Intravitreal Infliximab and Choroidal Neovascularization in an Animal Model

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Objective: To determine whether intravitreal infliximab can inhibit the growth of choroidal neovascularization (CNV) in an animal model of age-related macular degeneration.

Methods: Twenty-four brown Norway rats received 6 argon laser lesions of sufficient power to rupture the Bruch membrane in each eye. The right eye received a single intravitreal infliximab injection of 0.15 mg/mL, 1.5 mg/mL, or 15 mg/mL. The left eye received an injection of balanced saline solution. The animals were then euthanized at day 30, the eyes were enucleated, and the amount of CNV was quantified with digital analysis software.

Results: Intravitreal infliximab inhibited CNV growth in the rat laser-trauma model in a dose-response manner. In the 1.5-mg/mL group, there was an 11% reduction in CNV growth ($P = .01$). In the 15-mg/mL group, CNV growth was decreased by 68% ($P < .001$).

Conclusions: Infliximab can inhibit CNV in a rat laser-trauma model, implicating its target cytokine tumor necrosis factor α in the angiogenic stimulus for CNV. Suppression of inflammatory cytokines may prove to be another therapeutic target in the treatment of exudative macular degeneration.

Clinical Relevance: This study demonstrates in a model of macular degeneration an antiangiogenic effect of intravitreal infliximab, which provides a rationale for future human studies.

Arch Ophthalmol. 2007;125(9):1221-1224

Mediators of inflammation appear to play a role in the pathogenesis of exudative age-related macular degeneration (AMD). Tumor necrosis factor α (TNF-α) is one such cytokine known to promote angiogenesis. Studies have implicated TNF-α in neovascularization in the retina, cornea, and in a number of other systems. Tumor necrosis factor α has also been found to cause prolonged breakdown and increased permeability of the blood-retina barrier. Notably, TNF-α has been observed in human choroidal neovascularization (CNV) membranes.

Infliximab is a chimeric monoclonal antibody producing anti-inflammatory effects via blockade of TNF-α and -β isotypes and lysis of TNF-producing cells and is used clinically in the treatment of rheumatoid arthritis, Crohn disease, ankylosing spondylitis, psoriatic arthritis, and ulcerative colitis. Furthermore, it is particularly efficacious when administered systemically for uveitis, though adverse effects, such as blood dyscrasias, increased susceptibility to infection, and hepatotoxicity, may occur.

We report the findings of a study designed to assess the effects of intravitreal injection of infliximab on CNV in an animal model. Although there are no direct animal models of AMD-associated CNV, there are several animal models in which laser trauma induces fibrovascular proliferation arising from the choroid as a model of CNV. Our study employs the laser-trauma model, which has been extensively studied and validated by other researchers.

METHODS

LASER PHOTOCOAGULATION AND INTRAVITREAL INJECTION

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 and were approved by the University of Colorado School of Medicine, Aurora.
Colorado Animal Use and Care Program. Twenty-four male brown Norway rats (Harlan Inc, Indianapolis, Indiana) were anesthetized with intraperitoneal injections of 60 mg of ketamine and 10 mg of xylazine per kilogram of body weight. Both pupils were topicaly dilated with tropicamide, 1%. Laser photocoagulation was performed using a method similar to that described by Edelman and Castro. Briefly, a glass coverslip was placed over the eye and the retina was viewed with a slit-lamp laser system (Lumenis Inc, Santa Clara, California). Using an argon green laser at 150-MW power, a 50-µm spot diameter, and a 0.1-second duration, 6 laser spots were placed in the peripapillary area of each eye, avoiding major retinal vessels. Animals were then assigned to 1 of 3 experimental groups according to the concentration of infliximab (Centocor Inc, Horsham, Pennsylvania) injected: 0.15 mg/mL, 1.5 mg/mL, or 15 mg/mL. Prior to and immediately following injection, an ofloxacin ophthalmic solution (Allergan Inc, Irvine, California) was applied topically to prevent infection. A povidone-iodine solution (Allergan Inc, Irvine, California) was applied to the perimeters to prevent tissue desiccation. A povidone-iodine solution, 5%, was applied before any manipulations. Intravitreal injection was performed (generally) by using the method of Gao et al immediately following the laser procedure. A single dose of 5 µL of a sodium citrate and sodium chloride buffer was injected into the vitreous of the study eye using a Hamilton microinjector (Hamilton Co, Reno, Nevada), resulting in an infliximab dose of 0.75 µg in group A, 7.5 µg in group B, and 75 µg in group C. The injection was then visualized with the slitlamp to confirm proper placement. Animals with traumatic lens injury were excluded from the study. The fellow eye of each animal served as a control, receiving an intravitreal injection of 5 µL of a balanced saline solution. The animals were recovered from anesthesia and 1 drop of ofloxacin was given daily for 1 week following treatment.

FLUORESCEIN ISOTHIOCYANATE–DEXTRAN ANGIOGRAPHY AND FLAT-MOUNT VISUALIZATION OF CNV

Thirty days following laser and intravitreal injection, the animals were anesthetized intraperitoneally with 80 mg of ketamine and 10 mg of xylazine per kilogram of body weight. Fluorescein isothiocyanate (FITC)—dextran perfusion and flat-mount preparation were performed using the methods of McMenamin and Semkova et al. A cannula was introduced into the heart via a small incision in the left ventricle and the descending aorta was clamped. Using a constant flow pump (Harvard Apparatus, Holliston, Massachusetts), the animal was perfused with 120 mL of cold heparinized phosphate-balanced saline (1 IU of heparin per milliliter of phosphate-balanced saline) followed by 50 mL of phosphate-balanced saline with 5 mg of 2 million–molecular weight FITC-dextran per milliliter of saline (Sigma-Aldrich Inc, St Louis, Missouri). An incision in the right atrium was made to allow efflux of the perfusate. The eyes were then enucleated and placed in formalin, 10%, solution overnight. Subsequently, the globes were dissected at the equator and the anterior segment and vitreous were discarded. The neural retina was removed from the posterior cup and 4 radial cuts were made in the remaining retinal pigment epithelium choroid-sclera. The sections were flat-mounted on slides, epifluorescent microscopy–specific media (Biomeda Corporation, Foster City, California) was applied, weights were placed on the coverslips, and fingernail polish was applied to the perimeters to prevent tissue desiccation.

Flat-mounts were visualized, and FITC-dextran–labeled CNV was quantified in a manner similar to that described by Edelman and Castro. The flat-mounts were viewed under a 20X objective of an epifluorescent microscope, using the appropriate FITC filters. The areas of CNV were photographed with a camera operated by a personal computer with image capture and analysis software. The FITC-dextran–perfused vessels representing neovascularization were quantified by delineating the perimeter of the fibrovascular membranes.

STATISTICAL ANALYSIS

The areas of neovascularization were compared between treatment and control eyes using statistical analysis software (MedCalc Software, Mariakerke, Belgium). The data set for each group was run through the D’Agostino-Pearson test for normality, which indicated that the data have a normal distribution, indicating that the paired t test is the most appropriate parametric test. P ≤ .05 was considered statistically significant.

RESULTS

In our study, 2 million–molecular weight FITC-dextran angiography and epifluorescent microscopy were used
to visualize laser trauma-induced CNV. At day 30, the retinal pigment epithelium–choroid-sclera flat mounts demonstrated perfusion with FITC-dextran and areas of relative hyperfluorescence representative of CNV overlying the laser lesions (Figure 1).

There were 8 animals per treatment group. No eyes were excluded secondary to lens trauma. The area of each lesion was compared between treatment and control eyes to evaluate the efficacy of intravitreal infliximab in suppressing CNV. Figure 2 demonstrates the inhibition of CNV growth in an eye treated with intravitreal infliximab at a concentration of 15 mg/mL.

In the 0.15-mg/mL group, the mean CNV area per lesion was 152 901 µm² in the treatment group and 145 785 µm² in the control group (P = .49). In the 1.5-mg/mL group, the mean CNV area per lesion was 138 969 µm² in the treatment group, compared with a mean area of 156 080 µm² per lesion in the control group (P = .01), representing an 11% reduction in CNV membrane growth. Similarly, in the 15-mg/mL group, the mean CNV area per lesion was 45 663 µm² in the treatment eye, compared with 140 675 µm² per lesion in the control group (P < .001), a 68% reduction in CNV membrane growth (Figure 3).

**Figure 3.** Choroidal neovascular growth inhibition with intravitreal infliximab in a rat model of exudative macular degeneration. A, Control group vs infliximab dose of 0.15 mg/mL (group A). B, Control group vs infliximab dose of 1.5 mg/mL (group B). C, Control group vs infliximab dose of 15 mg/mL (group C). Asterisks indicate statistical significance (P ≤ .05); error bars indicate 95% confidence interval.

Age-related macular degeneration with CNV and/or geographic atrophy is currently estimated to affect 1.75 million Americans. Given the rapidly aging US population and that the prevalence of AMD increases with age, it is predicted that AMD may afflict as many as 2.95 million persons by the year 2020. Additionally, the Beaver Dam Eye Study found the prevalence of the exudative form in people older than 75 years to be as high as 5.2%. The natural history of neovascular AMD leads to a loss of both visual acuity and contrast sensitivity and, as such, a deteriorating quality of life. In a recent study, Williams et al reported that elderly patients with a visual acuity of 20/200 or worse in one or both eyes owing to AMD were more likely to show impaired performance on routine daily living tasks. Study participants also reported a quality of life index similar to those suffering from severe illnesses, such as AIDS and chronic obstructive pulmonary disease, and had emotional distress comparable with patients with melanoma, AIDS, and bone marrow transplants. In light of this, elucidation of the underlying pathologic mechanisms in AMD is an area of intense investigation.

Angiogenesis typically follows a well-ordered series of events: endothelial cell migration and proliferation, extracellular proteolysis, tube formation, and remodeling of the vessel wall. The process of angiogenesis is mediated by a host of cytokines, such as vascular endothelial growth factor, basic fibroblast growth factor, transforming growth factor β, and platelet-derived growth factor, among others.

It is currently believed that the pathogenesis of the CNV in AMD is, at least in part, an inflammatory process. Epidemiologic studies in support of this have found increased C-reactive protein levels to be associated with an increased risk of AMD. Macrophages have been postulated to stimulate angiogenesis in neovascular eye diseases, including AMD, and have been observed surrounding neovascular vessels. Vascular endothelial growth factor messenger RNA expression has also been noted to be increased proportional to the amount of surrounding inflammation in human subfoveal fibrovascular membranes. The anaphylatoxin complement components C3a and C5a are found in drusen, a hallmark lesion of AMD, and have been implicated in the formation of CNV. Most striking is the recent discovery that individuals homozygous for a variant of the gene for complement factor H, a regulator of the complement cascade, have a more than 7-fold increased risk of AMD.

A biological basis for the role of TNF-α in inflammatory disease has been established. Tumor necrosis factor and its related isoforms and receptors are involved in the immune response on many levels, including signaling of lymphoid neogenesis, augmentation of immune response via costimulatory signals, and termination of the response by way of proapoptotic signals. The effects of TNF-α on target cells of specific importance to the pathogenesis of AMD are numerous. Through ac-
tivation of nuclear transcription factor κB, endothelial cells up-regulate adhesion molecules and chemokine secretion, promoting the recruitment of inflammatory cells. Furthermore, TNF-α is believed to stimulate the production of angiopoietin-1, a growth factor specifically for neovascularization.

The exact mechanism by which the chimeric TNF-α antibody inhibits the progression of CNV is unclear. One possibility is that by inhibiting cell-signaling pathways and the concomitant inflammation, there is an overall reduction in endothelial cell reactivity and the activation and influx of macrophages and other inflammatory, pro-angiogenic cells is halted. Most likely, the antiangiogenic effect of TNF-α blockade on CNV is a combination of directly altering cell function, diminution of inflammation, reduced vascular endothelial growth factor levels, and other angiogenic growth factors.

The rat model of laser-induced CNV is the most widely used in ophthalmic research, though there is discussion as to whether this represents the true pathologic mechanism of AMD. Although the acute break in the Bruch membrane induced by laser is likely different from degenerative changes occurring across decades in patients, the basic principle is the same: neovascularization from the choroid occurs at the site of damage, making it a reliable and reproducible method to test the efficacy of drugs to inhibit CNV.

The current study demonstrates the ability of intravitreal chimeric TNF-α antibody to inhibit CNV in an animal model of exudative macular degeneration. Furthermore, we were able to demonstrate a dose-response relationship between drug and observed effect.

In conclusion, the development of CNV membranes is a complex interaction of cytokine cascades, inflammation, and emerging immunologic mechanisms yet to be determined. Nonetheless, TNF-α appears to be an attractive therapeutic target in exudative AMD. Further studies are needed to assess the efficacy of TNF-α antibody in treating pre-existing lesions, to elucidate any potential toxicity to native retinal cell function, as well as to determine optimal dosing.

Submitted for Publication: July 31, 2006; final revision received February 20, 2007; accepted February 21, 2007.

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Financial Disclosure: None reported.