Modulation of Major Histocompatibility Complex Class II Expression in Retinas With Age-Related Macular Degeneration

Philip L. Penfold,* Simon C. K. Liew,* Michele C. Madigan,* and Jan M. Provis*†

Purpose. To investigate antigenic and morphologic features of microglial and vascular elements in the neural retina associated with age-related macular degeneration (ARMD) compared with those features in age-matched normal and young adult retinas.

Methods. Adult eyes (n = 97) were classified histopathologically into normal and ARMD-associated groups. Peroxidase immunohistochemical examination of retinal flatmounts was used to visualize major histocompatibility complex class II (MHC-II) immunoreactivity; the intensity and distribution of labeling were quantified by image analysis. In histochemical investigation, reduced nicotinamide-adenine dinucleotide phosphate diaphorase and glial fibrillary acidic protein or MHC-II double labeling were used to detect vascular changes in some preparations.

Results. An increase in the proportion of the retina (percentage of total area) expressing MHC-II immunoreactivity was observed in age-matched retinas compared with that seen in normal retinas. A significant increase (P < 0.05) in the percentage of area immunoreactive for MHC-II was observed, primarily on vascular elements, in retinas with incipient ARMD compared with the area affected in the age-matched group. Increased MHC-II immunoreactivity on vessels in the normal-aged group observed with confocal microscopy was associated with irregularities in the organization of astrocytes. Hypertrophy of retinal microglia was also apparent, although the intensity of microglial MHC-II immunoreactivity was not significantly different between groups.

Conclusions. The results indicate that an increase in MHC-II immunoreactivity on retinal vascular elements is associated with normal aging. A further increase in MHC-II immunoreactivity on vascular elements and morphologic changes in microglia was associated with incipient ARMD. Immunologic responses in neural retinal microglia and vascular elements appear to be related to early pathogenetic changes in retinal pigment epithelium pigmentation and drusen formation. Invest Ophthalmol Vis Sci. 1997;38:2125–2133.
Adult human eyes \((N = 97)\) were obtained, subsequent to informed consent, within 5 to 16 hours after death through the New South Wales Eye Bank. Eyes with a known history of ocular or chronic neurologic disease were excluded from the study. After removal of the anterior segment, specimens were fixed in 2% paraformaldehyde–0.1 M phosphate-buffered saline (PBS; pH 7.4) for up to 24 hours at 4°C. Using a 7-mm trephine, discs comprising all ocular layers were cut from the macula and from peripheral locations at 8 mm nasal to the optic disc and 8 mm temporal to the macula on the horizontal meridian. The neural retina was dissected from the retinal pigment epithelium (RPE) and choroid and was prepared for immunohistochemistry or nicotinamide adenine dinucleotide phosphate (NADPH)–diaphorase histochemistry, as described later.

An additional three ARMD specimens were dissected as flatmount strips, which included the macula and ora serrata, and were examined for uniformity of labeling.

Histopathologic Survey. The RPE and choroid from each trephined macular sample were postfixed in 2.5% glutaraldehyde and 2% osmium tetroxide and were embedded in Epon–araldite resin. Semithin sections were cut and examined by light microscopy. Specimens were surveyed for signs of retinal pathology, including the presence or absence of drusen (Fig. 1C), pigmentary disturbance (Fig. 1D), abnormal collagen deposition (basal laminar deposit; BLD), and disciform (Fig. 1A) or atrophic (Fig. 1B) lesions. On the basis of this histopathologic assessment, eyes were classified in three groups: normal \((n = 37; \text{less than 50 years old, with an absence of RPE and choroidal changes})\), normal-aged \((n = 13; \text{more than 50 years old, with an absence of RPE and choroidal changes})\) and ARMD \((n = 47)\). The mean ages of the ARMD subgroups and normal-aged group (Table 1, ref. 20) were not significantly different \((P = 0.29; \text{Tukey--Kramer})\) and represent an age-matched series.

The ARMD eyes were further divided into five categories. Those with end-stage ARMD were classified as displaying atrophic (geographic atrophy; \(n = 3\)) or fibrovascular (disciform; \(n = 4\)) lesions. The presence of BLD is a common histopathologic feature of eyes with early ARMD. Therefore, eyes with early ARMD-related changes were classified according to the predominant histopathologic feature seen separately or associated with BLD and were assigned to the BLD group when BLD was the only histopathologic feature observed. On this basis, eyes were classified as having drusen \((n = 16)\), pigmentary disturbance \((n = 8)\), or BLD alone \((n = 16)\).

Staining and Labeling Techniques

Peroxidase Immunohistochemical Examination. Peroxidase immunohistochemical examination was performed, as described previously. Briefly, neural retinas were rinsed, soaked in 0.1 M Tris-buffered saline (TBS; pH 7.6)–0.2% Triton X-100 (BDH, Sydney, Australia) for 12 hours at 4°C and were incubated in primary antibody overnight at 4°C. Primary antibodies used were antihuman HLA–DR CR3/43, which reacts with the beta-chains of all products of the gene subregions DP, DQ, and DR (1:50 dilution; Dako, Sydney, Australia), and antiglial fibrillary acidic protein (anti-GFAP; 1:1000 dilution; Dako). After rinsing, specimens were incubated in biotinylated secondary antibody (1:50 dilution; Amersham, Sydney, Australia) overnight at 4°C. Primary and secondary antibodies were diluted in 2% fetal calf serum in 0.1 M TBS. Bound antibody was detected using the avidin–biotin peroxidase labeling technique (Vectastain, Vector Laboratories, Sydney, Australia) using a nickel-enhanced, 3,3′ diaminobenzidine tetrahydrochloride solution. After immunohistochemical procedures specimens were placed onto gelatinized slides, nerve fiber layer uppermost, air-dried in a humidified chamber, dehydrated in graded alcohols and xylenes, and mounted in DePeX (BDH). Nonspecific binding was excluded by substituting a nonimmune immunoglobulin G-1 (IgG-1) isotype of the same species (1:50 dilution; Dako) for the primary antibody; primary and secondary antibodies were omitted in some experi-
Major Histocompatibility Complex Class II Expression in Age-Related Macular Degeneration

FIGURE 1. Histopathologic features of specimens from the ARMD groups. (A) A large disclike scar overlying Bruch's membrane. The RPE above the scar has lost its pigmentation and is thinned and disrupted. (B) Atrophic changes in ARMD with loss of photoreceptors and RPE. (C) Large drusen along Bruch's membrane. The RPE overlying the drusen is disrupted and absent in areas. Calcification can also be seen within the drusen. (D) Disorganized layers of the RPE typical of pigmentary disturbance. Note the basal laminar deposit in Bruch's membrane. (E) In the macular region of ARMD retinas, the normal microglial topography surrounding the foveal pit was disorganized. In some specimens labeled cells were seen within the foveal pit. (F) Hypertrophic (arrows) and perivascular (arrowheads) microglia in an ARMD retina. ARMD = age-related macular degeneration; RPE = retinal pigment epithelium. Bar = 30 μm.

merits to exclude endogenous peroxidase activity as the source of positive labeling.

NADPH-Diaphorase Histochemistry. After fixing, retinas were washed in 0.1 M PBS (pH 7.6)–0.2% Triton X-100 for approximately 20 to 30 minutes and incubated in a solution containing 0.2% Triton X-100, 15 mM malic acid, 1 mM cobalt chloride, 0.5 mM NADP, and 0.2 mM nitroblue tetrazolium in 10 ml of 0.1 M PBS for 2 to 4 hours at 37°C. The reaction was halted by washing the retinas in several quick changes of 0.1 M PBS–0.2% Triton X-100. Whole retinas were then postfixed in 2% buffered paraformaldehyde (pH 7.4) overnight at 4°C before immunohistochemical double labeling.

Double Labeling. Specimens were washed in several changes of 0.05 M TBS (pH 7.6)–0.4% saponin (saponin–TBS) on a shaker table for 4 to 5 hours to remove excess fixative and preincubated overnight at 4°C in saponin–TBS–10% normal goat serum to reduce background labeling. Retinas were then incubated for up to 5 days at 4°C in a mixture of rabbit anti-GFAP and mouse antihuman HLA-DR at final dilutions of 1:500 and 1:250, respectively. After washing, specimens were incubated in a mixture of biotinylated swine antimouse IgG F(ab')2 (BioCell Research Laboratory, Cardiff, UK) and sheep antirabbit whole IgG conjugated with fluorescein isothiocyanate (Amerham), each at a final dilution of 1:50 in saponin–TBS, at 4°C for 2 to 3 days. Streptavidin-conjugated Texas red (1:100; Amerham) was used to visualize the biotinylated antirabbit secondary antibody. Retinas were mounted whole in glycerol and were viewed by dual scanning confocal microscopy (BioRad, Regents Park, Australia). Specimens were optically sectioned 2 to 3 times within the same field for each label. These sections were compressed into a single image and were stored in “raw” format. Combination of color images for each field was done using Adobe Photoshop. In some cases, double labeling was carried out after NADPH–diaphorase histochemistry.

Image Analysis

To measure the intensity of MHC-II immunoreactivity, peroxidase-labeled flatmounts were viewed through a Zeiss Axioplan microscope (Zeiss, Camperdown, Australia), using a ×10 objective lens and a Sony color video camera. Images were acquired by a Tracor Northern image analysis system (Sydney, Australia). The size of each field was 0.7 mm², and three different fields were selected from each specimen. A binary image was created to enable the computer to distinguish areas of immunoreactivity. Computer calculations
FIGURE 2. Microglia expressing MHC class II antigens are shown in three planes of focus in ARMD (A to C) and normal (D to F) flat-mounted retinas. In the nerve fiber layer of ARMD (A) and normal (D) retinas, microglia have an elongated morphology, apparently constrained by nerve fiber bundles. In ARMD retinas, labeled cells in the nerve fiber layer were also hypertrophied (B). Perivascular macrophages (arrowheads) were present on large and small vessels in ARMD (A,B) and normal (D,E) retinas. They had a rounded morphology and a reduced number of cell processes, which conformed to the vessel profiles and did not extend into the retinal parenchyma. In ARMD retinas, hypertrophied parenchymal microglia were visible (B) (see also Fig. 1F). These cells had an enlarged body with extensive multiple processes, which were thickened and vacuolated (B). The parenchymal microglia of the inner plexiform layer in normal retinas displayed typical dendritic morphology, with multiple smooth processes extending from a small oval cell body (E). Perivascular macrophages on the smaller vessels were less ramified but had cell processes extending into the parenchyma in ARMD (B) and normal (E) retinas. In ARMD retinas, paravascular microglia were most evident at the inner nuclear layer–outer plexiform layer border, closely apposed to intensely labeled blood vessels (C). These cells displayed higher levels of MHC class II antigen expression compared with that shown in parenchymal microglia (C). However, in the inner nuclear layer–outer plexiform layer border of the normal retina, parenchymal microglia with dendritic morphology were the predominant cell type (F). ARMD = age-related macular degeneration; MHC = major histocompatibility complex. Bar = 50 μm.

were made of the optical density of immunoreactive cells in each field, from which the average optical density of positive cells was calculated. The percentage of retinal area of MHC-II immunoreactivity in randomly selected fields was also calculated as described previously. The optical-density measurements and percentage-of-area estimates obtained from normal retinas and ARMD groups were compared statistically, using the Tukey–Kramer test.

Cell density (number of cells per square millimeter) was measured using a ×40 objective lens (field size 0.03 mm²) at comparable retinal locations. Fifty fields from each specimen (three normal and three ARMD retinas) were viewed on the television monitor and were manually counted in each of three layers.

We previously established that optical density measurements of MHC-II expression are not significantly influenced by postmortem delays of less than 16 hours. Additionally, it has been demonstrated that no significant difference in intensity of MHC-II expression is detected between normal infant and aged retinas.

RESULTS

Normal Retinas

In normal retinas, the area reactive to MHC-II antigens was less than 10% (Fig. 4B). Retinal vascular elements did not express significant levels of MHC-II antigens, and the endogenous peroxidase activity in intraluminal RBC was clearly visible (Fig. 2E, 2F). In control experiments, only endogenous reactivity of red blood cells was detected (not shown). Microglia immunoreactive to MHC-II were detected in all normal adult human retinas studied, as described pre-
Major Histocompatibility Complex Class II Expression in Age-Related Macular Degeneration

2129

Age-Matched Normal Retinas

The mean ages of the ARMD subgroups and normal-aged group (Table 1) were not significantly different ($P = 0.29$, Tukey–Kramer) and represent an age-matched series. However, there was a significant increase in the percentage of area of MHC-II immunoreactivity associated with the retinal vessels in normal age-matched retinas (15%), compared with that in normal retinas ($P < 0.05$, Tukey–Kramer; Fig. 4B). Diffuse immunoreactivity associated with the retinal vessels, in some cases, was attributable to MHC-II expression by vascular endothelial cells and perivascular macrophages. The distribution and morphology of microglia in age-matched retinas resembled that seen in normal retinas.

In triple-labeled specimens analyzed with confocal microscopy, increased MHC-II immunoreactivity was frequently associated with irregularities in the organization of astrocytes, particularly in the vicinity of large retinal vessels (Fig. 3A). These changes, together with the beading of astrocytic processes, are suggestive of astrocyte degeneration, as described previously. In specimens reacted for NADPH-diaphorase histochemistry before double labeling, it appeared that the increased MHC-II immunoreactivity was related to the presence of an increased population of perivascular macrophages in the perivascular space (Fig. 3A).

Age-Related Macular Degeneration Retinas

The most distinctive feature of ARMD retinas was the upmodulation of MHC-II immunoreactivity associated with retinal vessels. Optical densitometry indicated that there was no significant difference in the intensity of MHC-II immunoreactivity on microglia in ARMD retinas compared with that in normal and in age-matched normal retinas (Fig. 4A). However, analyses of the percentage of area of MHC-II immunoreactivity indicated significant differences in ARMD specimens compared with that in normal specimens and age-matched normal specimens (Fig. 4B). Retinas with drusen, pigmented disturbance, and BLD (associated with incipient macular degeneration) had the highest percentage of area of MHC-II immunoreactivity of the seven groups analyzed. These groups had a percentage of area of MHC-II immunoreactivity that was significantly different from that of the normal and the aged-matched normal groups ($P < 0.05$, Tukey–Kramer; Fig. 4B). The percentage of area of MHC-II immunoreactivity in various groupings was as follows (mean values ± SE): normal 8.4 ± 0.9%; age-matched normal 14.7 ± 1.5%; incipient ARMD (drusen, pigmentary disturbance, BLD) 22.68 ± 1.59%; and end-stage ARMD (disciform, atrophic) 15.99 ± 3.53%. The distribution of MHC-II immunoreactivity was similar in the end-stage ARMD groups and in the age-matched normal groups, but the small sample sizes of the former groups should be noted.

Upmodulation of MHC-II expression on vascular elements was uniformly distributed in the three retinal strips between the ora serrata and the margin of the optic disc, indicating that the modulation of MHC-II expression was not confined to the macular region.

Estimates of the mean total density of MHC-II immunoreactive microglia was (mean values ± SD) 319 ± 141 cells/mm² in normal retinas and 355 ± 140 cells/mm² in ARMD retinas.

In ARMD retinas (Figs. 2A to 2C) vessel-associated and parenchymal microglia were hypertrophic, although microglia retained the laminated distribution seen in normal retinas. Hypertrophy was most evident in the deep layer (inner nuclear layer–outer plexiform layer border), in which cells had numerous processes extending from a swollen cell body. Thickening of the cell processes and the presence of varicosities were also observed (Figs. 1F, 2C).

At the macula, small, rounded microglia with short processes were evident, in addition to the ramified population present in the normal macula. In some preparations, however, the macular microglia retained their normal characteristics, although aberrant MHC-II immunoreactivity associated with the macular capillaries was evident (Fig. 1E).

DISCUSSION

Microglia are the intrinsic immunoeffector cells of the brain, and a characteristic feature is their rapid activation in response to even minor pathologic changes in the central nervous system. The expression of MHC-II antigens by microglia has been reported in normal human adult and fetal retinas and in the normal human brain by a number of independent groups. In the current study, we report an increase in the proportion of retinal area immunoreactive to MHC-II antigens in ARMD-affected retinas, measured by optical densitometry.

Histologically, the increase in MHC-II expression was associated with a diffuse reactivity on vascular endothelial cells (Fig. 1E), whereas more intense MHC-II expression was evident on perivascular macrophages (Figs. 1F, 2A). Previously, we have shown that differential expression of MHC-II antigens is a feature of hu-
FIGURE 3. Dual scanning, confocal laser microscopy showing triple labeling of the retina using NADPH–diaphorase histochemistry (to stain endothelial cells) in combination with double immunolabeling using anti-GFAP (to label astrocytes) and anti-MHC-II (to label microglia, green–yellow). (A) Normal-aged retina from a 91-year-old donor with no recorded history of retinal pathology and no evident histopathology of the RPE or of Bruch’s membrane. In this specimen astrocytes (red) are distributed irregularly, in contrast to those in the normal retina (B). Intense MHC class II immunoreactivity is evident on vessel-associated microglia at the perivascular glia limitans (GL) and in the perivascular space (pv), separating the glia limitans from the vascular endothelium (VE). Bar = 35 μm. (B) Normal retina from a 54-year-old donor with no recorded history of retinal pathology and no evident histopathology of the RPE or of Bruch’s membrane. Astrocyte processes that are GFAP-positive show a characteristic association with retinal vessels and ganglion cell axon bundles, which traverse the field diagonally. Major histocompatibility complex class II immunoreactivity is restricted to isolated microglia (M); little MHC class II immunoreactivity is associated with the large vessel. ND = diaphorase-positive amacrine cells; ARMD = Age-related macular degeneration; GFAP = glial fibrillary acidic protein; MHC = major histocompatibility complex; NADPH = nicotinimide–adenine dinucleotide phosphate; RPE = retinal pigment epithelium. Bar = 30 μm.

man retinal microglia, whereby vascular-associated microglia are characterized by increased intensity of expression; perivascular macrophages have a condensed morphology and reside in the perivascular space. The appearance of greater numbers of perivascular cells and local shedding of MHC-II antigens in ARMD retinas may account for the increased proportion of retinal area expressing MHC-II antigens on vascular elements (Figs. 2A, 2C). Perivascular macrophages remain excluded from the parenchyma in normal neu-

FIGURE 4. (A) The intensity of MHC class II reactivity in normal, normal-aged, and ARMD groups. (B) The percentage of retinal area reactive for MHC class II in retinas of normal, normal-aged, and ARMD groups. Groups of retinas with ARMD associated with early or incipient macular degeneration (drusen, basal laminar deposit, and pigmentary disturbance groups) had significantly increased percentages of area reactive for MHC class II compared with those in the normal-aged retinas. ARMD = age-related macular degeneration; MHC = major histocompatibility complex. ■ = % area; *P < 0.05, Tukey-Kramer.
ral tissue, residing in the perivascular space. Induction of MHC-II antigen expression on microglial and vascular elements is a feature of a number of central nervous system pathologies, including Alzheimer’s disease. A morphometric analysis of brains with Alzheimer’s disease revealed a significant increase in MHC-II immunoreactive microglia. Modulation of MHC-II expression by microglial and vascular elements has also been described in the retinas of Alzheimer’s patients. In some neuropathies, for example, in brains infected with human immunodeficiency virus, perivascular macrophages are reported to increase in association with cytokine-mediated damage of endothelial cells.

Endothelial cells in the normal brain are reported to be unreactive to anti-MHC-II antibodies, although they express MHC-II antigens in aged and diseased neural tissue. In the current results, we have also observed an increase in MHC-II immunoreactivity on retinal vascular elements associated with normal aging. The expression of MHC-II antigens by nonleukocyte-lineage cells is, according to some reports, associated with the induction of immunologic unresponsiveness; therefore, MHC-II expression on vascular endothelial cells in ARMD-affected retinas may represent a means to restrict retinal inflammation.

Most pathogenetic studies of ARMD focus on changes in the outer retina and choroid, but the origin of the signals that provoke subretinal vessels to invade the neural retina remain to be defined. The current findings have shown that modulation of MHC-II expression by retinal vascular components and hypertrophy of microglia are related to incipient ARMD. It has already been suggested that early degenerative changes may occur in the neural retina associated with aging and RPE changes. Additionally, we have shown that human retinal microglia express phenotypical characteristics in common with leukocytes, which have an established capacity to elaborate angiogenic factors. Activated central nervous system-resident leukocytes, including microglia, are a potential source of angiogenic cytokines. The hypertrophic changes observed in resident leukocytes in ARMD retinas indicate activation and implicates microglia as a possible source of angiogenic cytokines, although this connection remains to be demonstrated.

Microglia and astrocytes are major constituents of the perivascular glia limitans of the human retina. Previous investigators have reported the occurrence of autoantibodies against astrocytes in sera from patients with incipient ARMD. We have observed the degeneration of astrocytes in ARMD retinas and, in the current findings, the disruption of astrocytes in aged retinas, particularly in the vicinity of large retinal vessels. Confocal microscopy images (Fig. 3) illustrate the relationship between astrocyte degeneration and increased MHC-II expression on vascular-associated microglia in normal-aged retinas. Taken together, these observations suggest that modulation of MHC-II expression and hypertrophy of retinal astrocytes and microglia contribute to the inception of ARMD. Increased MHC-II immunoreactivity on vascular elements and morphologic changes in microglia were, in particular, associated with incipient ARMD. Immunologic responses in neural retinal microglia and vascular elements appear to be related to early pathogenetic changes in RPE pigmentation and drusen formation.

**Key Words**

age-related macular degeneration, major histocompatibility complex class II, microglia, neural retina, vasculature

**Acknowledgments**

The authors thank Professor F. A. Billson for his continuing support, Professor J. Stone for critical discussions and advice on confocal images, Dennis Dwarte for advice on image and statistical analyses, Tania Balind for performing the histopathologic survey, Christine Tadros for performing the double labeling and confocal microscopy, and the staff of New South Wales Eye Bank for coordinating specimen donation.

**References**

9. Penfold PL, Provis JM, Billson FA. Age-related macular degeneration: Ultrastructural studies of the relation-


