEGF had direct effects on vascular permeability. VEGF has been found in neutrophils, monocytes, eosinophils, lymphocytes, and platelets.\textsuperscript{18,19} Leukocytes, \textit{via} their own VEGF, may indirectly amplify the direct effects of VEGF when they bind to endothelium. ICAM-1 levels also increased in the diabetic retina of humans and rodents.\textsuperscript{20,21} Miyamoto \textit{et al.} have shown that retinal leukostasis increases within days of developing diabetes and correlates with the increased expression of ICAM-1 in the streptozotocin-induced diabetic retinopathy.\textsuperscript{22} In diabetic retinopathy, the expression
56 μl, 321 50 μg of dexamethasone makes the final intravitreal concentration of 0.9 mg/ml. The dosage used on rats in this study is about five-fold higher than that of 0.18 mg/ml used in human. This high dose was chosen because the pharmacokinetics of the drug in the rat vitreous was unknown and because insufficient dosing was to be avoided.

Glucocorticoids is a promising tool in treating diabetic retinopathy by breaking down vascular endothelial barrier, a causative factor, but also having side-effects in treatment. However, understanding glucocorticoids function mechanisms will undoubtedly bypass these detrimental side-effects and lead to novel therapies.

Acknowledgments This research was supported by a project for standout of Science and Technology (2005-B-043) from Beijing Science and Technology Committee to IPCAS to JYG.

REFERENCES