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Macular and peripheral distribution of ICAM-1 in the human choriocapillaris and retina

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Purpose: In order to understand the extent of choriocapillary endothelial cell activation in different topographic regions of the eye, we sought to compare the localization of intercellular adhesion molecule-1 (ICAM-1) in macular and peripheral regions of human eyes.

Methods: Sections of sucrose-embedded human donor eyes that included the macula and ora serrata were evaluated for ICAM-1 and ICAM-2 immunoreactivity with monoclonal and polyclonal antibodies. Patterns of ICAM-1 labeling in peripheral and macular regions were examined in 20 eyes. Morphometric analyses of anti-ICAM-1 labeling intensity in the choriocapillaris were performed using ImageJ software on a series of macular and extramacular punches from nine eyes. Quantitative PCR analysis for ICAM-1 mRNA was performed on the RPE-choroid from the same regions from six of the same eyes, and Western blots of samples treated or untreated with N-glycosidase were performed to compare retinal and choroidal ICAM-1.

Results: ICAM-1 labeling of the choriocapillaris was typically more intense in the macula than in the peripheral choroid in human donor eyes (14/20). ICAM-2 was also detected in the choriocapillaris and retinal vessels. Morphometric measurements confirmed a significant macular-extramacular difference in ICAM-1 in six of nine eyes ($p<0.05$), with 1 of 9 eyes showing the opposite pattern. This pattern was not noted for endogenous alkaline phosphatase or ICAM-2. The opposite pattern was noted in the external limiting membrane (ELM), which exhibited more intense ICAM-1 labeling in the far periphery than in the macula. On Western blots, choroidal ICAM-1 exhibited a greater molecular weight than the retinal form, with most of the apparent weight difference due to N-linked carbohydrate chains.

Conclusions: The regional differences in ICAM-1 distribution in the choriocapillaris may indicate that this region is subject to increased leukocyte trafficking. In view of the role of inflammatory processes in age-related macular degeneration (AMD), we propose that the higher level of ICAM-1 protein in the macular choriocapillaris may impart greater susceptibility of the macula to immune cell-mediated damage in AMD.

Age-related macular degeneration (AMD) is a blinding disease, affecting millions of elderly individuals. In the most severe manifestation of AMD, blood vessels grow from the choroid into the subretinal pigment epithelial (subRPE) and subretinal space, leading to severe visual loss that can be sudden and permanent.

Recent data have emerged that show inflammatory processes are active in eyes with AMD. Data for the role of inflammation in this disease include the identification of cells of monocytic origin associated with Bruch's membrane of the eye and present in neovascular lesions in AMD [1-3], autoantibodies in the serum of AMD patients that recognize cells in the retina [4,5], elevated levels of C-reactive protein [6], a polymorphism in the complement-regulatory gene complement factor H [7-10] and inflammatory mediators as components of drusen [11-13], the hallmark lesions of early stage AMD. Human patients with specific immunological disorders affecting the kidneys in some cases also develop drusen similar to those observed in early AMD [14-17]. In addition, animal models for some aspects of AMD support the notion that inflammation may be important to the progression of this disorder. A mouse model that exhibits early and late AMD-like pathology has been described in which deficiencies in monocyte chemotactic protein-1 (MCP-1) or its cell surface receptor both lead to this phenotype [18]. Another area of support for the concept that inflammatory processes contribute to the development of AMD is that mice-specific deficiencies in immune function, such as those lacking monocytes [19,20], with genetically or experimentally impaired complement function [21], or lacking leukocyte-endothelial adhesion molecules [22], are relatively resistant to experimental choroidal neovascularization. These findings suggest that, at least in this mouse model, inflammatory molecules participate in the pathogenesis of choroidal neovascularization (CNV).

Intercellular adhesion molecule-1 (ICAM-1 or CD54) is a single pass transmembrane cell surface protein with five immunoglobulin superfamily domains. Like many adhesion molecules, ICAM-1 is distributed on endothelial cells (ECs) and leukocytes, and participates in the recruitment of leukocytes to sites of tissue injury and inflammation. In contrast to ICAM-2 (CD102), a related Ig superfamily EC surface protein, ICAM-1 does not show a static level of expression, but is upregulated or downregulated depending on conditions in the microenvironment [23].

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The level of expression of endothelial activation markers, such as ICAM-1, regulates the degree of leukocyte recruitment in inflammation. Endothelial ICAM-1 expression increases in response to a variety of different stimuli [24-29]. Based upon the apparent contribution of inflammatory processes to macular diseases, including the recruitment of monocytic cells to the choroid during the progression of AMD, it is feasible that molecules such as ICAM-1 play an important role in the pathogenesis of this disease.

Previous studies have described the distribution of ICAM-1 in the choroid and retina of human eyes [30], including eyes with diabetic retinopathy in which ICAM-1 expression is increased [31]. ICAM-1 has also been noted in choroidal neovascular membranes (CNVM) [32]. Little is known about the topographic distribution of this protein, however.

Topographically, the pathology of AMD is confined to a discrete region of the central retina termed the macula. It is not clear why the macula is at greater risk for the degenerative changes in AMD, including neovascularization. In light of the evidence for inflammatory events in AMD, differences in the expression of inflammatory mediators between the macular and extramacular regions of the eye might reasonably be expected to play a role in the relative susceptibility of the macula.

In the current study, we describe the topographic localization of ICAM-1 in human eyes. The observed labeling is frequently greater in the macular, as compared with extramacular, choriocapillaris vessels. This pattern is in contrast to the distribution of this adhesion molecule in the external limiting membane (ELM), which shows increased labeling from the macula to the far periphery. Quantitative PCR did not consistently show an increased level of ICAM-1 mRNA in macular punches of the RPE-choroid, suggesting that the increase in macular ICAM-1 is specific to the choriocapillaris, and not an overall difference in ICAM-1 expression by the RPE or other choroidal vessels. In addition to showing different distributions, retinal and choroidal ICAM-1 proteins were found to be of differing molecular weight, attributable to differences in N-linked oligosaccharides.

METHODS

Donor eyes and histology: Human donor eyes were obtained, following informed consent, from the Iowa Lions Eye Bank (Iowa City, IA). Eyes were dissected and photographed grossly. For morphometric and molecular experiments, processing of eyes was completed with an average time of 5.8 h (with a range of 5.3 to 6.3 h) after death. For nonquantitative morphological experiments, 20 eyes were utilized and the superotemporal quadrant was fixed in 4% paraformaldehyde, diluted in 1X phosphate-buffered saline (PBS), for 2-4 h. Sagittal wedges were then infiltrated in sucrose [33], prior to embedment in optimal cutting temperature compound and freezing in liquid nitrogen. Blocks were stored at -80 °C until sectioned. Sections (7 μm) were collected using a Microm H505E cryostat and were mounted on Superfrost plus slides (Ted Pella, Redding, CA). For morphometric analysis of ICAM labeling, 4 mm punches were collected from the macular and extramacular retina and RPE/choroid from nine donor eyes as depicted in Figure 1. Macular (T1, spanning from 2-6 mm from the foveal center) and extramacular (T3, 10-14 mm from the fovea) tissues were collected for analysis. In six eyes, these punches were adjacent to those used for either real time PCR or Western blotting or both (see below). Macular and extramacular punches were processed for light microscopy as above.

Immunohistochemistry: For immunohistochemical labeling, we utilized a mouse monoclonal anti-ICAM-1 antibody from the Developmental Studies Hybridoma Bank at the University of Iowa (clone P2A4) at a concentration of 2.7 μg/ml. Specificity of the antibody was confirmed by pre-incubating the monoclonal antibody with a ten fold excess of recombinant ICAM-1 (R & D Systems, Inc., Minneapolis, MN) for 10 min. In addition, a monoclonal antibody directed against ICAM-2 (Chemicon, Temecula, CA) was used for some experiments at a concentration of 25 μg/ml. Immunohistochemistry for ICAMs was performed using either immunofluorescence or colorimetric detection. Immunofluorescence was performed as described elsewhere [12]. For colorimetric detection, sections were blocked in 1% horse serum in PBS, and incubated with the diluted primary antibody in this same solution for 1 h in a humidified environment. Sections were then washed three times for 5 min in PBS, followed by incubation in the appropriate biotinylated secondary antibody (Vector Laboratories; Burlingame, CA) for 30 min. Following three 5-min-washes in PBS, the avidin-biotin-peroxidase complex was applied for 30 min. Sections were then washed three times for 5 min in PBS and incubated in the Vector VIP peroxidase substrate. For morphometric analyses (as follows), sections

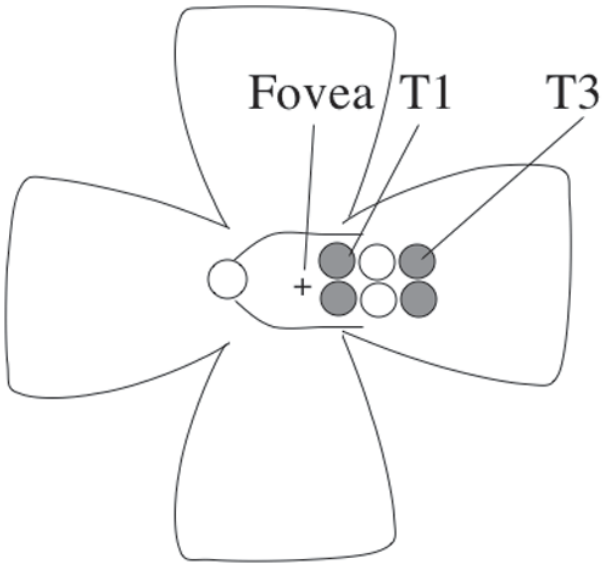

Figure 1. Collected areas of 4 mm punches from the temporal quadrant used for morphometric and molecular analyses. The RPE-choroid layer was collected from the indicated regions and was used for ICAM-1 immunohistochemistry and isolation of RNA or protein. The macular T1 and extramacular T3 punch were primarily used for these studies.

from different regions of human eyes were developed for the same length of time. Following formation of a purple reaction product, sections were washed for 5 min in running distilled water and were then dehydrated in alcohol and Clear Rite reagent (Richard Allan; Kalamazoo, MI) prior to coverslipping in Permount (Fisher Scientific, Pittsburgh, PA).

Immunoreactivity of ICAM-1 and ICAM-2 was also assessed in sucrose-embedded sections from two eyes with histopathologic evidence of CNV secondary to AMD. Sections were also labeled in some cases with peanut agglutinin, as described previously [34].

In order to confirm that macular-peripheral differences in ICAM-1 labeling were not due to a paucity of peripheral vessels, for some experiments adjacent serial sections were collected and stained for either (1) ICAM-1 (using the colorimetric method) or (2) endogenous vascular alkaline phosphatase with the NBT/BCIP solution (Vector Laboratories), prepared according to the manufacturer's instructions and diluted in 100 mM Tris-HCl, pH 9.5. Following 10-30 min incubation in this solution, sections were rinsed, dehydrated in graded alcohols, and coverslipped in Permount. Sections were viewed on an Olympus BX41 microscope, and digital images were collected with a SPOT RT camera (Diagnostic Instruments; Sterling Heights, MI). In some cases, To-Pro-3 (Molecular Probes) counterstained sections were viewed using a BioRad 1024 confocal miscroscope.

ICAM-1 morphometry: Morphometric measurements were collected to obtain numerical data for differential macular and extramacular ICAM-1 labeling. Grayscale digital photographs from the T1 (macular) and T3 (extramacular) punches (see Figure 1) of nine donor eyes were obtained, using exposure conditions that were standardized such that identical exposures were collected from each field within a donor. To minimize difficulties in quantifying histochemical data, we determined the linear range of the enzyme reaction by measuring capillary intensities at 30 s intervals of development in substrate from serial sections of two donor eyes. Based on these results, morphometric measurements for the entire data set were used at 150 s development, a duration at which reaction product generation was in the linear range with a correlation coefficient of 0.94-0.97 (Microsoft Excel 11.2.3). An average of 38 capillaries were measured from each region. Images were then analyzed using ImageJ software version 1.32j. Capillaries were traced using the polygon tool, and intensity values (maximum, minimum and average) were obtained for each capillary. Larger values obtained from these measurements correspond to more labeling and, indirectly, to more of the target antigen. Average values from all of the capillaries within a region were pooled for subsequent graphing and analysis.

ICAM-1 real time polymerase chain reaction: For experiments to evaluate the level of ICAM-1 mRNA, a series of

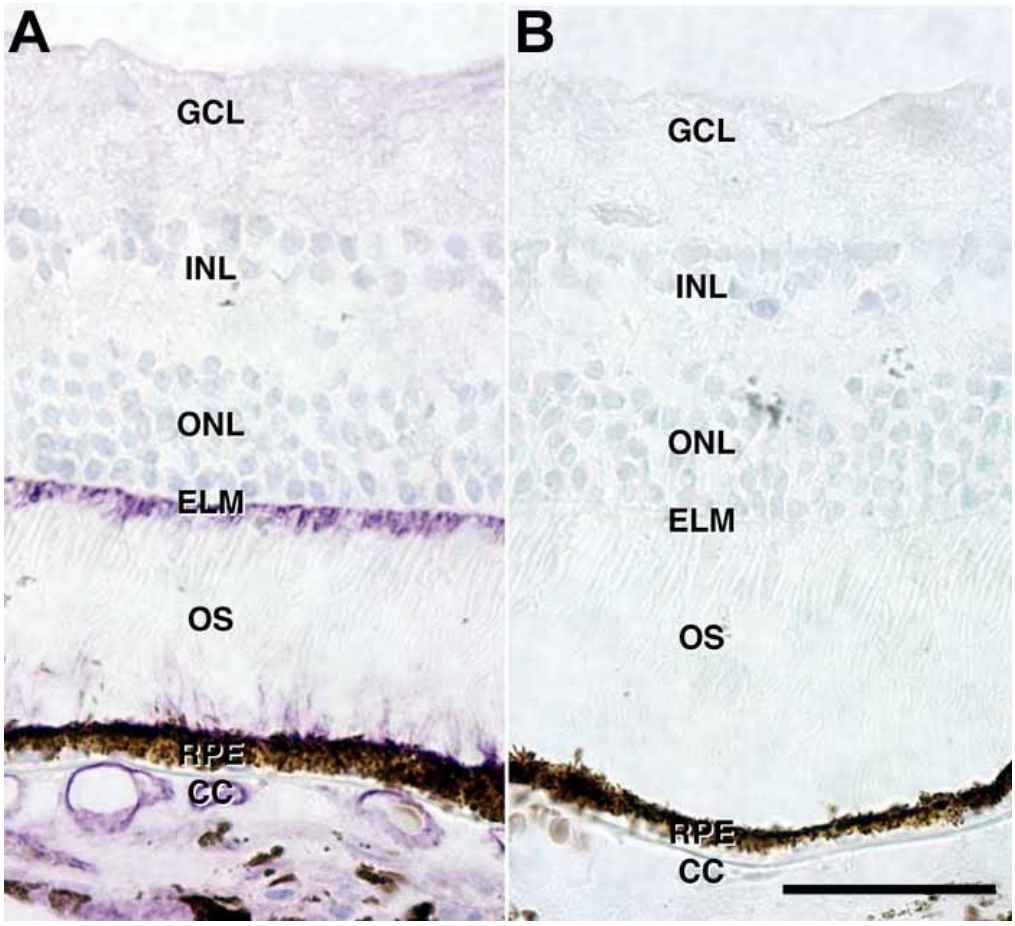

Figure 2. Specificity of the monoclonal ICAM-1 antibody used for morphometric experiments. The specificity of the monoclonal ICAM-1 antibody (**A**) was confirmed by blocking the monoclonal antibody with a ten fold excess of recombinant ICAM-1 (**B**). Note the loss of labeling in the external limiting membrane (ELM) and choriocapillaris (CC). The ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), and outer segments (OS) are also identified. Scale bar represents 50 μm.

4 mm punches of the RPE-choroid layer was collected from the temporal quadrant of eyes from six donors (Figure 1). Punches used for RNA experiments were snap frozen in liquid nitrogen and stored at -80 °C until used for RNA isolation. Total RNA was isolated from the RPE-choroid layer using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Punches were homogenized in lysis buffer and, following digestion with RNAse-free DNAse, RNA was eluted from the RNeasy column in RNAse-free water.

Total RNA was reverse transcribed in a random primed reaction using SuperScriptIII reverse transcriptase (Gibco, Grand Island, NY). Transcript levels were determined using real-time PCR (ABI Model 7700) and SybrGreen detection (QuantiTect PCR kit, Qiagen). All samples were analyzed in triplicate. To control for sample variations in RNA concentration, the cellularity of the different punches, and the efficiency of the RNA isolation and reverse transcription, ICAM-1 mRNA expression levels were normalized to those of Ubiquitin C (UBC) [35]. Primers used were: ICAM1-F: 5'-TGG GAA CAA CCG GAA GGT GTA T-3'; ICAM1-R: 5'-TTC AGT GCG GCA CGA GAA AT-3'; UBC-F: 5'-ATT TGG GTC GCG GTT CTT G-3'; UBC-R: 5'-TGC CTT GAC ATT CTC GAT GGT-3'. Melting curves confirmed that only a single reaction product was formed.

Western blotting: The retina and RPE-choroid was collected from two human eyes between 4 and 6 h of death. Tissues were frozen in liquid nitrogen and stored at -80 °C until utilized for protein preparations. Macular and extramacular tissues were homogenized in PBS with 1% Triton X-100 and complete protease inhibitor (Roche Diagnostic, Indianapolis, IN), prepared according to the manufacturer's instructions. Protein concentrations in supernatants were determined using the Lowry method (BioRad DC Assay, Hercules, CA), and 20 μg of protein were mixed with Laemmli buffer and separated electrophoretically on 7.5% polyacrylamide gels using methods described elsewhere [36]. Following electrophoresis, proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (BioRad).

Following brief treatment with methanol, membranes were blocked in 50% Napsure (G Biosciences, St. Louis, MO). Antibodies directed against ICAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used in 25% Napsure at a concentration of 1 μg/ml, followed by washing of the membrane, incubation with 50 ng/ml peroxidase-conjugated antirabbit secondary antibody (Amersham Biosciences, Buckinghamshire, UK) and detection using the ECL plus kit (Amersham).

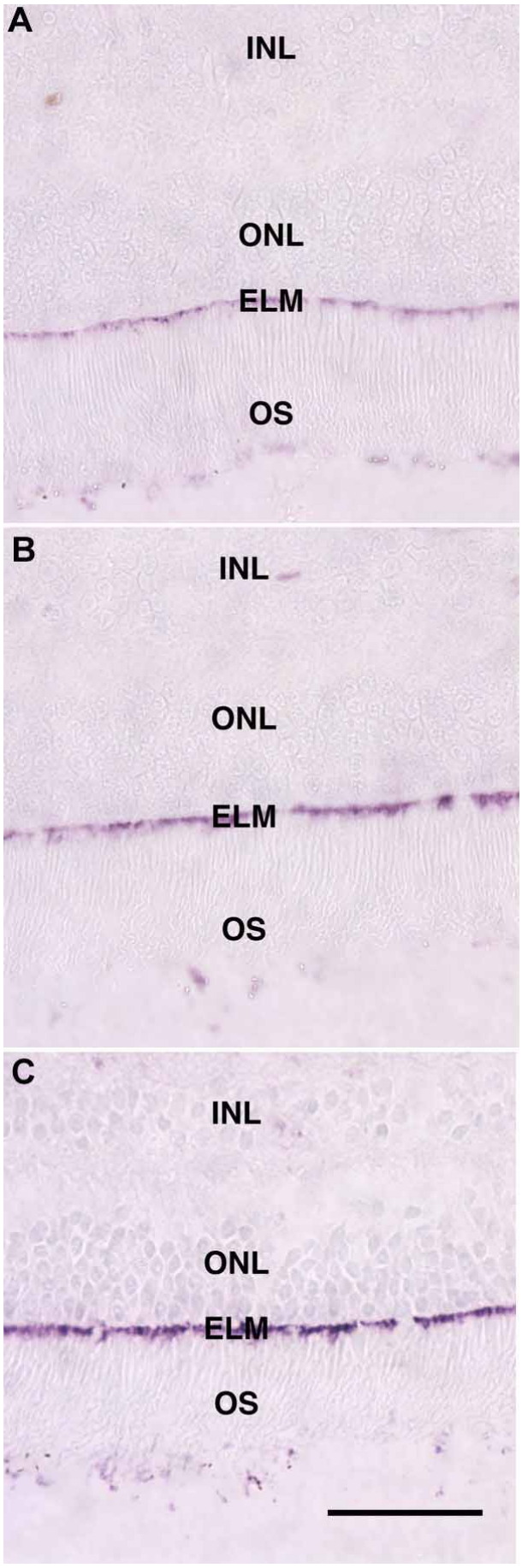

Figure 3. Topography of immunohistochemical labeling of ICAM-1 in the retina. Immunohistochemical labeling of the external limiting membrane (ELM; purple reaction product) in the macular (**A**), equatorial (**B**), and far peripheral (**C**) regions of the neural retina. Note the graded labeling from macula to periphery. This pattern was observed in 19 out of 20 examined. The inner nuclear layer (INL), outer nuclear layer (ONL), and outer segments (OS) are also identified. Scale bar represents 50 μm.

For one experiment, peripheral punches of retina and choroid were homogenized as previously described, and 10 μg of protein were incubated overnight in either 1 U/ml PNGase F (Sigma, St. Louis, MO) or control buffer with agitation at 37 °C. Western blots were prepared from each sample as already described, except that samples were separated on 10% polyacrylamide gels.

RESULTS

Immunohistochemical increase in ICAM-1 in the peripheral retina and macular choriocapillaris: To verify the specificity of the ICAM-1 monoclonal antibody, we pre-incubated this antibody with a ten fold excess of recombinant ICAM-1. Whereas strong ICAM-1 immunoreactivity is detected in the external limiting membrane and choriocapillaris (Figure 2A) [30], this labeling was completely abrogated when antibodies were preincubated with an excess of ICAM-1 protein (Figure 2B), confirming the specificity of the antibody.

Interestingly, we frequently observed a continuous gradient of ELM labeling topographically, with little or no labeling in the macula, moderate labeling in the equatorial retina, and intense labeling in the far periphery (Figure 3A-C). This pattern was observed in 19 of 20 eyes examined. The ELM in the fovea showed intense ICAM-1 labeling (data not shown). ICAM-2 showed no association with the ELM, but was localized to the retinal and choroidal vasculature (Figure 4A,B).

Whereas in the extramacular regions the ELM appeared to be more immunoreactive for ICAM-1, labeling of the choriocapillaris with ICAM-1 antibodies frequently showed a less dramatic but noticeable increase in the macular choroid (Figure 5A,C). This pattern was not invariable, but was noted in a majority (70%) of donor eyes (14/20). This macular-peripheral gradient was detected in the choriocapillaris but was not obvious in larger vessels, nor was it apparent when vessels were stained for endogenous alkaline phosphatase activity (Figure 5B,D).

Morphometric increase in macular choriocapillaris: Morphometric analyses were conducted on macular and extramacular tissue samples of human donor eyes to quantify the apparent difference in macular-extramacular labeling of ICAM-1 antibodies in the choriocapillaris. To determine that data were collected at a time during which the reaction product was being formed in a linear range, we evaluated the time course of the reaction by performing immunohistochemical

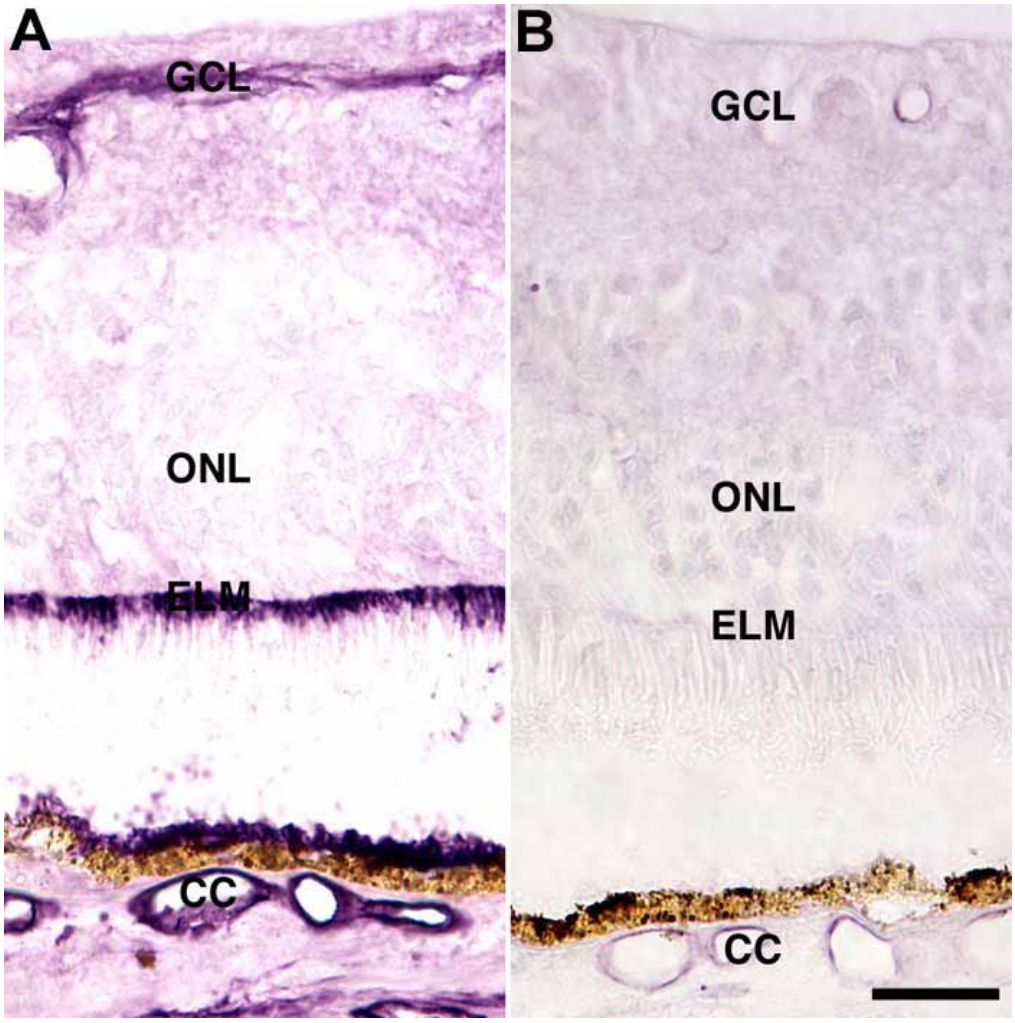

Figure 4. Comparison of ICAM-1 and ICAM-2 labeling in a healthy eye. **A**: ICAM-1 immunoreactivity is widespread, with labeling observed in the ganglion cell layer (GCL), external limiting membrane (ELM), RPE, choriocapillaris (CC), and other choroidal vessels [30]. **B**: ICAM-2 is restricted in its distribution to retinal and choroidal vasculature. The outer nuclear layer (ONL) is also identified. Scale bar represents 25 μm.

labeling of ICAM-1 and stopping the reaction at 30 s intervals. This was followed by morphometric measurement of capillary labeling intensity. We found during the first 180 s of development, the reaction product generation was in a linear pattern with a correlation coefficient of 0.94 and 0.97 in the two donors, respectively (Figure 6A). We therefore collected all the data for morphometric measurements at 150 s development.

Results from morphometry experiments on the choriocapillaris are depicted in Figure 6. While donor-to-donor variability was evident, six of nine measured eyes displayed a significant ($p<0.05$, Student's t-test) increase in labeling intensity in the T1 compared to the T3 punches (Figure 6B). In two cases, macular and peripheral labeling was identical and in one case the pattern was reversed ($p<0.01$). Measurements collected for topographic ICAM-2 intensity revealed that, unlike ICAM-1, ICAM-2 does not show a strong macular-extramacular predilection, with larger values more frequently in peripheral than macular choriocapillaris (data not shown), indicating that the observed increase in ICAM-1 labeling is not a consistent finding for all EC molecules or an artifact of the measurement technique. The fraction of eyes with a significantly greater labeling of ICAM-1 in the macula (66.7%) was similar to the number obtained from nonquantitative observation (70%).

Quantitative PCR: Quantitative PCR analysis was performed to assess whether the topographic differences in choroidal vessel labeling with ICAM-1 antibodies were attributable to differences in the quantity of ICAM-1 mRNA in these regions. Normalized ICAM-1 expression values in RPE-choroid punches from six donors varied greatly between individuals tested, and as great as a five fold difference could be detected between individuals. However, transcript levels measured in these full thickness RPE-choroid punches did not closely reflect the immunohistochemical labeling pattern observed in the choriocapillaris, as transcripts were generally as likely to be elevated in the peripheral as macular RPE-choroid. Levels are depicted in Table 1.

ICAM-1 localization in photoreceptor rosettes and ICAM-2 localization in choroidal neovascularization: Immunoreactivity of antibodies directed against ICAM-1 and ICAM-2 was also examined in two eyes with CNVMs. ICAM-1 labeling was noted in photoreceptor aggregates or "rosettes" (Figure 7A-C), which have been observed in some eyes with AMD [37]. In addition, ICAM-1 antibodies labeled the ELM as well as small and large vessels of the neovascular membrane (Figure 8A). In contrast, ICAM-2 localization in neovascular membranes was limited to the vasculature, and moderate labeling was observed in both normal and pathologic vessels in these tissues (Figure 8B).

ICAM-1 labeling of the RPE was variable, and did not show a strong macular-peripheral gradient in our samples. Some RPE cells overlying drusen showed increased reactivity, but this was not a consistent finding (data not shown). In CNVM, ICAM-1 labeling of the dystrophic RPE layer was intense, however (Figure 8A).

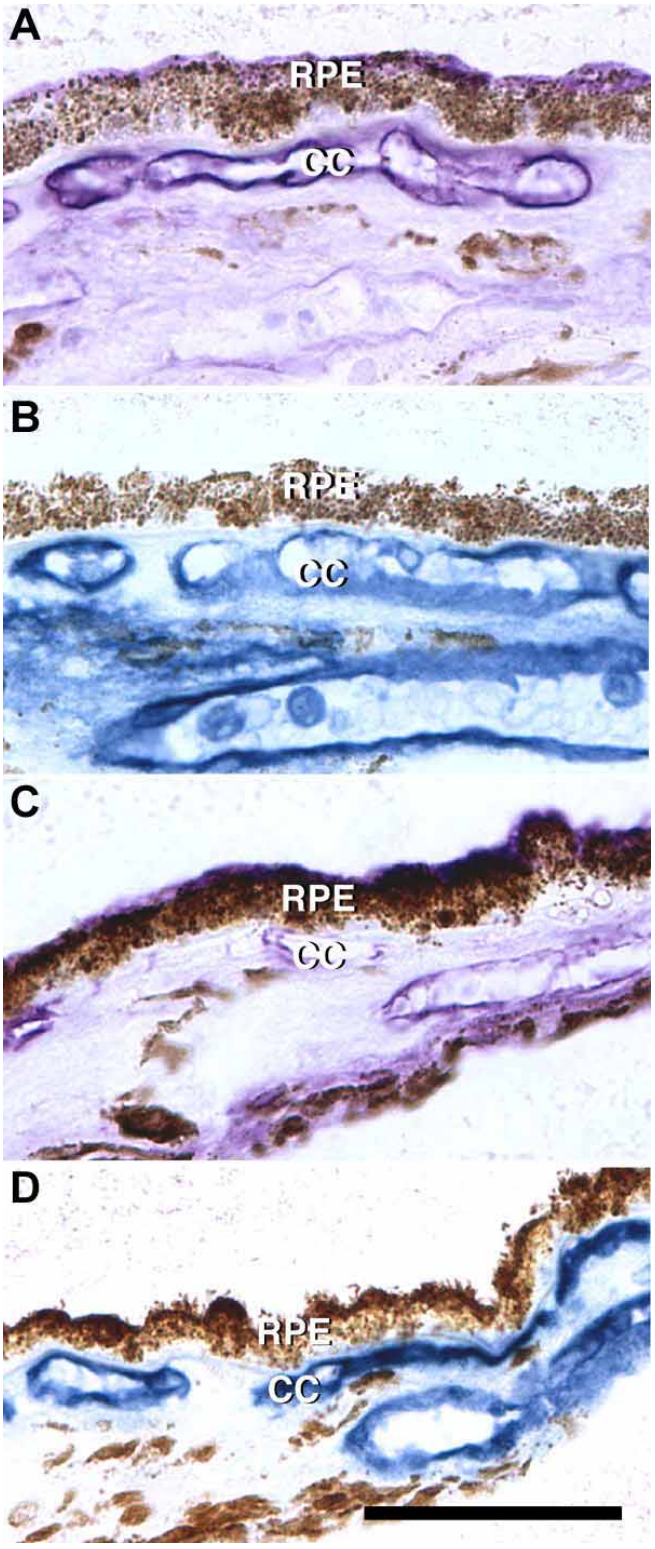

Figure 5. Topography of immunohistochemical labeling of ICAM-1 in choriocapillaris. Colorimetric detection of ICAM-1 (**A**,**C**) and endogenous alkaline phosphatase visualized by the NBT/BCIP alkaline phosphatase reaction product (**B**,**D**) in the macular (**A**,**B**) and peripheral (**C**,**D**) choroid in tissue sections containing both regions. An increase in labeling of the macular choriocapillaris (CC) was frequently noted in human eyes (14 out of 20 eyes). Scale bar represents 50 μm.

Western blots of retinal and choroidal ICAM-1: Western blots of ICAM-1 were performed on protein extracts from the retina and choroid. Interestingly, the apparent molecular weight of choroidal ICAM-1 was approximately 10 kDa higher than that of retinal ICAM-1 (Figure 9A). To determine whether this observed difference in size was due to post-translational modification, we treated retinal and choroidal homogenates with PNGase F. Both retinal and choroidal ICAM-1 showed increased mobility upon deglycosylation, with apparent molecular weights of 59 kDa, which is the predicted size of the unmodified protein (Figure 9B).

DISCUSSION

The macula is a small area of the posterior retina, occupying the region approximately within the vascular arcades. For reasons that are not well understood, AMD (and a number of other maculopathies) affects the macula of the eye, while leaving the majority of the posterior pole unaltered. This relatively small portion of the retina is responsible for visual acuity, and injury of the macula results in severe loss of vision.

In view of the emerging role of inflammatory processes participating in early and advanced AMD, we sought to determine whether variable distribution of EC activation molecules could explain in part the macular specificity of advanced lesions in AMD. In this study, we found an increase in the localization of ICAM-1 in the macular, as compared with the peripheral, choriocapillaris by immunohistochemistry and morphometry in a majority of donor eyes. This pattern was not noted for a related protein, ICAM-2.

As described elsewhere [30,38], ICAM-1 is present in the external limiting membrane. McLeod et al. [31] also noted that labeling of ICAM-1 in the ELM is elevated in areas of cystoid degeneration. Labeling in the ELM was qualitatively greater in the peripheral than macular retina and appeared to gradually increase from just outside the fovea to the ora serrata. The expression of ICAM-1 was also observed in photoreceptor aggregates in two eyes with neovascular AMD. Aggregates, or rosettes, in which photoreceptor cells respond to injury by forming invaginations with islands of interphotoreceptor matrix material, occur in a number of reti-

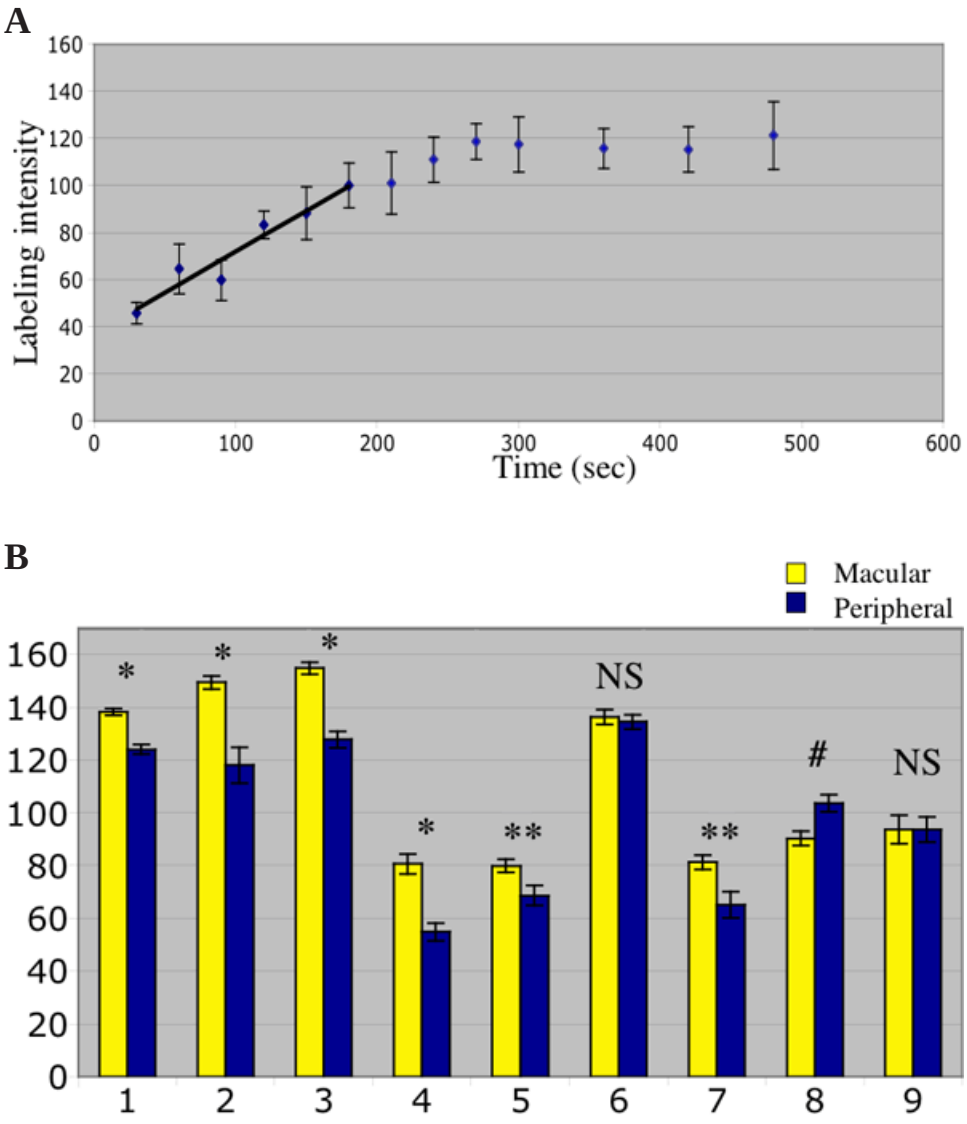

Figure 6. Graphs depicting ICAM-1 morphometry values. **A**: Mean capillary value measurements of macular serial sections incubated with ICAM-1 monoclonal antibody (clone P2A4) developed at 30 s intervals and measured as described in Materials and Methods. The 150 s time used for subsequent experimental measurements is within a linear range of values as depicted, with a correlation coefficient of 0.94 to 0.97. Error bars represent standard errors of the mean. **B**: Data from nine eyes used for morphometric measurements are shown. In six of nine eyes, a significantly greater labeling intensity was measured in the macular (yellow bars), as compared with extramacular (blue bars) choriocapillaris. In two cases, labeling was the same and in one of nine cases, the pattern was reversed. Asterisk (*) indicates $p<0.001$; double asterisk (**) indicates $p<0.05$; hash mark (#) indicates $p<0.01$ with peripheral values larger than macular values; nonsignificant comparisons are marked "NS".

nal degenerative conditions, including in human eyes with AMD [37]. The localization of ICAM-1 to these structures suggests that photoreceptor cells may express ICAM-1, at least in certain pathologic states, and that these cells may also be the source of ICAM-1 in the normal ELM. In contrast to ICAM-1, ICAM-2 labeling in eyes with AMD was restricted to normal vessels and neovascular ECs.

Unlike the majority of eyes evaluated histochemically, the mRNA levels assessed by quantitative PCR did not show a significant macular increase in the choroid. This discrepancy may be due to the fact that ICAM-1 is expressed by RPE, choriocapillaris, some circulating leukocytes, and large choroidal vessels. Hence, the relative contribution of the non-choriocapillary cell types may mask an increase in the choriocapillaris. Moreover, post-transcriptional regulation of ICAM-1 mRNA has been suggested as a mechanism for regulating ICAM-1 protein levels in other systems [39,40], such that transcriptional and translational control of ICAM-1 may be uncoupled. The finding that there were significant differences (up to five fold) in ICAM-1 mRNA expression levels between individuals might suggest that some eyes are inherently more at risk for inflammatory insults in AMD or other diseases.

First, what is the cause of the increased ICAM-1 expression in the macula? At least two hypotheses are presented. ICAM-1 expression is highly regulated in ECs, and if the microenvironment in the macula is more pro-inflammatory, enhanced ICAM-1 expression might result. Several environmental factors affect the level of ICAM-1 transcription in EC, including complement injury [24], oxidative stress [25], and exposure to vascular endothelial growth factor [26], advanced glycation endproducts [27,28], and infectious agents [29]. Many of these events have been proposed to be involved in the pathogenesis of AMD. It is also possible that the microenvironment in the choriocapillaris may become pro-inflammatory secondary to other events occurring in AMD, such as RPE and photoreceptor injury through other mechanisms. In this case, a secondary inflammatory response by the choriocapillaris may exacerbate the damage to the RPE and/or the photoreceptor cells. Another, nonexclusive, possibility

Table 1. Macular and peripheral normalized transcript levels of ICAM-1 mRNA (fluorescence units)

Sample	T1 (Macular)	T3 (Extramacular)	T1/T3
1	40.1	27.5	1.46
2	27	26	1.03
3	60.1	48.2	1.25
4	73.9	167.9	0.44
5	135.2	34.7	3.90
6	26.2	60.5	0.43

Results from quantitative PCR experiments performed on macular and extramacular punches of full-thickness RPE-choroid. Whereas a wide range of values was detected between donors, no consistent difference was observed between macular and peripheral regions.

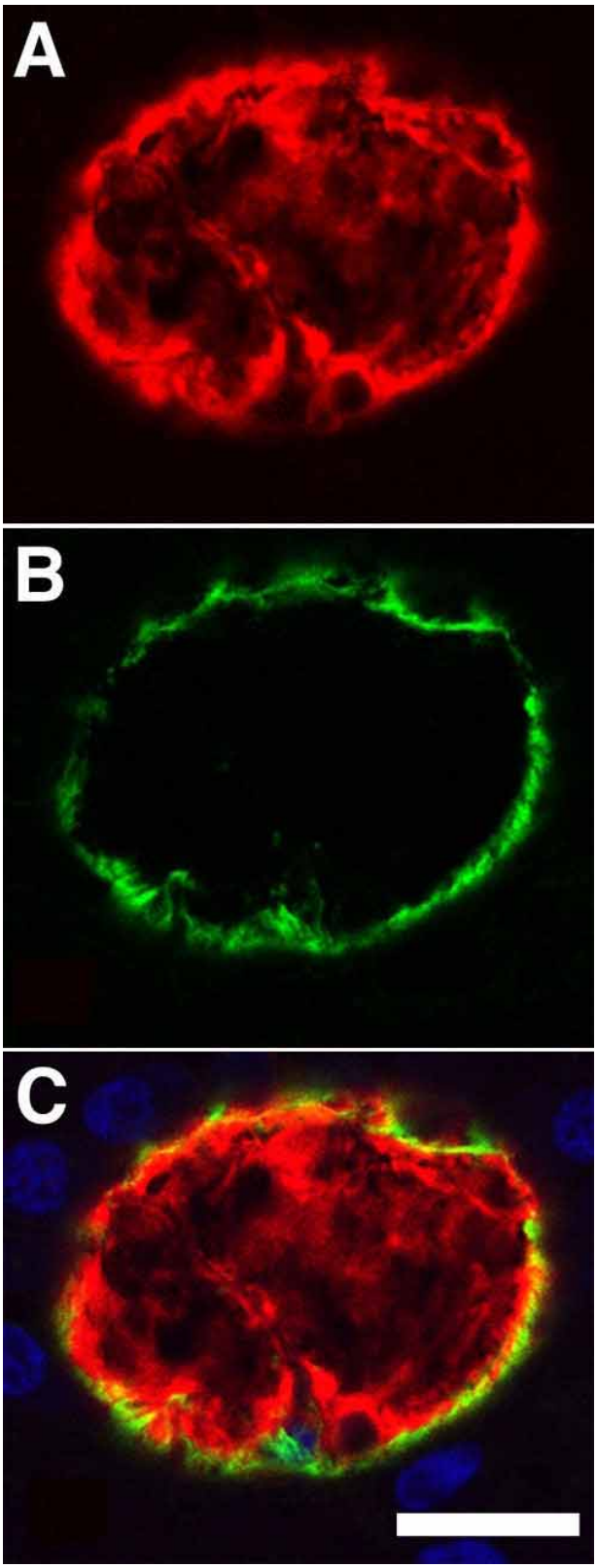

Figure 7. Labeling of ICAM-1 in photoreceptor aggregates. **A**: Photoreceptor aggregates present over a disficorm scar (not depicted) were labeled with peanut agglutinin (red). **B**: These aggregates also displayed a circumferential distribution of ICAM-1 (green). **C**: Merge of panels **A** and **B** with To-Pro-3 labeling of retinal nuclei (blue). Scale bar represents 20 μm.

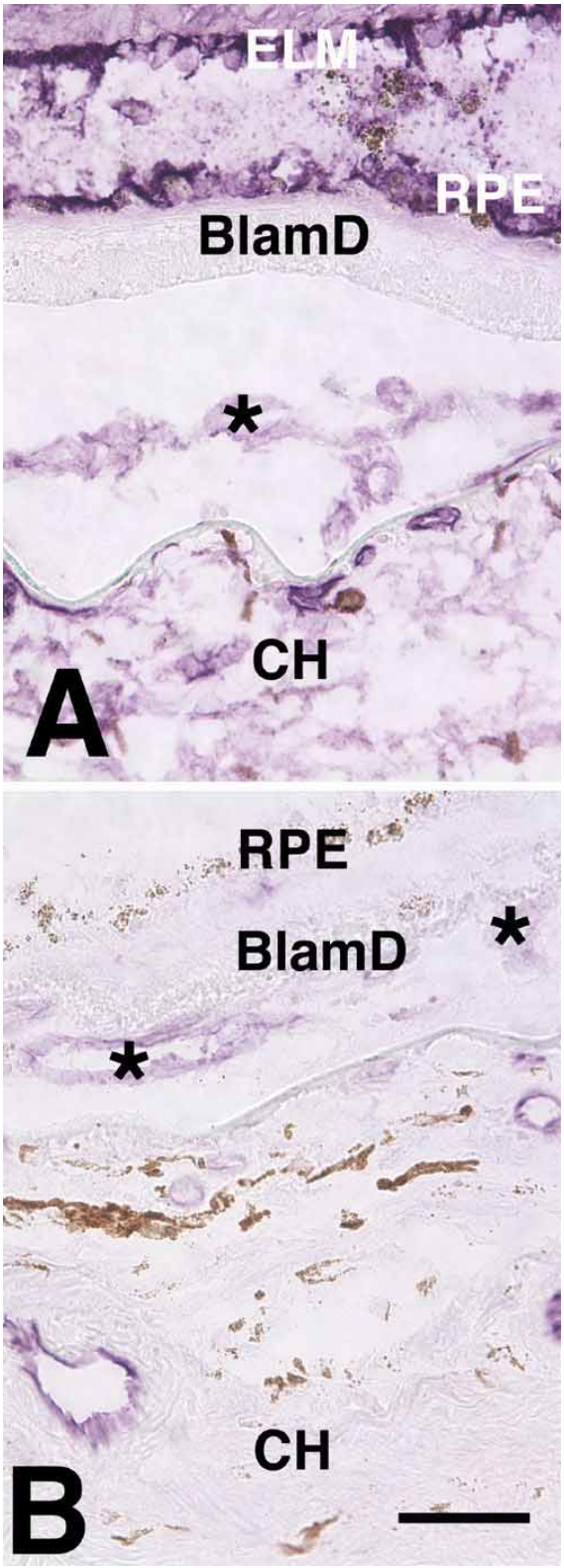

Figure 8. Comparison of ICAM-1 and ICAM-2 eyes with CNV. **A**: In eyes with choroidal neovascular membranes, ICAM-1 exhibits weak labeling throughout the dystrophic retina and in vascular elements within the choroidal neovascular membrane (asterisk). **B**: ICAM-2 is restricted to vascular elements (asterisks). The external limiting membrane (ELM), basal laminar deposits (BlamD), and choroid (CH) are identified. Scale bar represents 50 μm.

is that there are inherent differences in gene expression between macular and extramacular choroidal ECs. It is widely appreciated that ECs from different tissues exhibit distinct patterns of cell surface molecules [41]. It is therefore possible that ECs from various topographic regions of a tissue such as the choroid, with different supplying ciliary arteries [42], may show characteristic patterns of expression of EC activation molecules. Future studies on cultured human choroidal ECs from different topographic regions will assist in resolving this question.

A second question suggested by these data is what are the consequences of increased choriocapillary ICAM-1 expression to the macular region in health and disease? Although the

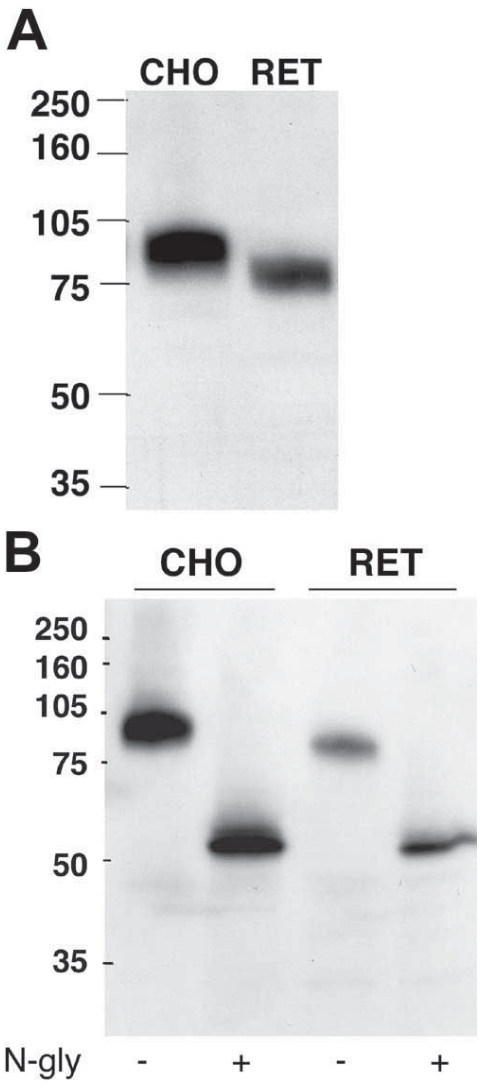

Figure 9. Western blots of ICAM-1 protein from retina and choroid. **A**: Total protein homogenates. Note the greater molecular weight of ICAM-1 in the choroid as compared with the retina. **B**: Contribution of N-linked carbohydrates to ICAM-1 molecular weight in the choroid and retina. Deglycosylation of retinal and choroidal protein results in the detection of a major band in both tissues at approximately 59 kDa. Each protein was examined with and without treatment of extract with PNGase F (N-gly).

measured difference in ICAM-1 labeling between the macular and extramacular choriocapillaris is relatively small (with the peripheral values ranging from about 79-92% of the macular values), we suggest that this slight difference may be important over the course of a lifetime, or during periods of increased cellular stress. Trafficking of leukocytes into and out of the microvasculature occurs to a certain degree in normal physiology. It has been estimated in the mouse that 50% of all monocytes leave the normal vasculature each day [43], with increased migration under inflammatory conditions. The extent to which leukocyte transmigration occurs in the normal choroid is not known. The transmigration of leukocytes across ECs does have consequences to the endothelium itself, and there is evidence that neutrophil-mediated EC injury occurs in several conditions, including ischemic reperfusion injury [44], nonsteroidal antiinflammatory drug-induced gastric injury [45], and acute respiratory distress syndrome [46]. It is conceivable that the macular choroidal endothelium is similarly challenged by the transcytosis of leukocytes, which, over the course of a lifetime, may result in capillary dropout and decreased perfusion-events that have been noted in early AMD [47-50]. Whether choriocapillaris ECs from eyes with early AMD exhibit increased macular ICAM-1 expression, compared with age-matched control eyes, will be an important extension of these studies.

The evidence for differential glycosylation of ICAM-1 between the retina and choroid is interesting in view of the known effects of glycosylation differences in ICAM-1 and its biological function. Jimemez et al. [51] recently showed that substrate recognition of the cell surface protein DC-SIGN/CD209 by ICAM-1 and ICAM-2 is modulated by the glycosylation state of these proteins. In addition, binding to CD11b/CD18 integrin can be modulated in vitro by altering the N-linked glycosylation of ICAM-1 [52]. Whereas the expression of ICAM-1 by endothelial cells of the choroid is expected to regulate leukocyte recruitment into this tissue, this adhesive glycoprotein is likely to have different binding partners in the retinal ELM, and this difference may be regulated in part by the glycosylation state of ICAM-1.

In summary, we describe a relative increase in the localization of ICAM-1 protein in the peripheral retina and the macular choriocapillaris. This finding was not observed for ICAM-2. We suggest that this increase in macular ICAM-1 may contribute to the incidence of lesions in AMD to the macula.

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