Vitreous Levels of Vascular Endothelial Growth Factor and Stromal-Derived Factor 1 in Patients With Diabetic Retinopathy and Cystoid Macular Edema Before and After Intraocular Injection of Triamcinolone

H. Logan Brooks, Jr, MD; Sergio Caballero, Jr, MS; Charles K. Newell, MD; Robert L. Steinmetz, MD; Debbie Watson, BS, CRA; Mark S. Segal, MD, PhD; Jeffrey K. Harrison, PhD; Edward W. Scott, PhD; Maria B. Grant, MD

Background: Diffuse macular edema (DME) and/or aberrant neovascularization (NV) can cause vision loss in diabetic retinopathy (DR) and may be modulated by growth factors and chemokines. The chemokine stromal-derived factor 1 (SDF-1) is a potent stimulator of vascular endothelial growth factor (VEGF) expression, the main effector of NV, and the key inducer of vascular permeability associated with DME. Circulating endothelial cell precursors migrating in response to SDF-1 participate in NV.

Objective: To investigate the relationship between SDF-1 and VEGF in vitreous of patients with varying degrees of DR and DME before and after intraocular injection of triamcinolone acetonide, used to treat refractory DME.

Methods: In this prospective study, 36 patients were included and observed for 6 months. Vitreous VEGF and SDF-1 levels were measured by enzyme-linked immunosorbent assay in samples obtained immediately before and 1 month after injection of triamcinolone.

Results: Both VEGF and SDF-1 were significantly higher (P<.01) in patients with proliferative DR than in patients with nonproliferative DR. Levels of SDF-1 were markedly increased in patients with DME compared with those without DME. Vascular endothelial growth factor correlated with SDF-1 levels and disease severity (r²=0.88).

Conclusions: Triamcinolone administration resulted in dramatic reductions of VEGF and SDF-1 to nearly undetectable levels, eliminated DME, and caused regression of active NV. Our results support a role for SDF-1 and VEGF in the pathogenesis of the adverse visual consequences of DR and suggest that the elimination of DME with regression and/or initiation of fibrosis of NV after triamcinolone injection may be due to the suppression of VEGF and SDF-1.


Proliferative Diabetic Retinopathy (PDR), the primary cause of blindness in the young to middle-aged population, affects many of the 7 million people with diabetes in the United States. The early stage of the disease, termed nonproliferative diabetic retinopathy (NPDR), is associated with vascular permeability and can lead to vision-threatening diffuse macular edema (DME) that is manifested as intraretinal and subretinal accumulation of fluid. In the later stages, the nonperfused ischemic retina produces angiogenic growth factors, such as vascular endothelial growth factor (VEGF), which stimulate new abnormal blood vessel growth. For 3 decades, laser photocoagulation has been the mainstay in the management of PDR. However, laser treatment for PDR breaks down the blood retinal barrier (BRB) and can cause or worsen DME.3,4

Thirty percent of patients with diabetes for 20 years or more have DME. More than half of these patients will lose 2 or more lines of visual acuity (VA) after 2 years of follow-up evaluations. The Early Treatment Diabetic Retinopathy Study (ETDRS) demonstrated a significant benefit of focal laser photocoagulation for the treatment of clinically significant DME, although 24% of patients had DME that persisted at 36 months and severely affected VA.5,6 Surgical treatment is appropriate for only a very small percentage of patients with DME and involves all the inherent risks, recovery time, and expense of surgery. In 2 recent studies, intravitreal injection of triamcinolone acetonide reduced retinal thickening, improved BRB function, and improved VA in patients with DME.7,8
The exact pathogenesis of DME has not been elucidated. Several mechanisms may be responsible, including altered BRB due to hemodynamic change, glycemic control, alterations in capillary basement membranes, and pericyte loss. The possibility that retinal-derived or blood-borne factors such as VEGF and chemokines play a role in DME should also be considered.

Chemokines participate extensively in mechanisms of leukocyte trafficking, immune surveillance, innate and adaptive immunity, and inflammation. Stromal-derived factor 1 (SDF-1), a member of the CXC chemokine subfamily, was initially identified as a bone marrow stromal cell–derived chemoattractant for hematopoietic progenitor (CD34+) cells. Stromal-derived factor 1 acts as an angiogenic agent in several model systems. The SDF-1 receptor CXCR4 is expressed on endothelial cells, and its expression is increased after treatment with VEGF or basic fibroblast growth factor. Animals deficient in either SDF-1 or CXCR4 have vascular defects. Endothelial precursor cells participate in both normal and pathological angiogenesis, express functional CXCR4, migrate in response to SDF-1, and express VEGF in response to SDF-1.

We postulated that patients with DME have activated leukocytes, microglia, Müller cells, and endothelial cells in their retina that produce cytokines and growth factors resulting in elevated vitreous levels of these factors. Because the expression of SDF-1 is increased during inflammation and because SDF-1 regulates cell trafficking and induces VEGF expression in multiple cell types, it was particularly appealing as a candidate factor to induce DME and angiogenesis. Thus, we asked whether intravitreal levels of SDF-1 were elevated in patients with DME and/or PDR, whether steroids could decrease SDF-1 and VEGF, and whether clinical improvement correlated with changes in vitreous levels of these factors.

### METHODS

#### PATIENT STUDIES

The institutional review board at the University of Florida approved the study protocol. Vitreous samples were obtained from 33 diabetic individuals with DME meeting the criteria of the ETDRS Report Number 12. Nineteen eyes (12 diabetic and 7 nondiabetic) were used as controls. Another group of patients included those with NVI (3 eyes). There were 15 patients (30 eyes) who had both eyes treated. No eyes had ocular hypertension, previous periocular steroid injection, steroid drop use within 3 months, vitreous hemorrhage within 2 months, or previous vitrectomy surgery. All eyes were observed for 6 months or were excluded from the study.

Snellen VA was used because one clinic did not have ETDRS charts for the study. Corrected VA was obtained on standard Snellen acuity charts and intraocular pressure measured at each monthly visit for the 6-month follow-up. Conversion of Snellen acuity to logarithm of the minimum angle of resolution values was performed. All patients received intravenous fluorescein angiography (IVFA) prior to steroid injection and then monthly for the duration of the study. Baseline IVFA showed the amount of DME, evaluated ischemia, and measured the amount of diabetic retinopathy in the periphery. The postinjection IVFA was evaluated in a subjective, unmasked manner. In 3 diabetic individuals, optical coherence tomography (OCT) was performed before and 1 month after triamcinolone injection to evaluate macular thickness.

All patients had complete physical examinations, laboratory tests, blood pressure, hemoglobin A1c, and urinalysis. Details of the medication being used were compared between groups, and no significant differences were observed regarding use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, angiotensin-converting enzyme inhibitors, calcium channel blockers, and oral hypoglycemics, including thiazolidinediones. Intravitreal injection of triamcinolone acetonide (Kenalog 40; Apothecon, Princeton, NJ) was offered to treat DME with NPDR, DME with PDR, and NVI. Topical proparacaine was applied to the ocular surface followed by 4% lidocaine at the injection site. Povidone iodine (5%) was applied to the ocular surface for 2 minutes. Liquid vitreous (0.2 mL) was removed using a 23-gauge needle, followed by injection of 4 mg (0.1 mL) of triamcinolone through the pars plana using a 30-gauge needle. In all patients, repeat intravitreal taps were performed after 1 month. The vitreous aspirates that were...
collected at the time of triamcinolone injection were frozen at –80°C until analysis.

MEASUREMENT OF SDF-1 AND VEGF IN VITREOUS

Stromal-derived factor 1 and VEGF in the vitreous samples were quantified using commercially available enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, Minn). The SDF-1 ELISA has a detection limit of approximately 160 pg/mL, whereas the VEGF ELISA has a sensitivity of approximately 15 pg/mL. The mean concentrations of SDF-1 and VEGF in the vitreous from diabetic patients with DME were determined and compared with those in normal control patients.

CHEMOTACTIC ACTIVITY OF VITREOUS SAMPLES

The functional activity of SDF-1 in selected vitreous samples was examined by chemotaxis of human retinal endothelial cells using a modified blind-well Boyden chamber (Neuroprobe, Gaithersburg, Md) assay as previously described.16-18 Human retinal endothelial cells were isolated and cultured as previously described.19 Cells from 2 separate donors were used. Vitreous samples from 3 patients showing high levels of both VEGF (approximately 22 to 220 µg/mL) and SDF-1 (approximately 6.5 to 11.8 ng/mL) were used in triplicate to stimulate cell migration across a collagen-coated, polyvinyl- and pyrrolidine-free polycarbonate membrane (Neuroprobe). Serum-free medium was used as a negative control, while complete growth medium for human retinal endothelial cells, containing numerous chemotactic factors (in the form of 10% vol/vol fetal bovine serum) as well as fibroblast growth factors (as part of the normal growth medium), was used as positive control. Selected cells were preincubated with AMD3100, a specific CXCR4 antagonist, prior to their being exposed to the chemotactic stimuli. The cells were given 12 hours to migrate through the membrane, after which nonmigrating cells were removed by scraping, and the remaining cells were fixed and stained with DiffQuick (Fisher Scientific, Atlanta, Ga). Individuals masked to the identity of treatment counted migrating cells in at least 3 high-power fields for each well.

STATISTICAL ANALYSIS

Statistical analysis was carried out using χ² analysis and rank analysis of data.

RESULTS

Intravitreal administration of triamcinolone typically appeared as a whitish suspension in the inferior portion of the vitreous cavity (Figure 1). Crystals settled preretinally in the vitreous cortex but did not interfere with vision. As shown in Figure 2 for a patient with NPDR and DME, IVFA 1 month after triamcinolone injection showed restoration of the BRB manifested as lack of hyperfluorescence and DME. Postinjection IVFA showed less fluorescein leakage than on preinjection angiograms in all 48 eyes (100%). At 3 months, 62% of eyes exhibited decreased hyperfluorescence and DME on IVFA. At 6 months, this value was 38%.

Figure 1. Triamcinolone appears as a whitish suspension located in the inferior portion of the vitreous cavity.

Figure 2. Intravenous fluorescein angiogram (IVFA) of diffuse macular edema (DME). A, A preoperative steroid angiogram with a transit time of 5 minutes shows hyperfluorescence, which is leakage of fluorescein dye into the retinal tissue from breakdown of the blood retinal barrier. B, An IVFA at the same transit time 1 month after triamcinolone injection showing restoration of the blood retinal barrier manifested by lack of hyperfluorescence and DME. Previous laser focal spots are clearly visible inferior temporal to fovea.
In the group with DME and NPDR, 3 patients having OCT showed a mean ± SD baseline central macular thickness of 487 ± 67 µm for the eyes measured. At 1 month, there was a mean ± SD reduction of 50% to 238 ± 24 µm. Figure 3 shows the OCT for the same patient shown in Figure 2. Figure 4 depicts the IVFA of a patient with active high-risk PDR showing venous segmentation, venous loops, and hyperfluorescence from aberrant neovascularization (NV) on the retina. One month after triamcinolone injection, the IVFA shows marked reduction of venous segmentation and loops and marked decrease in hyperfluorescence from NV.

No additional macular laser was done in any group during the follow-up. All 23 eyes with active high-risk PDR and/or NVI received additional peripheral laser a mean of 3.2 months after triamcinolone injection. The 3 eyes with NVI had media opacities preventing complete laser. After injection of triamcinolone, the hyphema or vitreous haze cleared, allowing complete laser 1 month after the second vitreous sample was obtained.

The VA of the 48 eyes with DME improved by 1.65 lines at 1 month, 1.25 lines at 3 months, and 1 line at 6 months. The regressed high-risk PDR group demonstrated the largest improvement in VA of 2.3 lines, 1.8 lines, and 1.6 lines at 1, 3, and 6 months, respectively, after triamcinolone. The improvement in lines of VA decreased inversely to the degree of PDR. Eyes with early PDR had 1.3 lines of improvement at 1 month, 0.9 lines at 3 months, and 0.7 lines at 6 months. Eyes with active high-risk PDR had 0.9 lines, 0.7 lines, and 0.5 lines of improvement in VA at 1, 3, and 6 months, respectively. Mean VA was unchanged in the group with NVI and macular ischemia. Measurement of the repair of the blood-ocular barrier by IVFA for all 4 groups together occurred in 100% of patients at 1 month, in 62% at 3 months, and in 38% at 6 months, with no significant difference between groups (P = .02).

During the study period, intraocular pressure was higher than 21 mm Hg in 16 (33%) of the 51 eyes and was normalized with topical antiglaucomatous medication. After 6 months of follow-up, 12 (23.5%) of the 51 eyes were still receiving topical antiglaucomatous medication. Three (6%) of the 51 eyes developed glaucomatous damage to the optic nerve. One eye required glaucoma surgery to control pressure.
One patient with active high-risk PDR experienced a traction/rhegmatogenous retinal detachment 4 months after triamcinolone injection and 2 months after extensive peripheral laser treatment. Subsequent retinal reattachment surgery was successful with ultimate improvement of visual acuity to 20/30 at 6 months. None of the eyes developed endophthalmitis.

Systemic factors such as duration of diabetes, hypertension, and hemoglobin A1c were documented for each group. A trend of longer duration of diabetes, hypertension, and higher hemoglobin A1c was found in the eyes with PDR or NVI, compared with those with NPDR; however, these differences were not statistically significant (P = .02).

The vitreous concentration of SDF-1 was increased in diabetic subjects with DME (Figure 5A), especially in active high-risk PDR, where VEGF levels were markedly elevated (Figure 5B) and SDF-1 uniformly decreased after steroids in all groups with DME (Figure 5A). Intravitreal levels of VEGF increased with the severity of the disease. Vascular endothelial growth factor levels uniformly decreased after steroid treatment in all groups (Figure 5B). Vascular endothelial growth factor levels correlated significantly with SDF-1 levels pretreatment (r²=0.88) (Figure 5C) but not posttreatment (r²=0.20) (Figure 5C). Patients with NVI responded with resolution of their NV after treatment with triamcinolone. All patients had a reduction of DME and resolution of macular cystic changes upon subjective clinical examination at 1 month. Pretreatment and posttreatment OCT was performed on 3 of the patients. Patients with active high-risk PDR also had regression of angiogenesis. In control subjects, SDF-1 and VEGF levels were below the limit of detection.

Vitreous from patients with high SDF-1 levels as shown by ELISA also demonstrated functional activity in the chemotaxis assay with human retinal endothelial cells (Figure 6). All 3 vitreous samples tested induced significant chemotaxis in retinal endothelial cells, at least as effective as the positive control. Preincubation of the cells with the selective CXCR4 antagonist AMD3100 resulted in significant reduction of chemotaxis to levels nearly comparable with unstimulated (negative control) cells.

**COMMENT**

In this study, triamcinolone injection reduced DME at 1 month and caused regression of preretinal NV and NVI. Intraocular triamcinolone injection in patients with active high-risk PDR and DME allowed peripheral laser to be used without worsening DME. Eyes with NVI and me-
The adverse effects of amcinolone has the physical advantage of being less soluble acting as a local depot. The coincident reduction of vitreous levels of both SDF-1 and VEGF and the clinical improvement by triamcinolone indicates that these molecules may contribute to the maintenance of the phenotype. The development of agents targeting SDF-1 and VEGF, and their respective receptors, may offer alternative therapeutic approaches to treating visual defects associated with diabetic retinopathy.

Further investigations are needed to clarify the ocular interaction between SDF-1 and VEGF, as well as the role of SDF-1 in the pathogenesis of DME. The simplest explanation for our results is that SDF-1 and VEGF participate in the aberrant NV observed in patients with diabetic retinopathy and DME. This hypothesis is supported by published data on the requirement of SDF-1 in vascular development, the angiogenic potential of SDF-1 in several adult model systems, and functional interactions between SDF-1 and VEGF. We showed that vitreous samples containing high SDF-1 levels induced migration in cultured human retinal endothelial cells. Furthermore, the migration-inducing activity of those same samples was almost completely abrogated by a selective inhibitor of the SDF-1 receptor, despite the presence of high concentrations of VEGF. Endothelial cells, including those from developing brain and heart, and endothelial precursor cells express CXCR4 and migrate in response to SDF-1. In an in vivo animal model of ischemic NV, SDF-1 augmented endothelial precursor cell recruitment. Increased homing of CD34+ progenitor cells to the liver after a stressful insult is also dependent on SDF-1.

Stromal-derived factor 1 stimulates VEGF expression in endothelial cells. Furthermore, VEGF has been shown to up-regulate CXCR4 on cultured endothelial cells. Thus, a synergistic interaction between these factors is likely occurring within the diabetic eyes, with SDF-1 promoting VEGF production and VEGF in turn enhancing the responsiveness of the endothelial cells to SDF-1 through increased surface expression of CXCR4. We are the first to demonstrate a link between reduction of vitreous levels of VEGF and SDF-1 and clinical improvement of DME, as well as regression of retinal NV and NVI after injection with triamcinolone. The mechanism by which decreased levels of VEGF and SDF-1 may mediate this clinical improvement is currently under investigation, but possible mechanisms include altering the BRB integrity, altering progenitor cell behavior, or affecting leukocytes and macrophage trafficking and modulation of tight junction and cell adhesion proteins. There is no published information available concerning the regulation of SDF-1 expression by corticosteroids. However, these potent anti-inflammatory agents are known to inhibit the production of other chemokines by both transcriptional and posttranscriptional mechanism.

The coincident reduction of intravitreal levels of both SDF-1 and VEGF and the clinical improvement by triamcinolone indicates that these molecules may contribute to the maintenance of the phenotype. The development of agents targeting SDF-1 and VEGF, and their respective receptors, may offer alternative therapeutic approaches to treating visual defects associated with diabetic retinopathy.

Submitted for Publication: November 21, 2003; final revision received May 17, 2004; accepted May 26, 2004.

Correspondence: Maria B. Grant, MD, Department of Pharmacology and Therapeutics, University of Florida, Box 100267, Gainesville, FL 32610-0267 (grantma@pharmacology.ufl.edu).


REFERENCES