Extensive Deposits of Complement C3d and C5b-9 in the Choriocapillaris of Eyes of Patients with Diabetic Retinopathy

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PURPOSE. To determine the presence of activated complement components in eyes affected by diabetic retinopathy.

METHODS. Eyes of 50 deceased donors with diabetic retinopathy and of 10 nondiabetic subjects with uveal melanoma (n = 6) or phthisical eyes (n = 4), as well as eyes of 16 deceased donors without diabetic retinopathy were subjected to immunohistochemical studies, using a panel of antibodies directed against candidate markers of complement activation.

RESULTS. Extensive deposits of complement C5b-9 complexes were detected in the choriocapillaris immediately underlying the Bruch membrane and densely surrounding the capillaries, in all 50 diabetic retinopathy specimens. Complement deposition was sometimes also observed around the larger choroidal vessels. Similarly intense staining for C5b-9 was absent in 25 of the 26 of the other donor eyes. Positive staining was observed in a case of systemic amyloidosis. Staining for C3d positively correlated with C5b-9 staining, corroborating the notion that complement activation had occurred in situ. Furthermore, positive staining was found for vitronectin, which forms stable complexes with extracellular C5b-9. When present, deposits under the pigment epithelium and drusen also stained positively for the activated complement components, independent of diabetic retinopathy. In contrast, there was no positive staining for C-reactive protein (CRP), mannose-binding lectin (MBL), C1q, or C4, indicating that complement activation did not occur through a C4-dependent pathway.

CONCLUSIONS. The presence of C3d, C5b-9, and vitronectin indicates that complement activation occurs to completion, possibly through the alternative pathway in the choriocapillaris in eyes affected by diabetic retinopathy. Complement activation at this site may evoke a spectrum of pathologic sequelae that could contribute to ocular tissue disease and visual impairment. (Invest Ophthalmol Vis Sci. 2002;43:1104–1108)

Diabetic retinopathy is among the leading causes of loss of visual function in middle-aged patients in industrialized nations and poses a number of important problems with regard to prophylaxis and therapy.1–4 From the very beginning, diabetic retinopathy is usually accompanied by thickening of the Bruch membrane, by the occurrence of PAS-positive deposits at this site, and by pathologic alterations in the choriocapillaris that have collectively been alluded to as diabetic choriodopathy or choriocapillaris degeneration.5–9 In some cases, visual impairment appears to be related solely to choriocapillaris degeneration, in the absence of detectable retinopathy.9 Choriocapillaris degeneration is characterized by loss of viable endothelial cells, as evidenced by reduction of alkaline phosphatase activity in the choriocapillaris. The increase in number of degenerative capillaries has been reported to correlate with increased thickness of basal laminal deposits in the Bruch membrane.9

The events leading to choriocapillaris degeneration are essentially unknown. If a gradual loss of endothelial cell viability occurs, as indicated by the report of Cao et al.,10 we reasoned that noxious processes might occur in their immediate vicinity. The absence of cellular infiltration in the choriocapillaris led us to focus our attention on humoral cytotoxic effectors. We were surprised that a literature search failed to disclose any existing immunohistochemical study on the presence of activated complement components in diabetic eyes. Herein, we report that complement activation occurred extensively and to completion in the choriocapillaris of donor eyes with diabetic retinopathy. Similar deposits were not found in 25 of 26 bulbi of eyes without retinopathy. Complement activation in the choriocapillaris may be one trigger of pathophysiological processes leading to choriocapillaris degeneration.

METHODS

Eyes (all pseudophakic) of 50 diabetic donors (mean age at death, 76 years) used in this study were most kindly supplied by David J. Apple (Storm Eye Institute Ophthalmology, Medical University of South Carolina). The decisive inclusion criterion was overt presence of diabetic retinal changes. The specimens represented a collection acquired from eye banks throughout the United States, and all bulbi had been preserved in 4% formaldehyde. The anterior segments had been cut away at the equator for analysis of the intraocular lenses. The primary diagnoses at death were: cardiovascular disease (n = 31), neurodegenerative disease (n = 6), metabolic syndrome (n = 4), genitourinary tract disease (n = 4), pulmonary disease (n = 3), and neoplasia (n = 2). Diabetic retinal changes had been diagnosed in all donors. Further information was not available.

As a control, 26 eyes of donors without diabetic retinopathy were examined. In six, enucleation was performed because of uveal melanoma. Four additional eyes had been enucleated because of phthisis. Sixteen eyes were from deceased donors (age range, 61–92) with neurodegenerative disease (n = 5), coronary heart disease (n = 5), sepsis (n = 1), HIV infection (n = 1), systemic amyloidosis (n = 1), or disease of unknown diagnosis (n = 3). Five of the patients had had type II diabetes mellitus. The decisive criterion for selection of these negative control specimens was the absence of overt diabetic retinal changes. These 16 deceased donor specimens stemmed from a collec-

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tion from the Department of Neuropathology, University of Mainz, and the bulbi had similarly been preserved in 4% formaldehyde for periods of months to years. The postmortem delay was 18 to 36 hours for these specimens. Information on the postmortem times of the specimens from the United States was not available. However, the bulbi from the United States showed no increased signs of autolysis that would have been expected with increased postmortem delay.

Freshly obtained bulbi were fixed in 4% paraformaldehyde and were then transferred to 100 mM sodium cacodylate, rinsed, and embedded in paraffin. Deceased donor specimens had been preserved in 4% formaldehyde. They were embedded in paraffin and the tissues were sectioned to a thickness of 6 to 8 μm. Immunolabeling was performed by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method using a commercially available kit from Vector Laboratories (Burlingame, CA) according to the manufacturer’s instructions. Counterstaining was with hematoxylin.

Polyclonal antibodies against C4c, C3d, and C1q were obtained from Dako (Carpinteria, CA) and used at working dilutions of 1:2000 to 1:3000. Murine monoclonal antibodies against CRP (clone CRP-8, IgG1) were from Sigma (Deisenhofen, Germany) and against mannose-binding lectin (MBL, clone 131-01) were from the State Serum Institute (Copenhagen, Denmark); monoclonal antibodies against C5b-9 and anti-vitronectin antibodies were kindly supplied by K. Preissner (University of Giessen, Giessen, Germany). Monoclonal antibodies were used at working dilutions of 1:300 (C5b-9, MBL), 1:500 (vitronectin), and 1:2000 (E-LDL).

Negative controls included replacement of the primary antibody by phosphate-buffered saline or an irrelevant isotype-matched monoclonal antibody (directed against Aspergillus niger glucose oxidase, clone DAK-GO-1, IgG1; Dako).

Specimens of coronary arteries with atheroma were from the Institute of Medical Microbiology, University of Mainz, and were used as the positive staining control for C-reactive protein, C3d, C5b-9, and modified LDL.10 Stainings for these molecules were always positive in the atheromas.

RESULTS

Only the posterior poles of diabetic eyes were available for study, and in most cases the retinas were detached and could not be examined. We performed immunohistochemical stainings for CRP, C3d, C5b-9, and modified LDL in six retinal specimens. Apart from occasional positive stainings for C3d and C5b-9 in the walls of retinal arteries, no conspicuous and consistent findings were made. The following report is confined to a description of findings made in the subretinal tissues.

Twenty-five of 26 eyes of donors without diabetic retinopathy showed negative immunohistochemical findings with the panel of antibodies used in the study. Figures 1A and 1B depict representative sections stained with antibodies against C3d and C5b-9, respectively. In eyes of older deceased donors (aged 61–92 years), weak staining for C5b-9 was occasionally observed in the choriocapillaris. A typical example is shown in Figure 2 (top). Whenever present, subpigment epithelium deposits (drusen) showed intense positive staining (Fig. 2, bottom). In the absence of diabetic retinopathy, however, staining of the underlying choriocapillaris remained essentially negative.

Evidence for massive complement activation in the choriocapillaris was obtained in a single control specimen from a donor with systemic amyloidosis. The staining pattern was essentially as found in the eyes with diabetic retinopathy, as described in the following.

Intense positive staining for C5b-9 complexes was noted without exception in all 50 eyes with diabetic retinopathy. Staining was strikingly focal and quite stringently localized and restricted to the choriocapillaris immediately underlying the Bruch membrane (Fig. 3). Staining was most intense immediately around the capillaries and did not extend into the pigment epithelial layer. Occasionally, complement deposits were also observed surrounding larger vessels (Fig. 3). The extensive, dense deposition of C5b-9 in the connective matrix of the choriocapillaris is shown at higher magnification in Figures 4 and 5. Most of the specimens examined were from the extramacular regions. A direct comparison between the patterns and extent of complement deposition in the macular versus extramacular regions was not undertaken. Whenever present, subpigment epithelium deposits and drusen on the Bruch membrane also stained strongly positive for C5b-9 (not shown). It is noteworthy that the endothelium and pigment epithelium appeared exempt from staining. Staining of the Bruch membrane could not be definitively assessed, because positivity often appeared to represent an artifact due to over-intense staining of the underlying tissue. On occasion, however, staining appeared to be genuine (Figs. 3, 4).

Immunohistochemistry with all other antibodies was performed on a smaller number of specimens (n = 20 for C3d) that were selected at random. Stainings for C3d were also strongly positive and found without exception in all bulbi from eyes with diabetic retinopathy. Staining for C3d was also localized and restricted to the choriocapillaris underlying the Bruch membrane.
membrane and did not extend to the pigment epithelial layer (Fig. 4B). Staining for vitronectin was similarly positive in all specimens and showed a similar localization as that of C5b-9 (Fig. 5). In contrast, there was never any positive staining for C1q or C4, early components of the classic complement pathway. We also noted no staining for CRP or MBL, potential activators of C1q and C4, respectively. Finally, we used an antibody directed against enzymatically degraded LDL (E-LDL), because this lipoprotein derivative is responsible for complement activation in atherosclerotic lesions.10 However, staining for E-LDL was always negative. These negative results at the same time excluded that the stainings for C3d, C5b-9, and vitronectin might have represented artifacts. Omission of the primary antibodies led to disappearance of immunolabeling in all control specimens.

**DISCUSSION**

Pathologic processes of noninfectious and nontumor origin are often mediated by perturbations in the homeostasis of the immune system. Diabetic choroidopathy is characterized by the presence of increased numbers of polymorphonuclear granulocytes in degenerative capillaries and by the appearance of protein deposits that possibly reflect endothelial leakage with insudation of plasma components to the Bruch membrane. Complement activation in the immediate vicinity of the capillaries could provoke such pathologic alterations.

The most direct method for determining whether complement activation has occurred to completion is to stain tissues for the presence of the assembled membrane attack complex C5b-9. Antibodies specific for C5b-9 complexes are directed against neoantigens that become exposed when terminal complex assembly occurs.11 The plasma concentrations of C5b-9 are essentially negligible, so the presence of C5b-9 can be equated with in situ complement activation. As an obligatory accompanying process, inflammatory mediators will be generated along the entire cascade.12

It was therefore not surprising that extensive complement deposition was not seen in 25 of the 26 control bulbi. On occasion, sporadic deposits were observed, but never to the same extent as seen in cases of diabetic retinopathy. Of distinct interest was that the characteristic staining of the choriocapillaris was also not observed in five eyes with type II diabetes mellitus but without severe retinopathy. It was also noteworthy that positive staining of drusen for complement components, as has been reported in the previous literature,13,14 occurred independent of choriocapillaris staining that was characteristic of diabetic retinopathy.

The finding that extensive C5b-9 deposition occurs in the choriocapillaris of patients with diabetic retinopathy bears high potential relevance. As was expected, C3d, the final C3 cleavage product that remains bound to the activation sites,12 was also detected. Furthermore, there was extensive codeposition of vitronectin, which is known to bind to C5b-9 complexes at the site of their generation. Once formed, C5b-9
complexes are long-lived, because they resist degradation by proteases. C5b-9 complexes have been detected in a large variety of diseased tissues (e.g., in atheromas, infarcted myocardium, and age-related and immune diseases of skin, muscle and joints, neural tissues, and kidney). In many cases, C5b-9 deposition has been found to bear relevance to the pathogenesis of the respective disease. Most recently, C5b-9 has been reported to be present in drusen associated with aging and age-related macular degeneration, in accord with the finding that C5 and vitronectin were present at these sites. We confirmed these findings in the current study.

Possibly in direct context with the present report, C3d and C5b-9 deposition has been shown to occur in endoneural microvessels of diabetic neuropathy. At this stage, it is not possible to speculate whether complement activation in the choriocapillaris is a cause or result of diabetic retinopathy. In any event, it may be that complement activation at this site represents an element that contributes to the vicious circle of events underlying progression of the disease. Thus, complement activation may provoke detrimental effects in neighboring cells through several mechanisms. Complement anaphylatoxins attract and activate neutrophils, and, indeed, increased numbers of neutrophils are present in diabetic choriocapillaris. Activated neutrophils may incur damage to the endothelium, accentuating choriocapillaris degeneration and augmenting insudation of plasma components into the connective matrix. Continued complement activation may stimulate bystander cells to provoke de novo synthesis of extracellular matrix, which would contribute to thickening of the choriocapillaris and the Bruch membrane. Deposited C5b-9 is apparently mainly extracellular and is thus complexed to vitronectin. Antibodies directed against clusterin, a second important component of extracellular C5b-9 complexes, were not available to us, but it is highly likely that clusterin is also present at these sites. With time, protein and lipid deposits possibly accumulating through choriocapillaris leakage may attain sufficient density to impede permeation of molecules to and from the pigment epithelium.

There have been a number of investigations into the expression and pathologic distribution of vitronectin in the normal and diabetic retina. Complement components were not sought in those studies, and it will be of interest to determine whether or how they relate to our present findings regarding the presence of complement activation products in the choriocapillaris.

A central question relates to the cause of complement activation. Our negative stainings for CRP, MBL, C1, and C4 essentially eliminate the participation of classic or related pathways involving C1 and C4. Alternative pathway activation therefore appears most likely, and a search is now under way to discover the trigger. It is of interest that transcripts encoding C3 and terminal complement components are synthesized by the retinal pigmented epithelium and choroid and that chronic low level activation probably occurs within the eye that is controlled by intraocular complement regulatory pro-

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933593/)

**Figure 4.** Diabetic choriocapillaris stained for C3d (A) and C5b-9 (B). (A) C3d-staining is mostly confined to the choriocapillaris (cc); (B) C5b-9 deposits appear somewhat more extensive and are also dispersed throughout the choroid (ch). There is also faint staining in the vicinity of larger scleral vessels (v). Arrowhead: retinal pigment epithelium. s, sclera. Original magnification, ×200.

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933593/)

**Figure 5.** Patterns of vitronectin (top) and C5b-9 (bottom) deposition in the diabetic choriocapillaris. Both antigens were mainly confined to the pericapillary matrix.
teins. Any disturbance in the homeostasis of these events could lead to gradual accumulation of activation products. Elucidating the mechanisms involved in complement activation at this unusual site may ultimately improve our understanding of the pathogenesis of diabetic eye disease. Studies are also called for to determine whether similar complement activation processes occur in other ocular diseases. Our observation that a similar pattern of deposition was found in the eye of a donor with systemic amyloidosis renders it evident that the present findings, although highly characteristic of diabetic retinopathy, may also be encountered in other diseases.

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