Angiography of Fluoresceinated Anti–Vascular Endothelial Growth Factor Antibody and Dextrans in Experimental Choroidal Neovascularization

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Objective: To determine if anti–vascular endothelial growth factor antibody and a range of dextrans with varying diffusion radii and molecular weights are permeable through experimental choroidal neovascularization (CNV).

Methods: Choroidal neovascularization was induced in 10 cynomolgus monkey retinas by means of argon laser injury. Digital fundus fluorescein angiograms were performed with fluorescein sodium, fluoresceinated IgG antibodies (anti–vascular endothelial growth factor and a control antibody), and fluoresceinated dextrans with molecular weights of 4, 20, 40, 70 and 150 kd. The 40- and 70-kd dextrans straddle the effective diffusion radius of IgG. For each reagent, early and late angiograms were performed in a standardized fashion, with follow-up images obtained to monitor residual fluorescence.

Results: Perfusion of retinal vessels and choroidal vasculature was seen with all reagents. Fluorescein and 4- and 20-kd dextran leaked rapidly from the CNV within the first minute. Angiography with the use of 40-kd dextran and fluoresceinated antibody, either anti–vascular endothelial growth factor or control IgG, showed fluorescence within the CNV that increased during the first 1 to 5 hours, with mild leakage from the CNV. By 24 hours, fluorescence in the CNV was minimal, although in some cases persistent fluorescence in the surrounding tissue was evident up to 2 weeks. The 70-kd dextran showed fluorescence within the CNV and leakage in 1 of 3 eyes. The 150-kd dextran showed fluorescence within the CNV but did not demonstrate leakage.

Conclusions: Fluoresceinated antibodies and dextran with smaller effective diffusion radii showed CNV perfusion and leakage. Dextrans with larger effective diffusion radii (70 kd and 150 kd) perfused into CNV but did not show leakage consistently.

Clinical Relevance: Determining the permeability of antibodies and molecules of similar size through CNV can help ascertain the feasibility of using intravenously administered antibodies against angiogenic growth factors as a future treatment for choroidal neovascularization.

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CHOROIDAL neovascularization (CNV) is the main blinding feature of age-related macular degeneration. Laser treatment of CNV is the standard therapy for neovascular age-related macular degeneration. Most patients are not eligible for laser photocoagulation by the current guidelines. Moreover, a large percentage of patients who have undergone photocoagulation still develop severe visual loss and/or recurrence of the CNV. An alternative strategy to laser therapy that uses intravenously administered antibodies against angiogenic growth factors has theoretical promise. To bind and inactivate angiogenic growth factors such as vascular endothelial growth factor (VEGF) found and produced in the extravascular space, antibodies must have access to this space. Neither the permeability of antibodies nor the molecular size permeable through CNV is known.

We performed fundus angiography with fluoresceinated dextrans and antibodies in a well-characterized experimental monkey model of CNV. In this model, leakage of fluorescein has been correlated with the presence of subretinal fluid overlying active neovascular membranes. Angiography was studied because the size of molecule that is functionally permeable through CNV cannot be visualized ultrastructurally. To determine the molecular size and diffusion radius capable of functionally leaking through neovascular membranes, we used fluoresceinated dextrans of various molecular weight and diffusion radii. Fluorescein isothiocyanate–labeled dextran-FITC-dextran have been
MATERIALS AND METHODS

ANIMALS

Cynomolgus monkeys were used in accordance with the guidelines of the Animal Care Committee of the Massachusetts Eye and Ear Infirmary, Boston. Monkeys (3-5 kg) were anesthetized for all procedures as previously described. Before enucleation, intravenous pentobarbital sodium solution was administered after the initial ketamine hydrochloride, acepromazine maleate, and atropine sulfate mixture had been used. Monkeys were killed with veterinary pentobarbital-based euthanasia solution (Vortech Pharmaceuticals, Dearborn, Mich).

CREATION OF CNV

Choroidal neovascularization was induced by laser injury in both maculae of 3 monkeys. Seven laser burns were placed in the macula with an argon green laser (514 nm) (Coherent Argon Dye Laser 920; Coherent Medical Laser, Palo Alto, Calif) with settings of 30-μm spot size, 0.1 second, and powers of 350 to 450 mW. Standard fluorescein angiography of every laser-treated eye confirmed the presence of CNV before testing the fluoresceinated reagents. The CNV developed 2 weeks after laser treatment and persisted for 6 or more weeks in all eyes.

REAGENTS

Dextran with molecular weights of 150, 70, 40, 20, and 4 kd (referred to as FITC-150, FITC-70, FITC-40, FITC-20, and FITC-4, respectively) (Genentech, South San Francisco, Calif) and human recombinant IgG monoclonal antibodies (anti-VEGF and control anti-GP120 antibodies) labeled with FITC (Genentech) were used for angiography. The control antibody was the same isotype as the anti-VEGF antibody. All solutions were determined to be nonpyrogenic with the rabbit pyrogen assay and the limulus lysate assay. Both antibody and dextran solutions were prepared in sterile fashion in tissue culture-grade phosphate-buffered saline at 120 mg/mL and were filtered through a 0.2-μm filter. Four milliliters of each solution was used for each angiogram, corresponding to a dose of 120 mg/kg. Solutions were injected intravenously via the saphenous vein during a period of 30 seconds.

PHOTOGRAPHY AND ANGIOGRAPHY

Color photographs were taken with a fundus camera (Canon Fundus CF-60Z; Canon USA, Inc, Lake Success, NY). A digital fundus camera connected to an image acquisition system (Topcon 501A and Imagent system; TopCon, Paramus, NJ) was used for fluorescein angiography. Standardized fluorescein angiography was performed by injecting 10% fluorescein sodium at 0.1 ml/kg of body weight and capturing an image every 5 minutes for at least 1 hour. The fundus camera had fluorescent exciting and barrier filters in place, and the digital camera gain was set at +18 and camera flash was set at 18 during early frames, increasing to 300 during late frames. Images were obtained 5 hours after injection in the antibody-injected animals only to determine the persistence of fluorescence. Images were obtained at 24 hours and then every 48 hours until fluorescence was undetectable in the animals injected with fluoresceinated antibody and FITC-dextran. Residual fluorescence was observed by using identical fundus and digital camera settings but with a camera flash setting of 300.

Choroidal neovascular membranes grew around individual laser burns and demonstrated hyperfluorescence and leakage with fluorescein angiography. The CNV typically measured approximately 1 to 3 disc areas (Figure 1).

The right eye of monkey 1 was studied before laser treatment by serial angiography after injection of fluorescein, FITC-4, FITC-20, FITC-40, FITC-70, and FITC-150. Serial angiography after injection of the same reagents was performed 2 weeks after laser treatment as well. The left eye underwent laser treatment and, after it developed CNV, FITC-40 angiography was performed.

In 2 other monkeys (monkeys 2 and 3), CNV was induced, and both eyes of monkey 2 were serially studied angiographically with the use of FITC-20 and then FITC-40. The use of FITC-40 and FITC-70 was serially studied in both eyes of monkey 3. All angiograms were taken between 2 and 6 weeks after laser treatment when CNV had developed. In contrast to the increase in flash settings from 30 to 200 for fluorescein angiography, FITC-dextran angiography was set only at 200 throughout both early and late frames. During all serial angiographic studies, no residual fluorescence was present before angiography with the second reagent.

FLUORESCEINATED ANTIBODY ANGIOGRAPHY

Both eyes of monkeys 4 and 5 were studied angiographically with fluoresceinated anti-VEGF and control antibody after CNV was documented by fluorescein angiography. All angiograms were taken between 2 and 6 weeks after laser treatment. Camera and flash settings were identical to those used for FITC-dextran angiography (Table 1).

used to visualize the choroidal circulation,8 to determine the permeability of retinal vessels in a primate model of uveitis,9 and to determine the permeability of the posterior blood-ocular barrier 1 to 7 days after xenon photocogulation in rabbits.10 To our knowledge, no one has studied the angiographic permeability of CNV to various sizes of fluorescein dextrans or fluoresceinated antibodies.

The permeability of anti-VEGF antibody and a fluoresceinated dextran with smaller and larger diffusion radius to IgG antibodies is of particular interest. Vascular endothelial growth factor has been correlated with both experimental11-13 and clinical14-16 ischemic retinopathies. Specific inhibition of VEGF with antibodies and antisense prevented and attenuated ocular neovascularization in animal models of ischemic eye disease.17,18 In addition, there is evidence that VEGF is involved in clinical choroidal neovascularization,19,20 and preliminary data in our laboratory correlates VEGF expression with experimental CNV in monkeys.21 Systemic administration of anti-VEGF may be a promising treatment for CNV in age-related macular
degeneration. In this study, we assessed the permeability of intravenously administered FITC-dextrans of small diffusion radii and IgG antibodies, in particular, anti-VEGF antibody, through experimental choroidal neovascularization in monkeys.

### RESULTS

#### COMPARISON OF FITC-DEXTRAN LEAKAGE

Baseline angiography by the various FITC-dextrans demonstrated perfusion of choroidal and retinal vessels within

![Figure 1](image)

**Figure 1.** Fluorescein angiogram of a monkey eye with laser-induced choroidal neovascularization.

![Figure 2](image)

**Figure 2.** One eye of 1 monkey studied angiographically with fluorescein isothiocyanate (FITC)–labeled dextran with a molecular weight of 40 kd 1 minute after injection (A) and 20 minutes after injection (B). The same eye was studied angiographically with FITC-labeled dextran with a molecular weight of 70 kd 1 minute after injection (C) and 20 minutes after injection (D). Hyperfluorescence was noted after 1 minute and leakage was noted 20 minutes after injection of FITC-labeled dextran with a molecular weight of 40 kd, but no leakage was noted 20 minutes after injection of FITC-labeled dextran with a molecular weight of 70 kd.

<table>
<thead>
<tr>
<th>Monkey No.</th>
<th>Eye</th>
<th>Reagent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OD</td>
<td>FITC-4,-20,-40,-70,-150</td>
</tr>
<tr>
<td></td>
<td>OS</td>
<td>FITC-40</td>
</tr>
<tr>
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<tr>
<td>4</td>
<td>OD</td>
<td>Anti-VEGF and control Ab</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>OS</td>
<td>Anti-VEGF and control Ab</td>
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</tbody>
</table>

*All angiograms were taken between 2 and 6 weeks after laser treatment. FITC indicates fluorescein isothiocyanate–labeled dextrans with molecular weights as shown (in kilodaltons); VEGF, vascular endothelial growth factor; and Ab, antibody.
the early phase of angiography or 30 seconds after infusion. The use of FITC-4 (diffusion radius of approximately 1.2 nm) demonstrated hyperfluorescence of CNV in 1 minute, with subsequent leakage by 15 minutes in 1 eye from 1 monkey. The CNV from 3 eyes of 2 monkeys studied with FITC-20 (diffusion radius of approximately 3.2 nm) showed hyperfluorescence in CNV by 1 minute, with subsequent leakage by 20 minutes. The CNV from 5 eyes of 3 monkeys demonstrated hyperfluorescence with FITC-40 (diffusion radius of approximately 4.5 nm) angiographically by 1 minute and showed subsequent leakage by 20 minutes (Figure 2, A and B). The CNV from 3 eyes of 2 monkeys showed hyperfluorescence angiographically with FITC-70 (diffusion radius of approximately 5.8 nm), but only 1 eye demonstrated progressive leakage from CNV by 20 minutes (Figure 3, A and B). One eye of 1 monkey showed only faint hyperfluorescence of CNV by 1 minute with FITC-150 (diffusion radius of approximately 8.5 nm). No leakage was noted after 25 minutes (Table 2).

When FITC-dextran angiography was compared with fluorescein angiography of the respective membranes, the amount of leakage was much less with FITC-dextrans, although the area of CNV that was delineated was similar. In the first animal studied, serial angiography by fluorescein dextrans resulted in accumulation of fluorescence in the subretinal space adjacent to the neovascular membrane but not in the center of the CNV lesion in 1 eye. This residual fluorescence remained for more than 2 weeks. Serial angiograms were not performed in subsequent animals after this was noted.

Table 2. Fluoresceinated Reagent Angiography Results

<table>
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<tr>
<th>Compound</th>
<th>Molecular Weight, kd</th>
<th>Diffusion Radius, nm</th>
<th>Total Eyes Studied, No.</th>
<th>CNV Hyperfluorescence, No.</th>
<th>CNV Leak, No.</th>
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<tbody>
<tr>
<td>FITC-4 4</td>
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<td>1.2</td>
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<tr>
<td>FITC-20 20</td>
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<td>3</td>
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<tr>
<td>FITC-40 40</td>
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<td>5</td>
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<tr>
<td>FITC-70 70</td>
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<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>FITC-150 150</td>
<td>150</td>
<td>8.5</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Anti-VEGF 146</td>
<td>5.5</td>
<td>5.5</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Control antibody 146</td>
<td>5.5</td>
<td>5.5</td>
<td>4</td>
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</tbody>
</table>

*CNV indicates choroidal neovascularization; FITC, fluorescein isothiocyanate–labeled dextrans; and VEGF, vascular endothelial growth factor.

FLUORESCEINATED ANTIBODIES

In 4 eyes of 2 monkeys, fluoresceinated anti-VEGF antibody (diffusion radius, 5.5 nm) perfused retinal and choroidal vessels. Hyperfluorescence of CNV was noted within 1 minute and leakage was noted within 1 hour on late-phase angiography. The intensity of fluorescence was less than the intensity noted for fluorescein angiography. Leakage into the center of the CNV was also minimal. Only a small amount of leakage from the CNV occurred after the early phases. After 5 hours, minimal but noticeable leakage occurred in 2 eyes of 1 monkey. In the other monkey, the leakage from CNV of fluoresceinated anti-VEGF was notable (Figure 4, A-C). Again, although the leakage in later phases continued, the degree of leakage was less than that seen with fluorescein angiography.

In the same 4 eyes in which anti-VEGF antibody was studied angiographically, control antibody was studied angiographically either before or subsequent to angiography by anti-VEGF antibody. In all 4 eyes, CNV demonstrated hyperfluorescence by 1 minute and persisted at the 20-minute
Leakage of CNV was demonstrated at 1 hour and up to 5 hours in 2 eyes (Figure 5, C and D). The intensity and area of leakage by fluoresceinated control antibody was severalfold less than that seen with fluorescein and more than but comparable in intensity with that seen with fluoresceinated anti-VEGF antibody.

In the monkeys injected with fluoresceinated anti-VEGF antibody, the phenomenon of retained fluorescence in the adjacent subretinal space was also observed. The length of time that fluorescence was retained was less than or equal to 5 days. If residual fluorescence was not localized in the CNV, immediately adjacent to the CNV, and was only barely perceptible, angiography was performed with another reagent.

Using fluoresceinated dextrans, this study demonstrated that molecules with diffusion radii equal to or less than FITC-40 can leak through choroidal neovascular membranes. All reagents perfused retinal and choroidal vessels, but only 40-kd molecular weight or smaller fluorescein dextrans and fluoresceinated anti-VEGF and control antibody consistently demonstrated leakage from CNV on angiography. The leakage of dextrans in the late-phase angiograms by molecules with a diffusion radius as large as FITC-70 and IgG implies that molecules of this size have access to the extravascular space surrounding CNV.

In addition, the reagents had access to the subretinal space of areas adjacent to the CNV, and egress from this subretinal space was slow. An explanation for the persistence and accumulation of fluoresceinated molecules in this adjacent subretinal space is the ability of large fluorescent molecules to pass through the leaky vessels of the CNV and the inability of these large molecules to pass through the tight junctions of undamaged retinal pigment epithelial cells to exit into the choroidal circulation in the areas adjacent to the CNV. In the area of CNV, persistent fluorescence rarely lasted longer than 24 hours, whereas fluorescence in the subretinal space adjacent to CNV persisted up to 2½ weeks after injection.

Vascular endothelial growth factor may play a role in the development of choroidal neovascularization in macular degeneration. Retinal pigment epithelial cells and choroidal fibroblasts, known components of CNV, express and up-regulate VEGF. Subfoveal fibrovascular membranes surgically removed from patients with age-related macular degeneration were found to express both VEGF messenger RNA and VEGF protein. Both messenger RNA expression and protein localization were found in extravascular space, in particular in the fibroblastlike cells and inflammatory cells interspersed within the fibrovascular stroma. Transdifferentiated retinal pigment epithelial cells that surround the vascular channels in CNV also show strong immunoreactivity for VEGF.

To inhibit VEGF with a neutralizing antibody, the antibody must reach the extravascular space surrounding the choroidal neovascular membrane. Although intravitreal injections were capable of inhibiting iris neovascularization in a primate model of branch retinal vein occlusion, the cells producing VEGF in the develop-
ment of CNV appear to be of retinal pigment epithelial origin and may be difficult to access from the vitreous cavity.

Intravenously administered antibodies may be unable to cross the intact blood-retinal barrier, but the increased permeability of newly formed vessels allows selective localization of intravenously administered molecules. This study demonstrates that antibodies and molecules similar in diffusion radii to antibodies have access to the extravascular space where VEGF has been localized.

The administration of intravenous anti-VEGF to inhibit choroidal neovascularization seems feasible and promising in light of these data. Further studies must be performed to determine if anti-VEGF antibody can inhibit CNV and if the concentration of antibody that can perfuse the CNV is sufficient to neutralize the VEGF localized there. The effects of systemic administration of anti-VEGF antibodies also need to be studied. The inhibition of constitutive production of VEGF by systemic administration of anti-VEGF poses a possible theoretical problem. On the other hand, if inhibition of endogenous steady-state VEGF does not cause severe adverse effects, intravenous anti-VEGF antibodies may hold promise for the treatment and prevention of CNV in the future.

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A look at the past . . .

Besides the actual pseudo-accommodation which the aphakic eye possesses, we are not to forget the artificial pseudo-accommodation which depends upon the patient’s holding the glasses nearer to or farther from the eye, or looking obliquely through the margin of the glass.