Effects of an Anti–VEGF-A Monoclonal Antibody on Laser-Induced Choroidal Neovascularization in Mice: Optimizing Methods to Quantify Vascular Changes

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PURPOSE. The purpose of this study was to evaluate different methods of detecting and quantifying experimentally induced choroidal neovascularization (CNV) and vascular changes induced on CNV by an anti–VEGF-A monoclonal antibody.

METHODS. Choroidal neovascularization was induced by 532-nm diode laser in C57BL/6 mice. Ten days after the laser, the following methods were used to detect the new vessels: high-resolution angiography with fluorescein isothiocyanate-dextran; immunohistochemistry with biotinylated isoelectin, rabbit anti–NG2, rabbit anti–CD105, rabbit anti–collagen IV, rat anti–ICAM-2, rabbit anti–desmin, and rat anti–MECA 32; and intravital injection of fluorescein-labeled Lycopestison esculentum (tomato) lectin. To verify the validity of these staining methods in the quantification of treated CNV, the authors applied the most effective of these techniques to three groups of mice after laser induction of CNV and treatment with an anti–VEGF full antibody (G6-31).

RESULTS. Fluorescein isothiocyanate-dextran angiography, rat anti–ICAM-2 immunostaining, and tomato lectin intravitral injection resulted in the most effective means of identifying choroidal neovascularization. A certain amount of nonspecific fluorescence was detected in the area of CNV for each method. This fluorescence appeared more intense when fluorescein isothiocyanate-dextran was used. Tomato lectin injection and rat anti–ICAM-2 immunostaining were the methods that better recorded the antiangiogenic drug effect.

CONCLUSIONS. Because of easy execution, low background fluorescence, and detailed visualization of new vessels, intravitreal injection of tomato lectin followed by a quantification based on threshold fluorescence represents the best technique for measuring CNV and the vascular changes induced by an anti–VEGF-A monoclonal antibody in mice. (Invest Ophthalmol Vis Sci. 2008;49:1178–1185) DOI:10.1167/iovs.07-1194

Choroidal neovascularization (CNV) is a feature of a number of eye diseases, such as age-related macular degeneration (AMD), pathologic myopia, angioid streaks, and ocular histoplasmosis syndrome. CNV reflects new blood vessel growth from the choroid that extends into the subretinal pigment epithelium, subretinal space, or a combination of both. Since the original study in the rhesus monkey performed by Ryan2 in 1979, the rupture of Bruch membrane using a laser has become a common technique to obtain CNV in different animal species.

Several studies have demonstrated that VEGF signaling plays a critical role in the development of CNV in this model. In fact, increased expression of VEGF-A has been demonstrated in experimentally induced CNV in rats,3 and the blockade of phosphorylation by VEGF receptors has been shown to cause dramatic, almost complete, inhibition of CNV in mice.4 In addition, significant advances in angiogenesis research during the past decade have led to the development of numerous, potentially promising, antiangiogenic drugs likely to have an impact on the future treatment of CNV. Currently, pegaptanib sodium injection (Macugen; OSI Pharmaceutical, Melville, NY) and ranibizumab (Lucentis; Genentech Inc., South San Francisco, CA) represent the first ocular anti-VEGF treatments evaluated in large, randomized, controlled clinical trials for the treatment of neovascular AMD. However, a number of other molecules targeting VEGF and its signaling pathway are in early stages of clinical development, including soluble VEGF receptor chimeric proteins, short interfering RNA molecules that inhibit the expression of genes encoding VEGF and VEGF receptor 1, and small-molecule VEGF receptor tyrosine kinase inhibitors.5

To demonstrate the success or failure of treatments, visualization, and quantification of CNV in animal models are crucial. The aim of the present study was to compare different methods to detect experimentally induced CNV in mice and to quantify vascular changes induced by an anti–VEGF-A monoclonal antibody (mAb).

MATERIALS AND METHODS

All animal experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee at Genentech.

To induce choroidal neovascularization, 6- to 8-week-old female C57BL/6 mice weighing 18 to 20 g each were anesthetized with intraperitoneal injections of 75 mg/kg ketamine and 7.5 mg/kg xylazine, and the pupils were dilated with 1% tropicamide. Three 532-nm diode laser spots (100 mW, 100 msec, 100 μm; OcuLight GL, Photo- coagulator, Iridex, Mountain View, CA) were applied to each fundus using a coverslip as a contact lens. The lesions were placed between retinal vessels 2 to 3 disc diameters from the optic nerve. Formation of a bubble at the time of laser application, which indicates rupture of Bruch membrane, is an important factor in obtaining CNV, so only burns in which a bubble was produced were included in the study. To detect the CNV, several methods were used 10 days after photocoagulation.

As control, we examined the following under the fluorescence microscope: unstained flatmounts with a CNV induced 10 days before microscopic evaluation, flatmounts containing laser scars induced a

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few hours before microscopic evaluation (i.e., before the growth of CNV) and stained with the CNV detection methods listed in this article, and flatmounts with CNV induced 10 days before microscopic evaluation of tissues stained with Alexa-594 –anti-rabbit or Alexa-488 –anti-rat (secondary antibodies used for immunohistochemistry).

Images obtained by fluorescence microscopy were digitalized using a three-color CCD video camera (RT Slider; Diagnostic Instruments, Sterling Heights, MI), and Image J software (National Institute of Mental Health, Bethesda, MD; http://rsb.info.nih.gov/ij/) was used to measure the total area (in µm²) of CNV associated with each burn. A calibration image was taken from a slide with a grating of known size. An established and constant threshold in pixels (corresponding to a threshold fluorescence) for each method was used to quantify the neovascularization.

Histologic evaluation of CNV was performed in two mice. The eyes were removed, fixed in formalin, embedded on edge in paraffin blocks, sectioned, and stained with hematoxylin and eosin. Approximately 191 sections, representing the entire globe, were examined per eye.

To validate the staining methods in the quantification of treated CNV, three groups of mice (10 animals per group) underwent intraocular administration of mAb G6-31 (5 mg/kg, 0.1 mL twice a week), initiated the day before induction of CNV for a total of three doses over the 10-day study. Full anti–VEGF antibody G6-31 is a novel anti–VEGF antibody that exhibits cross-species reactivity. It was synthesized as previously described. During this process, a number of antibodies that bind murine VEGF with high affinity were identified, including G6-31. Anti–ragweed antibody (lot #1428), intraperitoneally administered (5 mg/kg in 0.1 mL twice a week) served as control in the other three groups of mice (10 animals per group). Fluorescein iso-thiocyanate-dextran angiography, rat anti-ICAM-2 immunostaining, and tomato lectin intravitreal injection were used to detect the CNV in these six groups of animals 10 days after photocoagulation. For each mouse, the CNV area was averaged from the three lesions. This value was then used to calculate the mean CNV area in each group of animals. Because of the non-Gaussian distribution of the data, comparisons between the CNV areas of the different groups were performed with the Kruskal-Wallis test, followed by Dunn analysis as post hoc test. Statistical analysis software (JMP 5.1; SAS Institute, Cary, NC) was used for all analyses.

FITC-Dextran Angiography

Angiography was performed according to the method of D’Amato et al. and Edelman and Castro. Mice were anesthetized and perfused through their hearts with 1 mL phosphate-buffered saline containing 50 mg/mL fluorescein-labeled dextran (2 × 10⁶ g/mL average MW; Sigma, St. Louis, MO). One or 2 minutes later, the animals were killed with 100% CO₂, and the lasered eyes were removed and fixed in 10% buffered formalin for 1 hour. They were then sectioned at the equator, and the anterior half, the vitreous, and the retina were removed. The retinal pigment epithelium-choroid-sclera complex was dissected through four to five relaxing radial incisions and flatmounted on a slide.

Immunohistochemistry

Eyes were removed and fixed in 4% paraformaldehyde in PBS at 4°C overnight. After three washes with PBS, the anterior segment and retina were dissected out, and the remaining eye tissues were incubated with a blocking buffer (PBS + 0.5% Triton X-100 + 5% goat or mouse serum) for 2 to 16 hours at 4°C. After removal of the blocking buffer, they were subsequently incubated with a primary antibody overnight in Phbc buffer (PBS + 1% Triton X-100 + 0.1 mM CaCl₂ + 0.1 mM MnCl₂) on a rotator.

Nine different primary antibodies, usually a combination of rabbit and rat antibody, were used for each eye. We then studied the new vessels of CNV using several antibodies for correspondent antigens more or less specific for endothelial cells: biotinylated isoelectric B4 (isolectin B4 binds galactosyl epitopes on the membranes of several cell types, including endothelial cells; L2140, working concentration 20 μg/mL; Sigma); rat anti-Cd31 (CD31, or PECAM-1, is an adhesion molecule expressed by vascular endothelial cells, platelets, monocytes, neutrophils, and naive T lymphocytes; 550274, 1:50; BD PharMingen, San Diego, CA); rabbit anti–von Willebrand factor (VWF, a protein expressed by endothelial cells and platelets; A0082, 1:300, Dako, Carpinteria, CA); rat anti–CD105 (CD105, or endoglin, is a regulatory component of the TGF-β receptor complex and is expressed by endothelial cells, activated monocytes, and tissue macrophages, the stromal cells of certain tissues including bone marrow, pre-B cells in fetal marrow, and erythroid precursors in fetal and adult bone marrow; 555814, 1:500; BD PharMingen); rat anti–ICAM-2 (ICAM-2 is a cell-cell adhesion molecule mainly found on resting endothelial cells; 553325, 1:500; BD PharMingen); rabbit anti–desmin (AB907, 1:20; Chemicon, Temecula, CA): rat anti–MECA32 (MECA-32 is an antigen specific for endothelial cells; 550,563, 1:200; BD PharMingen).

Furthermore, we decided to attempt identification of CNV by staining the pericytes with rabbit anti–NG2 (NG2 is a chondroitin sulfate proteoglycan expressed on the surfaces of vascular mural cells during normal and pathologic angiogenesis; AB5320, 1:200; Chemicon) and the vascular basement membrane with rabbit anti–collagen IV (collagen IV is one of the several protein families included in the matrix components of vascular basement membrane; LB1403, 1:10.000; CosmoBio, Tokyo, Japan).

After six washes with PBST (PBS + 0.5% Triton X-100) for 1 hour each at 4°C, the eye tissues were incubated with the appropriate secondary antibody in PBST + 1% goat or mouse serum in a rotating plate overnight at 4°C. Alexa Fluor-488 – conjugated-streptavidin (1:50; Molecular Probes, Eugene, OR) was used for biotinylated isolectin B4, Alexa Fluor-594 – conjugated-anti-rabbit (1:800; Molecular Probes) secondary antibody was used for all the rabbit antibodies, and Alexa Fluor-488 – conjugated-anti–rat (1:800; Molecular Probes) secondary antibody was used for all anti–rat antibodies.

For each primary antibody, immunostaining was obtained with and without treatment with 0.1% osmium tetroxide in H₂O. After several 1-hour washes with PBST on the rotator at 4°C, the samples were fixed with paraformaldehyde in PBS, pH 7.4, for no more than 5 minutes and finally were rinsed several times with PBS. The eyeballs were then dissected and flatmounted as previously described.

Intravitral Intravenous Injection of Fluorescein-labeled Lycopersicon esculentum (Tomato) Lectin

Phosphate-buffered saline (0.1 mL) containing 1 mg/mL of fluorescein-labeled L. esculentum (tomato) lectin (Vector Laboratories, Burlingame, CA) was injected in the mice through a tail vein. Five minutes later, the animals were killed with 100% CO₂, and the lasered eyes were removed, fixed in 10% buffered formalin for 1 hour, sectioned, and flatmounted on a slide as previously described.

Microscopic Evaluation

All flatmounts were examined with the 20X objective of a fluorescence microscope (Eclipse E800; Nikon Instruments Inc., Melville, NY) using FITC and CY3 filters. Conditions for FITC were excitation at 480 nm and emission at 535 nm. Conditions for CY3 were excitation at 535 nm and emission at 610 nm. All digital images were taken under the same conditions.

Results

Fluorescence microscope observation of all flatmounts used as control revealed a certain amount of fluorescence in the area of the laser burn. In the flatmount obtained without the use of staining, this fluorescence (autofluorescence) was thought to be related to the formation of lipofuscinlike material or cell debris after laser photocoagulation-induced damage. It appeared greater in the flatmounts obtained a few hours after the laser (CNV not yet developed).
bars, 50 μm.

An additional nonspecific (background) fluorescence was observed when the area of laser burn was studied by perfusion with FITC-dextran (Fig. 1a). This fluorescence probably emanated from the underlying sclera, where the retinal pigment epithelium and the choroid had been thermally ablated. High concentration (50 mg/mL) of FITC-dextran, commonly used for CNV detection, obviously promoted this fluorescence. Unlike FITC-dextran, neither immunohistochemistry nor intravital tomato lectin injection showed this additional fluorescence in the laser injury (Fig. 1b).

There appeared to be a significant variability among CNV size, not only in different mice but also in the same mice. This may be explained by individual variability, differences in the focus of the laser spot, and differences in the quality of the laser beam. This variability must be taken into account, especially when comparing different drugs, because a small difference in their inhibitory effect is expected. Histologic evaluation confirmed the presence of CNV (Fig. 2).

**FITC-Dextran Angiography**

CNV was well visualized by fluorescein-labeled high molecular-weight dextran (Figs. 3a, 3b). Nonetheless, the thick network of new vessels did not appear as easily detectable as with other techniques, at least in part because of the confounding fluorescence deriving from the large choroidal vessels.

**Immunohistochemistry**

Results varied greatly among the different primary antibodies. Only images obtained with rat anti–ICAM-2 were able to show the typical morphology of blood vessels inside the CNV (Figs. 3c, 3d). Although biotinylated isoelectin, rat anti–CD31, rat anti–CD105, rabbit anti–collagen IV, rabbit anti–desmin, and rat anti–MECA 32 stained the lesion, they were unable to clearly identify a vascular pattern inside it (Figs. 3e–j). Rabbit anti–NG2 and rabbit anti–VWF failed completely to identify the CNV (Figs. 3k, 3l). Furthermore, similar fluorescence was noted for all these antibodies in CNV stained, as control, with only secondary antibodies. In addition, 1% osmium tetroxide treatment did not reduce the background fluorescence.

**Intravitral Tomato Lectin Injection**

A thick network of new vessels was identified in the area of the laser burn after intravitral injection of fluorescein-labeled *L. esculentum* (tomato) lectin (Figs. 3m, 3n). The vasculature extended through various planes, and the background fluorescence was very low.

**CNV Detection in Treated Mice**

All mice treated with mAb G6-31 showed marked inhibition of the CNV compared with controls, irrespective of the detection method used. However, two different patterns were noted: in some animals total inhibition was observed (Figs. 4a–c), whereas in others the inhibition was only partial (Figs. 4d–f). Quantification of CNV revealed a mean area of 681.15 (±749.58), 130.46 (±153.40), and 102.12 (±146.04) μm² using fluorescein isothiocyanate-dextran angiography, rat anti–ICAM-2 immunostaining, and tomato lectin intravitral injection, respectively (Table 1, Fig. 5). In the control animals, the mean CNV area detected with the three methods was 2214 (±1359.22), 782.88 (±1030.72), and 657.96 (±919.75), respectively (Table 1, Fig. 5). With each method, the difference in the mean area of treated and untreated CNV was statistically significant (P < 0.05).

The percentage inhibition was 69.24%, 83.33%, or 84.47% when the CNV area was detected by FITC-dextran angiography, rat anti–ICAM-2 immunostaining, or tomato lectin intravitral injection, respectively. A statistically significant difference (P < 0.05) was noted between the mean CNV area in treated and untreated animals that underwent fluorescein isothiocyanate-dextran angiography compared with animals that underwent the other two staining methods.

**DISCUSSION**

Several lines of evidence indicate that VEGF-A is a critical stimulus for the development of CNV. Earlier studies identified high levels of VEGF or its mRNA in surgically removed human CNV membranes and in animal models of CNV (Husain D, et al. *IOVS* 1997;38:ARVO Abstract 2310). Anti-VEGF agents suppress the development of experimental CNV. In addition, clinical trials have demonstrated that intravitreal injections of an aptamer (pegaptanib sodium; Macugen, OSI Pharmaceuticals) or an antibody fragment that binds VEGF-A (ranibizumab; Lucentis, Genentech Inc.) provides visual benefits in patients with neovascular AMD. Furthermore, another antibody fragment that bevacizumab (Avastin), though approved only for the treatment of metastatic cancer of the colon/rectum and of nonsquamous non–small cell lung cancer, is nevertheless being used off-label for this condition.

The inability of ranibizumab and bevacizumab to neutralize murine VEGF-A precludes a meaningful testing of these antibodies in murine models. In the present study, we evaluated in a murine model of CNV a novel anti–VEGF-A antibody, G6-31, that, unlike ranibizumab and bevacizumab, binds and potently blocks human and murine VEGF-A. Inhibition of the CNV was achieved by G6-31 in all animals, though in some of them few new vessels were still observed in the lesion after treatment (partial inhibition). In addition, we noted a significant difference in the percentage inhibition in relation to the method used for the detection of the neovascularization.

**FIGURE 2.** Histologic image of a choroidal neovascularization in a mouse eye 10 days after the laser-induced injury (10×, hematoxylin and eosin).
Several methods have been used by different authors for the visualization of CNV, including FITC-dextran angiography, immunohistochemical visualization of platelet endothelial cell adhesion molecule (PECAM)-1, visualization of intravascular lumens by peroxidase in histologic analysis, histochemical representation of alkaline phosphatase in endothelial cells, fluorescein angiography, histologic cross-sections, and choroidal flatmounts stained with isolectin B4. We performed FITC-dextran angiography and immunostaining of endothelial cells of CNV using seven primary antibodies (biotinylated isolectin, rat anti–CD31, rabbit anti–VWF, rat anti–ICAM-2, rabbit anti–desmin, and rat anti–MECA32). Furthermore, we decided to attempt identification of the CNV by staining associated pericytes with rabbit anti–NG2 and the vascular basement membrane with rabbit anti–collagen IV. Finally, we also tried to detect the new vessels through the intravitral intravenous injection of fluorescein-labeled *L. esculentum* (tomato) lectin injection. Intravitral fluorescein-labeled *L. esculentum* (tomato) lectin injection at higher magnification. Scale bars: (a, c, e–m) 25 μm; (b, d, n) 10 μm.

We found that tomato lectin intravitral perfusion and ICAM-2 immunostaining enable a detailed microscopic evaluation of choroidal neovascularization with a very low amount of background fluorescence. Compared with FITC-dextran, these techniques were able to better visualize the thick network of new vessels. These findings are especially important when a quantitative analysis of the image is required. Even if lectin were subject to measurable processing in the form of uptake, transport, and transcytosis, we did not observe leakage of fluorophore from the new vessel wall.

Different methods have been used to quantify CNV. Bora et al. used a simple score assessing lesions with new vessels versus lesions without new vessels. In contrast, Lambert et al. proposed a quantitative morphometric assessment of the thick-
ness of choroidal new vessels on microscopic images of hematoxylin-stained eye sections. Histological examination of CNV may have some advantages over measurements of lateral spread, which potentially can be influenced by the angiogenic influence of neighboring CNV sites. However, if quantification of the vascular changes induced by an antiangiogenic drug is required, assessing these changes only by the variation of thickness of the lesion (as suggested by Lambert et al.) may not be entirely accurate. As shown in Figure 2, laser-induced CNV is not exclusively constituted by new vessels; it is, rather, a plaquelike focus of increased cellularity and matrix material associated with disordering of retinal pigment epithelial cells, Bruch membrane, and the underlying pigmented choroidal layer. Its thickness is therefore related not only to vessels but also to other structures that may or may not be influenced by the antiangiogenic treatment.

Other methods for quantification of CNV have been reported. Saishin et al.11 used fluorescence microscopy to analyze flatmounts obtained from mice perfused with 1 mL phosphate-buffered saline containing 50 mg/mL fluorescein-labeled dextran. They then measured the total area of CNV associated with each burn. Sakurai et al.25 described two different methods: grading of lesions by fluorescein angiography on an ordinal scale based on the spatial and temporal evolution of fluorescence leakage and calculation of CNV volume by summation of the whole fluorescent area in flatmounts incubated with FITC-isolectin B4 and examined under a scanning laser confocal microscope. In a similar way, Campos et al.29 quantified CNV volume from an animated 3-D reconstruction built by the confocal microscope z-series of the CNV complex detected by FITC-dextran.

However, none of these studies clarified whether they were able to subtract the background fluorescence from the whole fluorescence of the lesion when measuring the CNV. The question may be relevant because, as we demonstrate in this report, a significant amount of background fluorescence (unrelated to new vessels) may be present in the lesion, which may consequently be responsible for an overestimate of the CNV size. We found a statistically significant difference in the mean CNV area (for treated and untreated CNV) when different staining methods were used. This difference may only be explained by differences in the CNV measurements because we used the same antiangiogenic compound.

CNV quantification based on threshold fluorescence, as described, appears a more suitable method, especially if the aspecific fluorescence is at a low level. When higher levels are present, in fact, setting the correct threshold fluorescence may be challenging, and consequently the whole analysis may be inaccurate. This may explain, in our opinion, the difference we found in the percentage of CNV inhibition obtained by the same drug when CNV was detected with different staining methods. On the other hand, we must emphasize that the choice of a specific threshold is arbitrary and that even slight differences in this setting might lead to significant differences in the calculated CNV areas.

Among the different techniques used to stain a CNV, only immunofluorescent staining and intravital tomato lectin were characterized by a minimal amount of background fluorescence. Immunofluorescent staining, however, is a tricky and time-consuming technique. Among the several antibodies tested, only ICAM-2 showed an ability to identify the new vessels comparable to *L. esculentum* (tomato) lectin. Finally, intravital injection has the advantage of staining only functioning blood vessels.

A potential limitation of all the methods reported in the present study lies in the procedure of tissue preparation. During removal of the retina for direct visualization of the choroid and the CNV, the CNV itself may be affected. In some cases, in fact, the new vessels infiltrate the retina and may become detached, peeling away the retina. This phenomenon can confound the qualitative and quantitative interpretation of the data, regardless of the vascular labeling method. Unfortunately, double-layered vascularization of the retina made difficult the

### Table 1. CNV Area Quantified by Three Methods of Detection in Mice Treated with Anti–ragweed (Controls) and mAb G6-31

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<th>FITC-Dextran Angiography</th>
<th>Rat Anti–ICAM-2 Immunostaining</th>
<th>Tomato Lectin Intravital Injection</th>
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<tr>
<td>Anti–ragweed</td>
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All values are expressed in μm². Values in parentheses are SD.
simultaneous evaluation of choroidal vasculature, forcing us to perform mechanical removal of the retina.

In conclusion, we propose intravital injection of *L. esculentum* (tomato) lectin as a quick, easy, and accurate method of detecting and quantifying laser-induced CNV and vascular changes produced in the CNV by an anti-VEGF-A monoclonal antibody in mice.

Acknowledgments

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References