Decreased Circulation in the Feline Choriocapillaris Underlying Retinal Photocoagulation Lesions

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PURPOSE. To investigate the effects of argon laser photocoagulation on the choroidal circulation in cats.

METHODS. Three sizes of argon laser lesions designed to damage the outer retina were created in six cats: larger than 1 mm, 500 μm , and 200 μm . At least 1 month after the lesions, damage to the choroidal vasculature was studied in two ways. First, scanning laser ophthalmoscopy was used to obtain infrared reflectance (IR) photographs and indocyanine green (ICG) angiograms. Second, fluorescent microspheres (15 μm) were injected into the left ventricle. The globes were fixed, the choroid was flat mounted, and images were taken with a fluorescence microscope. Retinal histology was assessed in comparable lesions.

RESULTS. Histology showed that the inner retina was preserved, but the choroid, tapetum, and outer retina were damaged. ICG angiograms revealed choriocapillaris loss in large lesions and in some 500- μm lesions, whereas the larger vessels were preserved; in 200 μm lesions, choriocapillaris loss was not detectable. However, in all lesions, the distribution of microspheres revealed little if any choriocapillaris flow. In larger lesions, the damaged region was surrounded by an area in which the number of microspheres was higher than in the lesion but lower than in the normal retina.

CONCLUSIONS. Under lesions that destroyed photoreceptors, the choriocapillaris was also compromised, even when no change could be detected with ICG angiography. Panretinal photocoagulation is designed to increase retinal Po_2 by allowing choroidal oxygen to reach the inner retina, but its effectiveness may be limited by damage to the choriocapillaris. (*Invest Ophthalmol Vis Sci.* 2011;52:3398–3403) DOI:10.1167/iovs.10-6560

Proliferative diabetic retinopathy is found in about half of type 1 diabetics and in approximately 10% of type 2 diabetics who have had the disease for 15 years.¹ Panretinal photocoagulation (PRP) has been the most effective treatment,^{2,3} but the disease may continue or worsen in a substantial fraction of patients,^{4,5} and in one study, retreatment was

effective in only approximately half the patients.⁵ Although PRP damages the retinal pigment epithelium and photoreceptor cell layer, it leaves the inner retina relatively unaffected^{6–9} and helps preserve vision. The leading hypothesis for the effectiveness of PRP is that the destruction of photoreceptors reduces the oxygen consumption of the lesioned area, allowing an increased rate of choroidal oxygen delivery to the inner retina.¹⁰ There is considerable indirect evidence supporting this hypothesis,^{11–13} as well as direct evidence from microelectrode measurements that show increased vitreal Po_2 ^{6–7,13–16} and intraretinal Po_2 ¹⁷ after photocoagulation in the retinas of animals.

Choroidal Po_2 is normally higher than inner retinal Po_2 .^{18–22} In order for the choroidal circulation to provide oxygen to the inner retina, the Po_2 at the choroid must remain high after photocoagulation. However, we found that photocoagulation reduces choroidal Po_2 ,¹⁷ and we hypothesized that the choroidal circulation is damaged by the lesion. Several investigators have provided histologic evidence that the choriocapillaris is damaged and does not heal completely after photocoagulation in humans,^{9,23,24} but the functional implications of this are rarely discussed. There have been only two studies of choroidal blood flow after photocoagulation. Chandra et al.²⁵ measured blood flow with radioactive microspheres after photocoagulation. The flow in the retina and choroid of rabbits was reduced at times ranging from a few hours to several weeks after photocoagulation, and in monkeys, flow was reduced immediately, the only time point measured. However, their technique did not separate retinal and choroidal blood flow, and they did not determine whether the changes were local or global. In contrast, a recent study with laser Doppler flowmetry reported increased foveal choroidal blood flow 1 month after photocoagulation for proliferative diabetic retinopathy.²⁶ We therefore reinvestigated the hypothesis that the choroid is damaged by photocoagulation, after both the large lesions used in our earlier study of retinal Po_2 ,¹⁷ and smaller lesions more similar to those used clinically. We used in vivo scanning laser ophthalmoscopy (SLO) and microsphere counts in choroidal flat mounts in cats, techniques that could localize changes in choroidal blood flow.

METHODS

All animals were managed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and institutional guidelines regarding animal experimentation.

Photocoagulation

To produce lesions, adult female cats were preanesthetized with 2.2 to 4.4 mg/kg ketamine and 0.4 mg/kg butorphanol followed by 0.35 mL/kg 5% pentothal sodium (IV) or with 11 mg/kg ketamine and 0.4 mg/kg butorphanol (IM). In either case, 5% pentothal sodium was administered as needed during the laser procedure to maintain anes-

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thesia. A retrobulbar injection of sterile 2% lidocaine HCl solution was administered to retract the nictitating membrane and to prevent the eye from rotating downward during the procedure. The pupil of one eye was dilated with topical ophthalmic solutions of 2.5% phenylephrine (Neosynephrine; Abbott Laboratories; Abbott Park, IL) and 1% tropicamide (Bausch & Lomb; Rochester, NY). Tetracaine (5%; Bausch & Lomb; Rochester, NY) was used as a local anesthetic.

For all lesions, a slit-lamp mounted argon laser (Ultima 2000; Coherent Inc., Santa Clara, CA) was set at 532 nm with a 0.4 seconds pulse duration. The power was initially set at 100 to 400 mW and was increased until a successful laser burn was detected on the retinal surface. Spot diameters were nominally 500 and 200 μm . In two cats, large lesions 3 to 5 mm in diameter were created by placing 500- μm spots confluent. In the four remaining cats, 500- and 200- μm lesions were separated by at least one lesion diameter. In all cases but one, the lesions were all located on the tapetal part of the retina. The lesions were photographed with a fundus camera (Genesis; Kowa Optimed, Inc., Torrance, CA) and 100 ASA color film.

Scanning Laser Ophthalmoscopy

Four to 5 weeks after laser photocoagulation, scanning laser ophthalmoscopy (SLO) was performed in two cats with large lesions and two with small lesions. The cats were anesthetized, and their eyes dilated as described above. The SLO (Heidelberg Retina Angiograph, 2000; Vista, CA) was used to take infrared reflectance (IR) images and indocyanine green (ICG) angiograms. For the ICG angiograms, 0.5 mL of ICG (12.5 mg/mL) was injected intravenously. Angiogram videos and still photographs were captured digitally.

Microsphere Injection

Five cats were anesthetized for a final time for microsphere injections. The sixth cat was diagnosed with cardiomyopathy when it died at the end of the SLO procedure and was used for histology. In two cats, a cannula was inserted through a femoral artery into the left ventricle, with the amplitude of the pressure pulse used to indicate when the cannula had entered the ventricle. In the other three cats, it was not possible to position the cannula inside the ventricle, so it was advanced until it met substantial resistance. We assume that this was in the aortic arch, because microspheres injected at this location did reach the eye. The cannulation and microsphere injection techniques were the same as those in a previous study.²⁷

Microsphere injections contained an average of 4.8×10^6 (range, 3.8×10^6 to 7×10^6) 15- μm diameter fluorescent polystyrene microspheres (Invitrogen Corporation, Carlsbad, CA). These microspheres are large enough to become impacted in the choriocapillaris, and the local density of microspheres is directly related to blood flow (e.g., Ref 27). Two injections, each with microspheres of a different color, were performed in each cat. Two injections gave twice as many microspheres to count, and the use of two colors was convenient but not necessary.

After the microsphere injections, the cat was euthanatized. The eyes to be used for microsphere analysis were enucleated and fixed with 10% formalin for 48 hours. The retina was removed, and the choroid was then flat mounted on slides with 50% glycerol for fluorescence microscopy, which allowed visualization of all the microspheres that had localized in the choroid.

Image Acquisition

Choroidal microspheres were imaged with a fluorescence microscope (Olympus BX-30, Olympus America, Inc.; Center Valley, PA), with 40 $\times$ magnification. Red, green, and blue fluorescence images were acquired with a CCD camera (CoolSNAP; RS Photometrics, Downingtown, PA) and an image-acquisition program (MetaMorph, Universal Imaging Corporation; Downingtown, PA). The red and green fluorescence images were used to image microspheres and were added using ImageJ (<http://rsb.info.nih.gov/ij/index.html>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Individual microscope

fields were stitched together (Photoshop ver. 7.0; Adobe Systems; San Jose, CA). A blue fluorescence image was used to show the location of the lesions. Red and green fluorescence images were overlaid on the blue image and set at an opacity level of 40%, to ensure microsphere visualization.

Microsphere Analysis

In eyes with large lesions, microspheres were counted in equal-sized retinal sections of 0.23×2.31 mm (50×500 pixels on the image), starting from the center of the lesion. Because the lesions were not exactly circular, microsphere distribution was measured along several axes, at approximately 0° (horizontal), 90° (vertical), -45°, and 45°.

Assessing the impact of single small lesions on the choroidal circulation required a different approach. Circles were placed around the lesion edges, and the microspheres in each circle were counted. The average diameters of small lesions were found by selecting the best circular approximation for each lesion. For lesions that were nominally 500 μm in diameter, the average actual diameter was 1.00 ± 0.19 mm ($n = 22$ lesions in three cats); for 200- μm lesions, the average diameter was 0.64 ± 0.09 mm ($n = 22$ lesions in three cats). Circles of these diameters were also placed randomly in nonphotocoagulated regions, and the number of microspheres in 30 control regions was compared to the number in lesioned areas.

Histology

One eye with 500- μm lesions was used to evaluate the retinal damage caused by the lesions. It was fixed immediately after removal in 10% formalin for 24 hours and then in Davidson's fixative for 48 hours. It was rinsed with water for 30 minutes and returned to 10% formalin. The eye was halved at the equator, the vitreous was removed, and the remaining tissue was placed in formalin for 2 hours. It was then dehydrated by immersion in increasing ethanol concentrations and finally in xylene before embedding in paraffin. The globe was sectioned in 5- μm sections every 50 μm and stained with hematoxylin and eosin. The identical procedure was used for histologic analysis of large

FIGURE 1. (A) Histologic section of a portion of a large laser lesion approximately 5 weeks after the lesion (cat 368). The lesion caused loss of the outer retina extending through most of the section, but the inner retina was preserved. Photoreceptors were present outside the edge of the lesion on the right. (B) Histology of a 500- μm lesion (cat 4). Photoreceptors were absent in the lesion and begin to reappear at the lesion edges. The retina was not appreciably thinner in small lesions. Scale bar, 200 μm .

FIGURE 2. Infrared (IR) images (*left*) and corresponding indocyanine green (ICG) angiograms (*right*) of two large lesions (A, B, cat 380), six 500-μm lesions (C, D, cat 4), and seven 200-μm lesions (E, F, cat 388).

lesions in a previous series of cats.¹⁷ Figure 1A shows histology after a large lesion from one of those cats, in which identical laser parameters were used.

Statistical Analysis

A two sample *t*-test with unequal variance was used to determine whether the number of microspheres found in the 500- and 200-μm lesions were significantly different from microsphere counts in non-lesioned regions. Because there were multiple lesions of each size in each animal, a *t*-test was performed on each choroid and each lesion size separately.

RESULTS

The large lesions were made as reported previously.¹⁷ Histology of these lesions revealed that photoreceptors were destroyed (Fig. 1A; also see Fig. 2 of Ref. 17) and the inner retina was closer to the choroid, reducing the thickness of the retina. The ganglion cell and inner nuclear layers were still present, although they were disrupted.

In the 500-μm lesions, the damage to the photoreceptor layers was similar to that seen in large lesions, although not as extensive (Fig. 1B). The tapetal layers were considerably thinner in the lesioned areas, but were not completely obliterated, and the photoreceptors were replaced by a glial scar. The other retinal layers were mostly intact. It was not possible to identify a 200-μm lesion in the available histologic sections, but Budzynski et al.¹⁷ illustrated a 250-μm lesion that has similar properties to the 500-μm lesion in Figure 1B.

Four to 5 weeks after the lesions were made, the choroid was visualized with the SLO. Figure 2 shows examples of IR images of different size lesions (large lesions in Fig. 2A, 500-μm lesions in Fig. 2C, and 200-μm lesions in Fig. 2E). The lesions

appear dark on IR images, which indicates tapetal damage confirmed by histologic analysis (Fig. 1). To visualize choroidal perfusion, ICG angiograms were obtained (Fig. 2B, large lesion; Fig. 2D, 500-μm lesions; and Fig. 2F, 200-μm lesions). The punctate appearance of the choriocapillaris is believed to reflect the distribution of feeder vessels penetrating the tapetum from the larger choroidal vessels.

The choriocapillaris damage was obvious in the large, confluent lesions (Fig. 2B). It exposed the large choroidal vessels, which did not appear noticeably damaged. For the large lesions (Figs. 2A, 2B), the dark regions in the IR images corresponded to a loss of fluorescence from the choriocapillaris in the ICG angiograms.

It was more difficult to detect changes in choroidal vessels in the ICG angiograms of the 500- and 200-μm lesions (Figs. 2D, 2F, respectively). In Figure 2C, a cluster of six 500-μm lesions can be seen in the IR image. In the corresponding ICG image (Fig. 2D), only the lower row shows reduced perfusion in the choriocapillaris. In Figure 2E, a cluster of seven 200-μm lesions can be seen in the IR image, but in the ICG angiogram (Fig. 2F), the choroidal circulation appears normal.

Figure 3 shows color fundus photographs and the corresponding choroidal flat mounts with microsphere distributions. The tapetum was thinned or destroyed in large lesions, and so these areas appeared dark on fundus photographs. From the histologic sections,¹⁷ it appeared that the tapetum in the lesion was lost, and therefore the dark choroidal pigmentation was visible. Bright spots inside some of the larger lesions, as in Figure 3A, probably represented areas in which some tapetum remained. Very few microspheres were present in these lesions (Fig. 3B). Figure 3C shows a retina in which 500- and 200-μm lesions were made. These produced dark burns surrounded by a lighter gray halo in

FIGURE 3. Color fundus images (*left*) and corresponding microsphere distributions in flat-mounted choroids (*right*) after a large lesion (A, B, cat 378) and small lesions (C, D, cat 390). (C, D) The top two rows of lesions are 200 μm; the bottom row is 500-μm. The microsphere composite images shown are at a lower power than was used to make quantitative measurements.

FIGURE 4. (A) Total number of microspheres as a function of distance from the lesion center ($x = 0$), along a single axis in the most superior lesion in cat 378. The values are the number of microspheres in each counting region (adjacent 0.23×2.31 -mm rectangles across the lesion). (B) The number of microspheres per counting area as a function of distance from the edge ($x = 0$) of five large lesions (three in cat 378 and two in cat 380). Each plot shows the mean and SEM along eight directions from the center of the lesion. Logarithmic fits approximately represent the increase in the number of microspheres as a function of distance from the lesion edge.

the fundus photographs. No microspheres were present in the center of these lesions (Fig. 3D).

The quantitative effect of the lesions on microsphere distribution was evaluated differently for large and small lesions. The large lesions were divided into subregions. Because the number of microspheres next to the lesion also appeared to be

reduced, the analysis extended outside the lesioned area itself. Figure 4A illustrates the microsphere count as a function of distance for one of the large lesions along one axis. Virtually no microspheres were found in the lesioned area, and the reduction in microsphere density extended outside the lesion. For each of the five large lesions in two cats, the number of microspheres as a function of distance from the edge of the lesion was averaged over eight different radial directions at intervals of approximately 45° from the edge of the lesion. The result is shown in Figure 4B. These are fitted with a logarithmic function to show the trends. The number of microspheres, and therefore the choroidal blood flow, was affected by the lesion for 1 to 2 mm from the lesion edge.

Smaller lesions were treated by comparing the number of microspheres in lesions to the number of microspheres in nonlesioned areas of the same size. Table 1 compares these counts for the 200- and 500- μ m lesions in three cats. The mean microsphere counts in the lesions were consistently much lower than the mean counts in the nonlesioned areas ($P < 0.005$ in all cases). All but one of these were on the tapetal retina, but the result was the same for a set of 500- μ m lesions on inferior nontapetal retina in cat 388. The ratio of the counts in lesioned areas to nonlesioned areas (Table 1) shows that the choroidal blood flow was markedly reduced, even in the small lesions.

DISCUSSION

PRP is thought to stop or reduce the progression of diabetic retinopathy by relieving hypoxia in the inner retina.^{10,13,16} Direct evidence that inner retinal P_{O_2} increases after photocoagulation was provided by Budzynski et al.,¹⁷ who made intraretinal oxygen measurements in cats after photocoagulation. These measurements reinforced the idea that oxygen from the choroid diffused into the inner retina in the absence of photoreceptors. Lesions in cats were made to mimic human lesions as closely as possible, so that the photoreceptors were destroyed, but the inner retina was largely intact.^{8,9} However, whereas inner retinal P_{O_2} increased, the P_{O_2} at the choroid decreased after photocoagulation to an average of approximately 85% of control, but sometimes much more, which was unexpected.

We hypothesized that the lesions may have damaged the choriocapillaris, and undertook the present study to test this hypothesis. For technical reasons, P_{O_2} measurements were made in large lesions, and so it was important to understand how lesions of that type affected the choroid. The measurements made showed clearly that, when assessed by either SLO ICG angiography or counts of microspheres in the choroid,

TABLE 1. Average Number of Microspheres Found in Lesioned and Nonlesioned Areas

Cat	Lesion Size* (μ m)	No Lesion†	Lesion†	Lesions (n)	Ratio of Lesion to Nonlesion
388	500	8.9 (6.1)	0.8 (1.5)	4	0.08
	200	3.1 (3.0)	1.0 (1.2)	7	0.32
	500	1.8 (1.9)	0.0 (0.0)	6	0.00
389	500	2.0 (2.1)	0.0 (0.0)	6	0.00
	200	1.1 (0.9)	0.0 (0.0)	10	0.00
390	500	8.9 (5.8)	0.3 (0.5)	6	0.04
	200	4.0 (3.5)	0.8 (0.9)	5	0.20

Student's *t*-tests were performed to determine whether the average microsphere counts in the lesions were different from those in 30 randomly selected nonlesioned areas of the same size in the same choroid. The differences were significant for all counts ($P < 0.005$).

* All lesions were made on the tapetal retina, except those in the third row in cat 388, which were in the inferior nontapetal retina.

† Mean microsphere count $\pm$ SD.

photocoagulation essentially eliminated the choriocapillaris. This effect was not confined to the lesioned area, but extended outside of it by at least 1 to 2 mm. With such severe choroidal damage, it may be surprising that choroidal Po_2 was not reduced more than it was. We suggest that the choroidal arterioles and venules, which move closer to the retina after destruction of the tapetum and choriocapillaris, took over the role of retinal oxygenation. Although we could not obtain quantitative measurements of total choroidal blood flow, the SLO images suggest that these larger choroidal vessels were intact, consistent with earlier results.²³

Human PRP is performed with smaller lesions, and so it was important to determine whether the loss of the choriocapillaris was solely the property of large lesions. There is reported to be histologic damage to the choriocapillaris in humans in such lesions.^{9,23,24,28} However, the only measurements of human choroidal blood flow after PRP are, on the surface, not consistent with this damage. Foveal choroidal blood flow, measured with laser Doppler flowmetry, was reported to increase 1 month after PRP.²⁶ Although this is surprising, it is unlikely that the blood flow measurements were localized to the sites of PRP, and the measurements may have included flow in the larger vessels, so they are difficult to interpret in the context of local choriocapillaris function. In animals, histologic measurements have generally shown the same pattern of choroidal damage as in humans, but previous work on cats indicated that the choriocapillaris, after initial damage, was almost healed by 1 month.^{29,30} The only previous measurements of blood flow in animals²⁵ showed decreased blood flow in the retina and choroid of rabbits at times ranging from a few hours to several weeks after photocoagulation and reduced blood flow in monkeys acutely after the photocoagulation. However, the technique used in that study did not separate retinal and choroidal blood flow and did not localize the effects to particular regions.

Because of the limitations of previous measurements in localizing functional changes in the choroid, we used the SLO and microsphere techniques to investigate lesions that were nominally 500 and 200 μm in diameter. Infrared reflectance photographs made with the SLO, standard color fundus photographs, and blue fluorescence imaging showed precisely where these lesions had been made, allowing a correlation of lesion location and microsphere density. Using essentially the same laser parameters and the same recovery time as the previous work on cats (compare Refs. 30 and 17), we found that choroidal damage was still present at 1 month. The SLO images clearly showed filling defects in the choriocapillaris under the 500- μm lesions. No lack of ICG fluorescence could be detected in 200- μm lesions, but microsphere counts were much lower in both the 200- and 500- μm lesioned areas than in randomly selected control regions of the same sizes, in each of three cats. At most, lesioned areas contained 30% of the microspheres in nonlesioned areas, but it was more common to find no microspheres in the lesioned areas. Thus, choriocapillaris blood flow was much lower in all lesioned areas, but the SLO ICG measurements could not resolve this in the smaller lesions.

The severity of choroidal damage could be somewhat different in cats and humans, because the structures absorbing the laser energy are different. In cat, the photoreceptors, tapetum, and choroidal pigment must absorb most of the energy, because the RPE is unpigmented in most of the areas we lesioned. In humans, the melanin in the RPE absorbs a great deal of the energy. However, argon laser parameters similar to those used here have been reported in humans (432 ± 116 mW and 0.2 ± 0.2 second).³¹ In both humans and cats, the energy is absorbed in close proximity to the choriocapillaris and is likely to cause choroidal heating and damage. In one set of 500- μm lesions that we made in inferior cat retina, which is

nontapetal and pigmented, the loss of choriocapillaris was just as striking as that in the tapetal retina, and the histology in humans^{9,23,24} is consistent with choriocapillaris loss. As a result, we suggest that PRP often reduces local choroidal blood flow in humans. Although it would be feasible to make ICG measurements in humans after PRP to compare the present work to the human situation, ICG angiography probably does not have the resolution to detect the small filling defects that would be expected.

Photocoagulation is not always effective in stopping neovascularization,^{1,4,5} for several possible reasons. First, the choroid can be damaged by diabetes itself,³² and so it may have a low blood flow and may be unable to provide enough oxygen for the inner retina. Second, PRP could stimulate choroidal neovascularization,^{33,34} causing additional complications. Ophthalmologists may be able to avoid this effect by reducing laser power. However, the present results suggest that the failure of moderate PRP to stop neovascularization also stems from the damage that PRP causes to the choroid. If choroidal damage leads to a reduced Po_2 , as it does in the larger lesions in cats, then the expected benefit of PRP in elevating inner retinal Po_2 would be reduced or eliminated.

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