Correlation of Histologic 2-Dimensional Reconstruction and Confocal Scanning Laser Microscopic Imaging of Choroidal Neovascularization in Eyes With Age-Related Maculopathy

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Objective: To topographically localize vascular channels, macrophages, and retinal pigment epithelium and other components of choroidal neovascularization (CNV) associated with age-related maculopathy.

Methods: Two postmortem eyes with age-related maculopathy and CNV were evaluated. The formalin-fixed CNV complex was excised and processed for confocal scanning laser microscopy including immunostaining for factor VIII–related antigen and incubation with Ig fluorescein isothiocyanate. After confocal microscopy, the specimens were serial step sectioned, stained, and 2-dimensional topographic reconstructions were made. The confocal images were compared with the 2-dimensional reconstructions.

Results: Both specimens contained central disciform scars surrounded by areas of intact retinal pigment epithelium. The first specimen was more atrophic and contained fewer choroidal neovascular channels than the second specimen. The topographic arrangement of the CNV and retinal pigment epithelial changes in the confocal images corresponded with the 2-dimensional reconstructions. Macrophages were concentrated around areas of vascularization.

Conclusion: Confocal scanning laser microscopy of excised CNV simulates fluorescein angiography and topographic localization of the components of CNV provides insight into the pathogenesis of CNV.

Arch Ophthalmol. 2000;118:625-629

AGE-RELATED maculopathy (ARM) is the leading cause of irreversible blindness in the United States. The neovascular form of ARM, choroidal neovascularization (CNV), occurs in at least 0.6% to 0.7% of the population.1,2 The pathological characteristics of CNV in ARM have been studied in postmortem eyes that were untreated3-7 or treated with laser photocoagulation8,9 or surgery.10 Surgically excised CNV specimens in patients with age-related macular degeneration have been examined by light microscopy, electron microscopy,11 and with immunohistochemical staining.12 Two-dimensional reconstructions of the histologic findings of CNV in postmortem eyes8,9 and surgically excised CNV13 have been described. The 2-dimensional reconstructions have been compared with fluorescein angiograms performed before enucleation.8,9 Some studies have compared Macular Photocoagulation Study fluorescein angiographic “classic” and “occult” patterns of CNV14 with the histopathologic findings in surgically excised CNV15 and 2-dimensional reconstructions.10

One problem with comparison of fluorescein angiographic findings with histologic 2-dimensional reconstructions is that there is often a prolonged interval between the angiogram and 2-dimensional reconstruction. This may be partially circumvented by using an adenosine diphosphatase technique,17 in which histologic and topographic findings may be compared. Another method of obtaining topographic images of tissue sections is confocal scanning laser microscopy.18 In this study, we examined CNV in 2 eyes with ARM by confocal scanning laser microscopy and histologic 2-dimensional reconstruction. We determined the vascular, retinal pigment epithelium (RPE), and macrophage distribution in the CNV. We chose to determine the RPE and macrophage distribution since these 2 cell types are often found in CNV specimens15,19,20 and may be involved with the topographic orientation of the CNV.21

The first pair of eyes were obtained from an 84-year-old woman. Histologic examination of the right eye showed basal laminar deposits, soft drusen, calcified drusen,

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MATERIALS AND METHODS

EYE BANK EYES

Two pairs of formalin-fixed eyes obtained from the Georgia Lions Eye Bank, Atlanta, were studied. Historical data other than patient age and sex were not available. The eyes were grossly examined and determined to have ARM according to the Alabama age-related macular degeneration grading system. These eyes had pigment clumping 500 µm in diameter or larger and at least one drusen larger than 125 µm. The right eye of each pair was routinely processed and histologically determined to have ARM and CNV. Under the direction of a dissecting microscope, the grossly visible CNV complex of the left eye of each pair including a 7 × 7-mm section of the retina centered around and including the macula and adherent underlying tissue was dissected and immersed in phosphate-buffered saline (PBS). The topographic orientation of the CNV complex was maintained during the dissection, confocal microscopy, and histologic 2-dimensional reconstruction.

CONFOCAL SCANNING LASER MICROSCOPY

The excised CNV complex was permeabilized with 0.2% Triton X for 4 hours, blocked with 5% goat serum in 0.2% Triton X for 30 minutes, and washed 3 times and soaked in PBS for 1 hour. The CNV complex was incubated for 12 hours with the primary antibody (factor VIII, 1:100; Dako, Carpente- ria, Calif) in PBS. The secondary antibody (Ig fluorescent isothiocyanate, 1:100; Dako) was applied for 1 hour and the tissue was washed and soaked in PBS. Confocal microscopy was performed using excitation and emission filters of the appropriate wavelength for fluorescein isothiocyanate labeling. Serial step confocal scanning sections at 10-µm increments were obtained from the inner (retinal) surface to the outer (choroidal) surface of the specimens and 3-dimensional reconstruction was performed with a confocal scanning laser microscope (Biorad MRC 1024; Biorad Inc, Hercules, Calif).

2-DIMENSIONAL RECONSTRUCTION

After confocal scanning microscopy, the specimens were wrapped in tissue paper with their orientation marked on the paper and submitted in 4% neutral buffered formalin for histologic processing through increasing concentrations of alcohol, cleared with xylene, and embedded in paraffin with the orientation maintained. Serial step sections were obtained every 200 µm for the first specimen and every 50 µm for the second specimen. Alternate slides were stained with hematoxylin-eosin and periodic acid–Schiff. Representative sections from the centers of the specimens were immunostained for the macrophage marker CD68 (1:100; Dako). The histologic findings were recorded and the CNV, choroidal vessels, macrophages, RPE defects, breaks in Bruch membrane and disciform scarring were identified and mapped using the known orientation of the specimen and a microscopic reticule.

RPE atrophy, photoreceptor atrophy, and CNV. Confocal scanning laser microscopy of the excised CNV complex in the left eye showed a central clear area traversed by large choroidal vessels and scattered delicate vessels peripherally (Figure 1). Histopathologic findings in the CNV complex included photoreceptor atrophy and CNV within Bruch membrane and between the RPE and neurosensory retina forming a disciform scar. The disciform scar was present centrally, where there were basal laminar deposits, overlying atrophy of the RPE and underlying large choroidal vessels (Figure 2). The RPE was intact peripherally, where there were 2 defects in Bruch membrane (Figure 3). Macrophages confirmed by CD68 positivity and giant cells were present (Figure 4). The 2-dimensional reconstruction showed central diffuse atrophy of the RPE overlying the disciform scar (Figure 5). Large choroidal vessels, as seen in the confocal image, were present in the central area underlying the disciform scar. Foci of vascular channels were present near the periphery of the disciform scar. Macrophages were concentrated nasally and in the area of the large choroidal vessels.

The second pair of eyes were from an 82-year-old man. Histologic examination of the right eye showed CNV...
between the neurosensory retina and Bruch membrane with atrophy of the overlying photoreceptor cell layer. Scattered drusen were present and basal laminar deposits were identified. Confocal scanning laser microscopy of the excised CNV complex from the left eye showed a clear circular area centrally with tufts of vascular channels in the center of the circular area (Figure 6). Peripherally, there were numerous tufts of vascular channels and there was diffuse staining for factor VIII. Histologic examination showed a central disciform scar with associated basal laminar deposits and atrophy with associated acinar configurations of RPE and atrophy of retinal photoreceptors (Figure 7). The disciform scar contained components between the RPE and Bruch membrane and between the RPE and neurosensory retina. The RPE and overlying photoreceptors were intact in the peripheral portion of the specimen (Figure 8). Macrophages confirmed by CD68 positivity and giant cells were present (Figure 9). Unlike the first specimen, choroidal tissue was not present. The 2-dimensional reconstruction showed that the CNV was concentrated in the center of the disciform scar and more diffusely scattered under the intact RPE (Figure 10). Macrophages were concentrated around the vascular channels.

Comparison of the 2-dimensional reconstruction and confocal image in the first case (Figures 1 and 5) showed that large choroidal vessels were present centrally underlying a thin disciform scar and the RPE was intact surrounding the central disciform scar, thus partially obscuring visualization of underlying structures in the confocal image. The choroidal neovascular channels were centrally absent and present along the periphery of the disciform scar. Comparison of the 2-dimensional reconstruction and the confocal image in the second case (Figures 6 and 10) showed that the choroidal neovascular channels were concentrated in the center of the disciform scar and diffusely present under the intact RPE peripherally. There were more macrophages and more CNV channels in the second than in the first specimen.
COMMENT

The pathological characteristics of CNV in eyes with ARM have been described in surgical and postmortem specimens. These studies have shown that the neovascularization arises in the choroid, traverses defects in Bruch membrane, and is associated with the presence of basal laminar deposits. Although the presence of basal linear deposits may be a specific marker for ARM, basal linear deposits are only detectable by electron microscopic examination and are likely included in what is identified by light microscopy as thick basal laminar deposits. Correlations between the topography of CNV in eyes with ARM and fluorescein or indocyanine green angiography have been made. These studies have shown that CNV is clinically underrecognized, “classic” CNV is surrounded by a demarcating rim of hyperplastic RPE, and “occult” CNV is diffusely present between the RPE and Bruch membrane. It has been postulated that the histologically determined location of the CNV between the RPE and Bruch membrane (type 1 pattern) or between the neurosensory retina and RPE (type 2 pattern) corresponds to the preoperative clinical fundus appearance.

Various growth factors including fibroblast growth factors (FGF) aFGF and bFGF, transforming growth factor β, and vascular endothelial growth factor have been demonstrated in surgically excised CNV. The RPE in CNV expresses vascular endothelial growth factor and transforming growth factor β. Factor VIII highlights vascular endothelium in surgically excised CNV. Bynoe and coworkers used factor VIII immunostaining to 2-dimensionally reconstruct surgically excised CNV specimens, of which were from patients with ARM. Those authors found a nonuniform distribution of blood vessels and large avascular areas in the membranes. We found a disciform scar with a central avascular area in our first case and a disciform scar with a centrally located vascular channels in our second case.

Histologic, ultrastructural, and immunohistochemical studies have shown that macrophages are a major component of CNV. These macrophages, presumably derived from the choroid, are characteristically in the stroma...
of the CNV and express tumor necrosis factor α.\textsuperscript{21,30} We have postulated that CNV is a dynamic process with sequential initiation, maintenance, and involutional stages and that macrophages play an important role in the evolution of CNV.\textsuperscript{21} The fluorescein angiographic topographic classic vs occult and fundus topographic appearance of type 1 vs type 2 may be established during the maintenance stage of CNV evolution, with the pattern of macrophage distribution correlating with the topographic appearance of the CNV.\textsuperscript{21} There is experimental evidence that macrophages produce tumor necrosis factor α, which stimulates macrophage colonization protein-1 production by RPE and macrophage colonization protein triggers adhesion of moving monocytes to vascular endothelium, thus perpetuating the maintenance stage of CNV development.\textsuperscript{31,32}

Since there is often a prolonged interval between the most recent fluorescein or indocyanine green angiogram and histologic evaluation of a postmortem eye, we attempted to simulate an angiogram by staining the CNV complex for factor VIII and evaluating the specimen by confocal scanning laser microscopy. In addition to evaluation of the topographic location of the blood vessels in the CNV, we identified the topographic location of macrophages. In both of our cases, there was RPE atrophy in the center of the specimen in the area of the disciform scar. The disciform scar was surrounded by intact RPE. Our first specimen was more atrophic than the second, with the CNV only present in the periphery of the disciform scar and present to a lesser extent than in the second specimen. The topographic location of the CNV demonstrated by confocal microscopy correlated to the location shown in the 2-dimensional reconstruction in both cases. Macrophages were present in close proximity to choroidal vessels in our first case and the neovascular channels in both cases, although fewer were present in our first compared with our second specimen. This is consistent with macrophage migration into the CNV from the choroid and greater involutional changes in the first than the second case, suggesting that our first case was in the involutional stage and our second case was in the maintenance stage.

Our study demonstrates that the topography of vascular channels in postmortem eyes with CNV may be determined by confocal scanning laser microscopy and this topography correlates with histologic 2-dimensional reconstruction. Confocal scanning laser microscopy may be applied to surgically excised CNV and compared with 2-dimensional reconstruction, thus allowing for examination of CNV with less involutional change than in eye bank eyes.

Accepted for publication November 27, 1999.

This work was supported in part by an unrestricted departmental grant from Research to Prevent Blindness Inc., New York, NY, and departmental core grant EY06030 and NIH U10-12662 from the National Institutes of Health, Bethesda, Md.

We would like to thank the Georgia Lions Eye Bank, Atlanta, for providing eyes for this study.

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