8-2010

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Interferon γ–Inducible Protein-10 (IP-10) and Eotaxin as Biomarkers in Age-Related Macular Degeneration

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Purpose. To analyze serum cytokine levels in subjects with different stages of AMD and to study the expression of salient cytokines in postmortem eyes with AMD.

Methods. A suspension array system was used to analyze sera (n = 18 to 20/group) from control subjects and those with early AMD (AREDS stage 1), intermediate dry AMD (AREDS stage 3), advanced AMD with geographic atrophy (GA), or neovascular AMD (CNV). Postmortem eyes with AMD or control eyes were examined immunohistochemically for expression of IP-10 and eotaxin (n = 4 to 8/group).

Results. Serum eotaxin and IP-10 levels were significantly elevated in all stages of AMD, except for eotaxin levels in neovascular AMD ($P < 0.07$). The peak of serum IP-10 concentration was at intermediate dry AMD. In donor eyes, IP-10 and eotaxin expressions were increased in the RPE of eyes with early AMD, GA, and CNV. Eotaxin accumulated within the layer of basal linear/laminar deposits in all stages of AMD, while IP-10 was mainly in eyes with GA and CNV. IP-10 was abundant in the connective tissue matrix associated with CNV, and eotaxin was usually present but more focally and with less intense staining. Both IP-10 and eotaxin were expressed by neovascular endothelial cells. Both IP-10 and eotaxin were expressed in the neurosensory retina, but there was no detectable difference in staining between eyes with or without AMD.

Conclusions. IP-10 and eotaxin may be early biomarkers in AMD. The authors hypothesize that the relative balance between levels of IP-10 and eotaxin is critical in regulating the neovascular response. (*Invest Ophthalmol Vis Sci.* 2010;51: 4226–4236) DOI:10.1167/iovs.09-3910

Age-related macular degeneration (AMD) is a chronic disease that develops over decades and may lead to severely damaged vision.[1] This process is initially heralded by accumulation in the aging retinal pigment epithelial (RPE) cells of lipofuscin granules from long-term turnover and recycling of photoreceptor outer segments[2] and induction of oxidative stress.[3–5] Early phenotypic findings in AMD include the appearance of hard and soft drusen.[6] Some drusen constituents identified in proteomic studies include proinflammatory stimuli.[4,7,8] It is currently accepted that inflammation plays an important role in the pathogenic progression of AMD.[1,5,9] Thus, control of chronic inflammation may retard the progression to the advanced form of AMD and may limit visual loss from this disease.

Chronic inflammation consists of a series of biological responses to harmful stimuli that include activation of subsets of immune cells, which are recruited to inflamed areas, angiogenesis and scar formation.[10,11] Inflammatory cells are regulated by a multitude of cytokines.[12,13] Inhibition of cytokine release may affect the disease outcome.[14–17] For example, mice deficient in monocyte chemotactic protein (MCP)-1 or its receptor develop cardinal features of AMD.[14] Some cytokines have been implicated in AMD, including vascular endothelial growth factor (VEGF) which supports choroidal neovascularization.[18] Interleukin (IL)-1β and tissue necrosis factor (TNF)-α are also detected in AMD tissues.[19,20] However, the systemic cytokine profile of subjects with AMD has not been studied. Understanding the cytokine profile, especially with respect to inflammation, may further elucidate the underlying pathogenic mechanisms that participate in AMD.

Thus, we hypothesized that the cytokine profile in AMD is changed in response to inflammatory signals that could be manifested in the course of the disease and be detected in the circulation. In this study, we screened 27 serum cytokines in subjects with AMD and in age-matched control subjects with no phenotype or family history of AMD. This cytokine panel included Th-1- or -2–associated cytokines, other inflammatory cytokines, as well as angiogenic and angiostatic factors. After screening, we identified two serum cytokines that were significantly elevated in subjects with AMD: interferon γ–inducible protein-10 (IP-10) and eotaxin. We performed statistical tests to validate that these two cytokines could be biomarkers for detection of the early stage of AMD. We then studied the expression of IP-10 and eotaxin in ocular tissues from eyes with various stages of AMD obtained at autopsy and in control specimens.

IP-10 belongs to a group of α-chemokines in which the first two cysteine residues are separated by an intervening amino acid (CXC) and the ELR tripeptide motif is absent.[11,21] The ELR-lacking α-chemokines possess high affinity for the CXCR3 receptors found on activated T cells and natural killer (NK) cells.[11,22] IP-10 is a known chemoattractant to Th1 lymphocytes and monocytes. It has been considered a pathogenic factor in cerebral malaria,[23,24] multiple sclerosis,[25–27] Graves' disease,[28] and proliferative glomerulonephritis.[29,30] In addition to its role in chemotaxis, IP-10 is angiostatic and antifibrotic.[31,32] Eotaxin is a member of the cc cytokine family.[11] This Th2-associated chemokine is a potent chemoattractant for eosinophils.[11,33] The pathophysiological roles for eotaxin in gastrointestinal allergy and allergic pulmonary disease have been reported.[34–36]

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Supported in part by a grant from the Novartis Institute for Biomedical Research, Cambridge, MA, and in part by the Stone Scholar Fund, Schepens Eye Research Institute.

Submitted for publication April 24, 2009; revised December 1, 2009 and January 26, 2010; accepted February 6, 2010.

Disclosure: **F.M. Mo**, None; **A.D. Proia**, None; **W.H. Johnson**, None; **D. Cyr**, None; **K. Lashkari**, None

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Investigative Ophthalmology & Visual Science, August 2010, Vol. 51, No. 8

Materials and Methods

AMD and Control Subjects

This study was approved by the Institutional Review Board of the Schepens Eye Research Institute. Seventy-eight subjects with phenotypic and clinical evidence of AMD were recruited into the study and were classified according to the Age-Related Eye Disease Study (AREDS)[37] into AREDS stage 1 (early AMD), AREDS stage 3 (intermediate dry AMD), geographic atrophy (defined as geographic loss of photoreceptor-RPE-choriocapillaris), and neovascular AMD that includes classic and occult lesions in active stage. All subjects were examined by slit-lamp biomicroscopy, optical coherence tomography, fundus photography, and fluorescein angiography (when indicated). Eighteen age-matched subjects with no phenotype of AMD and no family history of AMD were recruited as control subjects and were examined by using similar methods. All subjects were given a simple questionnaire regarding their smoking history, and whether they had or were treated for hypertension and hypercholesterolemia. Inclusion criteria included minimum age of 55 years and willingness to participate in the study. Subjects with the following systemic conditions were excluded from the study: any cancer, inflammatory conditions including collagen vascular disease, arthritis, use of nonsteroidal or steroidal anti-inflammatory or immune-modulating agents, diabetes mellitus, kidney or liver disease, vascular diseases such as stroke, blood dyscrasia, or recent surgery (<90 days). The following ocular conditions were also excluded: history of glaucoma or suspected glaucoma, use of topical anti-inflammatory products; history of central or branch retinal vein occlusion, diabetic retinopathy, retinal detachment, other chronic macular disease; and recent cataract extraction (<90 days). Subjects were asked to sign a detailed consent form, and blood samples were obtained for plasma and serum collection. The protocols conducted in human subjects complied with the Declaration of Helsinki.

Serum Samples Preparation and Suspension Array Assay for Cytokine Measurement

Blood samples were allowed to clot for at least 30 minutes at room temperature or at 4°C overnight, and then centrifuged 1000 *g* for 10 minutes to remove cellular components. Sera were collected and stored at −20°C. Suspension array assay components, including the validation kit (Bio-Plex, V4), calibration kit, and human 27-plex were purchased from Bio-Rad Laboratories (Hercules, CA). Samples (50 μL) were diluted fourfold with human serum diluent, and the results were adjusted for the dilution factor. Each sample was run in duplicate on the multicytokine suspension array system (Bio-Plex; Bio-Rad), according to the manufacturer's protocol. The eotaxin detection antibody in the cytokine assay recognizes eotaxin-1.

Tissue Specimens

Eyes of the patients who had died recently and were undergoing autopsy were enucleated and fixed in 4% neutral-buffered formaldehyde solution, embedded in paraffin, and sectioned at Duke University Hospital, Department of Pathology. H&E and PAS (periodic acid-Schiff reagent) stained sections were examined by one of us (ADP) and evaluated for subretinal lesions characteristic of AMD. Early-stage AMD was diagnosed by the presence of confluent drusen or granular and/or striated, eosinophilic, PAS-positive material typical of basal linear/laminar deposit between the RPE and Bruch's membrane. The RPE layer and choriocapillaris remained intact although the RPE was sometimes irregular. GA was characterized by degeneration of photoreceptors and RPE and the presence of a transition zone. CNV exhibited the presence of new vessels on the inner side of the Bruch's membrane with associated RPE and/or photoreceptor degeneration. The control group consisted of age-matched eyes that had normal Bruch's membrane and RPE anatomy with the absence of basal deposits or confluent drusen within the macular area. The use of the donor eyes for research was approved by the Institutional Review Board of the Duke University Health System and the eyes were obtained and managed in compliance with the Declaration of Helsinki.

Immunohistochemistry

Histologic sections were heated at 60°C for 30 minutes, deparaffinized in xylene solution, and rehydrated through a graded ethanol series. For IP-10 staining, tissues sections were treated with a universal blocking reagent (Background Sniper; Biocare Medical, Concord, CA), and then were incubated with mouse monoclonal anti-human IP-10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1.5 μg/mL for 1 hour at room temperature. Slides were washed in TBS-T (Biocare Medical) and incubated with mouse AP-polymer kit (Biocare Medical), stained with a fast red chromogen kit (Vulcan-2; Biocare Medical), and counterstained with hematoxylin (Vector Laboratories, Burlingame, CA). For eotaxin staining, antigen was retrieved by incubation with 0.5% pepsin in 10 mM HCl at 37°C for 10 minutes. Tissue sections were blocked with 10% goat serum and avidin for 1 hour and then incubated with rabbit polyclonal anti-human eotaxin antibody (Abcam, Inc., Cambridge, MA) at a concentration of 4 μg/mL for 2 hours. This antibody recognizes all three known forms of eotaxin. Tissue sections were washed with TBS-T, incubated with biotinylated goat anti-rabbit antibody (1:250), stained by AP-linked substrate kit (Vector Laboratories), and counterstained with hematoxylin. Negative control slides were treated identically except that corresponding nonimmune IgG isotype was substituted for the primary antibody.

Statistical Analysis

GraphPad Prism 4 and Prism statistics (GraphPad Software, Inc., La Jolla, CA) were used to plot graphs and perform statistical analyses. Since serum samples were not distributed in a Gaussian fashion, a Mann-Whitney test was used to test the statistical difference between the AMD and control groups. A nonparametric age-matched paired Wilcoxon signed-rank sum test was also used to compare the signifi-

Table 1. Clinical Profile of Study Subjects

	Control	AREDS I	AREDS III	GA	CNV
Total subjects, *n*	18	20	19	20	19
Men	9	6	7	6	5
Women	9	14	12	14	14
Mean age in years ± SD	69 ± 2	72 ± 2	78 ± 1	83 ± 2	81 ± 1
Smoker					
No	12	15	9	11	12
Former	5	4	9	5	5
Yes	1	1	1	4	2
Hypertension					
No	8	9	5	10	7
Yes	10	11	14	10	12
High cholesterol					
No	11	12	12	16	11
Yes	7	8	7	4	8

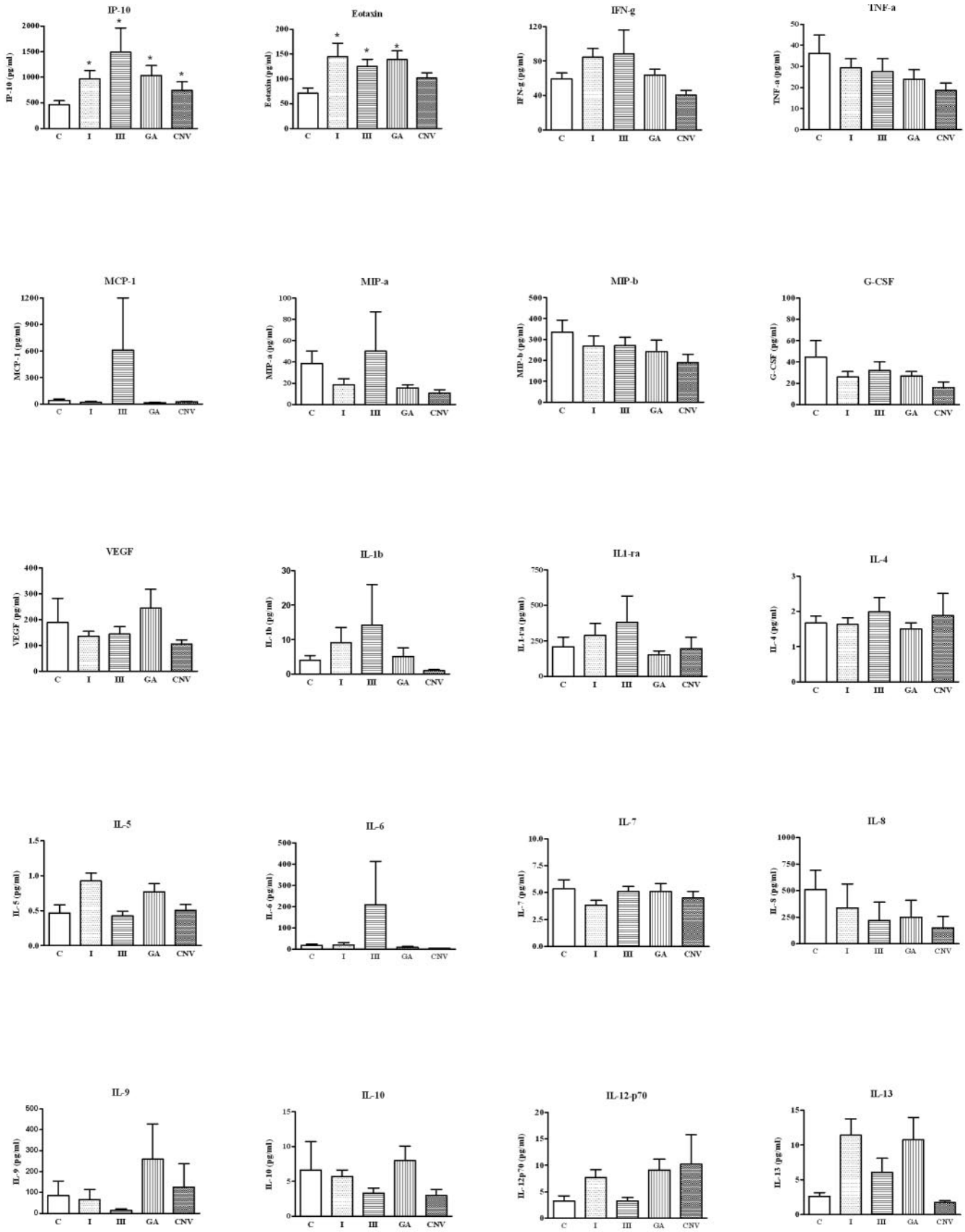

FIGURE 1. Twenty serum cytokine concentrations are represent mean and SEM. *Significant differences between control subjects and those with each stage of AMD by the Mann-Whitney test.

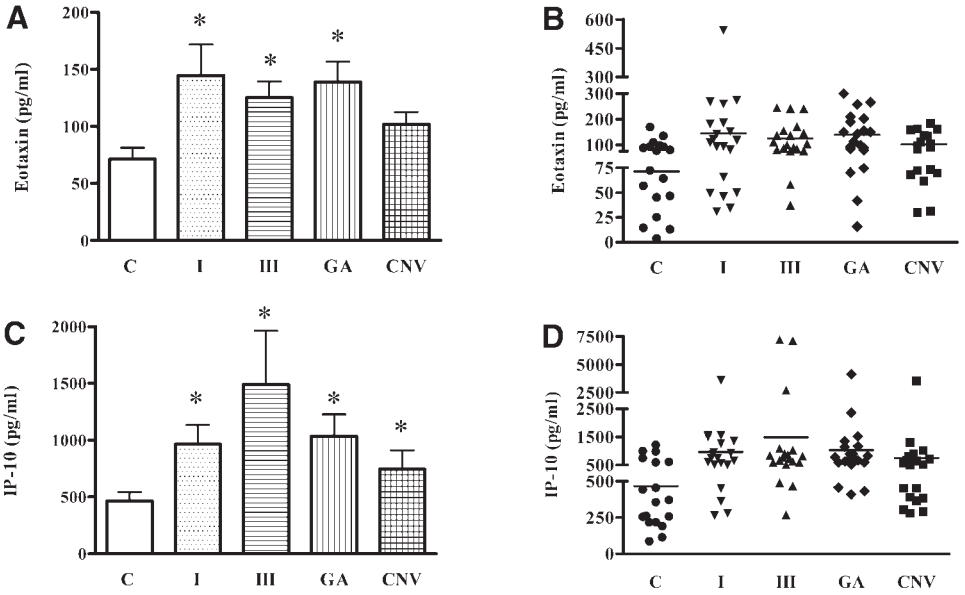

FIGURE 2. Mean serum concentrations and individual serum distributions of eotaxin and IP-10 are shown for subjects with various stages of AMD and for control individuals. (**A**) Mean serum eotaxin concentration; (**B**) individual distribution of serum eotaxin concentration; (**C**) mean serum IP-10 concentration; (**D**) each individual distribution of IP-10. Error bars represent SEM; *statistically significant differences between control subjects and those with each stage of AMD (Mann-Whitney test).

cant differences in cytokine levels between the AMD and control groups; the result excluded age as a confounding factor.[38]

RESULTS

Serum Cytokines and AMD

Table 1 summarizes the clinical data of all 96 subjects. There were between 18 and 20 subjects in each group. The distribution of smokers, hypertension, and cholesterol levels in the study groups are indicated.

The results of the analysis of levels of 20 cytokines in these samples are shown in Figure 1. The concentrations of five cytokines (IL-2, IL-15, IL-17, FGF, and GM-CSF) were below 0.2 pg/mL, the lowest limit of detection for these analytes. These cytokines were therefore regarded as undetectable. The levels of RANTES and PDGF-bb exceeded the upper limit of the standard curves and were excluded from the study. Of the 20 cytokines studied, only IP-10 and eotaxin were significantly elevated in the AMD study group. These two cytokines were selected for further study and analysis.

Serum Eotaxin and IP-10 Levels in Subjects with AMD

The mean serum concentrations and individual distributions of eotaxin and IP-10 are shown in Figure 2. Eotaxin was significantly increased in AREDS stages 1 and 3 and in GA ($P < 0.02$, < 0.007, and < 0.005, respectively), but not in neovascular AMD (CNV; $P < 0.07$). There were no differences in eotaxin concentrations among AMD subgroups.

IP-10 was increased in AREDS stage 1 ($P < 0.004$) and remained high in all stages of AMD. In AREDS stage 3, IP-10 levels reached a peak ($P < 0.002$). In advanced AMD, IP-10 levels were lower in subjects with neovascular AMD than in AREDS stage 3 and GA ($P < 0.05$ and $P < 0.03$, respectively).

Age-Matched Pair Analysis of IP-10 and Eotaxin in Subjects with AMD

Subjects in the control group ranged between 59 and 85 years of age. There was a similar wide span of ages in the four groups of subjects with AMD. However, mean ages were higher in the groups with more severe AMD. To illustrate, we assigned a numeric code for the stages of AMD, with 0 assigned to no AMD, 1 to AREDS stage 1, 3 to AREDS stage 3, 4 to neovascular AMD, and 5 to GA (Fig. 3). The strong correlation between age and level of AMD necessitated the use of statistical methods to remove age as a factor when studying cytokine levels and AMD stage. To accomplish this adjustment, age-matched groups were formed and levels of eotaxin and IP-10 were compared only in subjects of the same age (within 1 year). To avoid any assumptions about normality, we used the nonparametric matched pair Wilcoxon signed rank sum test. The null hypothesis states that for subjects of the same age, the level of cytokine (eotaxin or IP-10) is the same for control subjects and those with any stage of AMD. Twelve age groups, each containing control subjects (AMD = 0) and subjects with AMD (AMD > 0) were formed, and the average cytokine level was calculated for each age group. The difference between these two average cytokine levels was then determined according to the following equation:

$$\text{Difference}_{\text{same age}} = \text{mean cytokine (AMD} > 0) - \text{mean cytokine (AMD} = 0).$$

Results of a comparison of cytokine levels in age-matched AMD subjects and control subjects are shown in Table 2. The

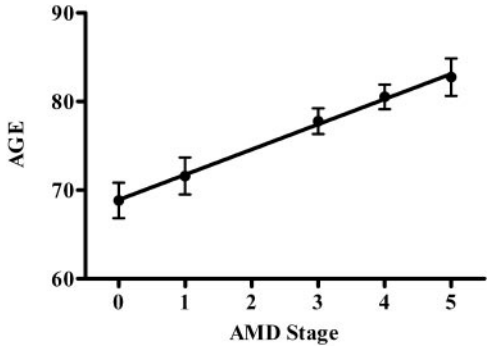

FIGURE 3. The linear increase in the mean age of subjects against the severity of AMD. Each stage of AMD was assigned a numeric code: 0, AREDS 0 (no AMD); 1, AREDS stage 1; 2, AREDS stage 2; 3, AREDS stage 3; 4, neovascular AMD; and 5, AMD with GA. Error bars represent SEM.

TABLE 2. Wilcoxon Signed Rank Sum Test for Serum Eotaxin and IP-10 in Age-Matched Subject Pairs

Mean Concentrations of Eotaxin

A. Comparison of Mean Eotaxin Concentrations in Control Group and in Subjects with AMD

Age Group (y)	Control Group (AMD = 0)	Subjects with AMD (AMD > 0)	Difference (AMD > 0 − AMD = 0)
58–59	13.7	93.3	79.6
60	45.8	202.4	156.5
62	152.0	121.0	−31.0
65	95.0	129.8	34.8
67	61.4	241.6	180.2
68–69	97.4	119.7	22.3
70	72.4	177.0	104.5
72	45.2	151.8	106.5
74	25.2	71.2	46.0
80	74.7	129.1	54.4
83	92.9	126.6	33.8
85	64.5	183.8	119.3
			$P < 0.005$

B. Comparison of Mean Eotaxin Concentrations in Control Group and in Subjects with Stage 1 AMD

Age Group (y)	Control Group (AMD = 0)	Subjects with Stage 1 AMD (AMD = 1)	Difference (AMD = 1 − AMD = 0)
58–59	13.7	93.3	79.6
60–62	98.9	86.6	−12.3
65	95.0	129.8	34.8
67–69	73.4	46.0	−27.4
70	72.4	267.9	195.5
72	45.2	151.8	106.5
73–74	25.2	260.0	234.8
80	74.7	49.6	−25.1
83	92.9	34.4	−58.5
85	64.5	543.0	478.6
			$P < 0.05$

Mean Concentrations of IP-10

C. Comparison of Mean IP-10 Concentrations in Control Group and in Subjects with AMD

Age Group (y)	IP-10 (AMD = 0)	IP-10 (AMD > 0)	Difference (AMD > 0 − AMD = 0)
58–59	166.9	527.6	360.7
60	264.6	432.0	167.4
62	505.8	791.8	286.0
65	808.2	1029.4	221.2
67	676.9	509.0	−167.8
68–69	190.2	717.0	526.8
70	1228.0	463.3	−764.7
72	355.6	564.7	209.1
74	254.3	4510	196.7
80	404.5	1405.4	1001.0
83	455.1	1007.6	552.5
85	261.9	1563.8	1301.9
			$P < 0.025$

D. Comparison of Mean IP-10 Concentrations in Control Group and in Subjects with Stage 1 AMD

Age Group (y)	IP-10 (AMD = 0)	IP-10 (AMD > 0)	Difference (AMD = 1 − AMD = 0)
58–59	166.9	529.6	360.7
60–62	385.2	86.6	220
65	808.2	1029.4	221.2
67–69	514.6	753.4	238.8
70	1228.0	657.1	−570.9
72	355.6	564.7	209.1
73–74	254.3	3600.0	3345.7
80	404.5	1429.1	1024.6
83	455.1	1558.0	1102.8
85	261.9	893.4	631.5
			$P < 0.025$

absolute values of the differences were ranked and the ranks of the negative differences were summed. Most of the differences among age-matched groups were positive (AMD > 0 − AMD = 0) indicating that the level of the cytokine in any AMD category was higher than those in the control group. Table 2A shows the concentrations of eotaxin in control subjects (AMD = 0), in subjects with any stage AMD (AMD > 0), and their respective difference, in an age-matched manner. The Wilcoxon statistical test[38] indicates that the probability of such a distribution is <0.005. We concluded that eotaxin levels at any stage of AMD were higher than in control subjects with no AMD.

To determine whether elevated levels of eotaxin could be used as a biomarker for early-onset AMD (AREDS stage 1), age-matched groups of subjects with AMD = 0 were compared with those with AMD = 1. The difference between eotaxin levels for AREDS stage 1 (AMD = 1) and control (AMD = 0) was calculated according to the following formula:

$$\text{Difference}_{\text{same age}} = \text{eotaxin (AMD = 1)} - \text{eotaxin (AMD = 0)}.$$

Of the 10 groups in which the age in each group was the same (within 2 years; Table 2B), differences were dominantly positive with the largest differences being always positive. The Wilcoxon test shows the probability of such a distribution is <0.05. We concluded that eotaxin levels in subjects with AREDS stage 1 AMD are significantly higher than those in control subjects with no AMD.

A similar analysis was performed for IP-10 using the same age-matched groups. Table 2C shows the concentrations for IP-10 for control subjects (AMD = 0), subjects with any AMD (AMD > 0), and their differences. The concentrations of IP-10 in subjects with any stage of AMD were significantly higher than those in the control group. The Wilcoxon test shows the probability of such a distribution is < 0.025.

Table 2D shows the concentrations of IP-10 for age-matched control subjects (AMD = 0), subjects with AREDS stage 1 (AMD = 1) and their differences and shows significantly higher IP-10 levels for subjects with stage 1 AMD than in the control group with no AMD ($P < 0.025$).

Immunohistochemical Localization of IP-10 and Eotaxin in Eyes, with or without AMD

The expression of IP-10 and eotaxin in eyes obtained at autopsy of patients with different stages of AMD and in age-

matched non-AMD control eyes was determined with immunohistochemical staining. Staining intensity was graded on a scale of 0 to 3: 0, no expression; 1, very faint staining (visible only at high magnification); 2, moderate staining (easily visible at intermediate magnification); 3, strong staining (visible at low magnification). Results in all the eyes are presented in Table 3 (IP-10 scores) and Table 4 (eotaxin scores). Staining intensity was scored by two investigators (FMM, ADP), and the results are a consensus of their observations.

Both IP-10 and eotaxin were expressed in the neurosensory retina in the nerve fiber layer, ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), and photoreceptor outer segments (POS); staining intensity was variable among the eyes within each group, and there was no consistent difference in staining among the eyes with or without AMD, as shown in Tables 3 and 4 and Figures 4 and 5. For all eyes, we performed negative controls for IP-10 and eotaxin using nonimmune IgG isotype in place of the primary antibody. The negative control slides uniformly lacked stain (results not shown).

Despite the variation in staining intensity for IP-10 among eyes within each group, there were definite differences in IP-10 expression in control eyes and in those with AMD. As shown in Figure 4, there was enhanced expression of IP-10 in the RPE of eyes with early AMD, GA, and CNV. IP-10 accumulated focally within the layer of basal linear/laminar deposit in GA and CNV, and it was uniformly and strongly expressed by neovascular endothelial cells and within the connective tissue matrix associated with the CNV.

There was also variation in staining intensity for eotaxin among eyes within each group, but again, there were distinct differences in eotaxin expression in control and AMD eyes. Figure 5 illustrates enhanced expression of eotaxin in the RPE of eyes with early AMD, GA, and CNV. There was accumulation of eotaxin within the basal linear/laminar deposit in all stages of AMD, though the staining was patchy (focal) and less than that observed for IP-10. Similar to our results for IP-10, we consistently observed eotaxin expression by neovascular endothelial cells. There was often eotaxin within the connective tissue matrix associated with CNV, but the expression of eotaxin was more focal and the staining less intense than for IP-10 in this tissue compartment (for example, compare the staining for IP-10 in Fig. 4H with that for eotaxin in Fig. 5H).

Discussion

AMD has been proposed as an inflammatory disease.[5] Our findings implicate IP-10 and eotaxin as two inflammatory cyto-

Table 3. Distribution of IP-10 in the Macular and Subjacent Choroid

Case	NFL	GCL	IPL	INL	OPL	ONL	POS	RPE	BLD	Choroid EC	Ch. Stroma
Control											
1	2	2	1	0	1	1	3	0		0	1
2	1	2	0	0	1	0	2	Focal 1		0	1
3	2	2	1	Focal 1	0	0	3	Focal 2		Focal 1	Focal 1
4	2	3	2	1	1	0	2	0		1	2
5	Focal 2–3	3	1	1	1	1	3	Focal 2–3		1	2
6	1	2	1	0	1	0	1	Focal 1–2		0	0
7	0	3	1	1, Focal 2	0	Focal 1	2	0		0	2
8	1	3	1	1	0	1	2	Focal 1–2		0	Focal 1
Early AMD											
11	2	3	2	1	1	1	3	3	0	2	3
12	2	3	1	Focal 0–1	0	1	2	Focal 3	Focal 2	0	Focal 1
13	3	3	2	0	1	1	3	Focal 2	0	0	2
14	2	3	1	1	0	0	2	2	0	0	1
15	2	3	2	1	2	1	3	2	0	1–2	2
16	1	3	2	2	2	1	3	1	0	1	1
17	2	3	2	1	2	2	2	3, Focal 4	Focal 1	0	1
18	2	3	1	Focal 1–2	1	1	2	Focal 1	0	0	2
GA											
21	2	2	1	0	0	0	—	1	0	0	2
22	2	3	2	1	0	—	—	2	Focal 1–2	0	0
23	2	3	2	1	1	1	3	3	Focal 3	1	1
24	2	3	2	1	2	0	3	2	Focal 3	Focal 1	1

Case	NFL	GCL	IPL	INL	OPL	ONL	POS	RPE Outside	RPE CNV Area	BLD	Choroid EC	Neovasc EC	Ch. Stroma
CNV													
27	1	3	2	1	2	1	3	3	Focal 2	Focal 3	1	2	1–2
28	3	3	1	2	2	Focal 1–2	2	3, Focal 4	Focal 1–2	Focal 2	1	3	2
29	3	3	1	1	1	1	3	3	Focal 1–2	Focal 3	1	3–4	2
30	2	3	1	1	1	Focal 1	1	2–3	Focal 2–3	2	1	2	2
31	1	2	1	1	1	Focal 1	2	1	Focal 3	1, Focal 3	1	3	3
32	2	3	1	1	0	Focal 1	2	1, Focal 2	1, Focal 2	Focal 3	1	2, Focal 3	1

Grading scale for the expression of IP-10 in the macula and subjacent choroid. 0, no expression; 1, very faint expression; 2, moderate staining; 3, strong staining; Focal, focal expression; NFL, nerve fiber layer; IPL, inner plexiform layer; OPL, outer plexiform layer; BLD, basal linear/laminar deposits; Choroid EC, choroidal endothelial cells; Ch. stroma, choroidal stromal cells; Neovasc EC, neovascular endothelial cells; RPE outside, RPE cells outside the area of choroidal neovascularization; RPE CNV area, RPE cells within the area of choroidal neovascularization.

Table 4. Distribution of Eotaxin in the Macular and Subjacent Choroid

Case	NFL	GCL	IPL	INL	OPL	ONL	POS	RPE	BLD	Choroid EC	Ch. Stroma
Control											
1	3	3	1	1	2	1	1	0		1	2
2	3	3	1	1	2	1	2	0		Focal 1	2
4	0	3	1	1	1	1	3	1		2	
6	0	2	0	1	0	0	2	0		2	
7	0	3	1	1, Focal 2	0	1	2	0		1–2	1
8	0	1	0	1	0	0	0	0		0	0
9	1	2	1	Focal 1	1	Focal 1	1	0		1	1
10	1	2	0	1	0	1	3	1		1	
Early AMD											
12	0	2	0	1	0	1	0	Focal 1	Focal 2	1	2
13	0	3	1	2	1	1	3	2	3	2	2
14	0	0	0	0	0	0	0	0	3	1	0
16	0	3	0	1	0	0	3	3	Focal 1	2	2
19	0	3	1	2	1	0	3	2	3	2	1
20	0	3	1	2	2	2	3	2	3	2	2
GA											
22	2	2	0	1	1	1	3	1	Focal 2	2	2
24	0	3	0	2	0	1	3	3	Focal 3	2	2
25	0	1	0	0	0	0	0	1	Focal 2	1	1
26	0	0	0	0	0	0	0	0	1–2	0	0

Case	NFL	GCL	IPL	INL	OPL	ONL	POS	RPE Outside	RPE CNV Area	BLD	Choroid EC	Neovasc EC	Ch. Stroma
CNV													
27	0	1	0	1	0	0	0	1	Focal 3	0	Focal 2	2	0
29	2	3	1	2	2	3	3	2, Focal 3	1	0	2, Focal 3	3	2
30	0	3	2	2	1	2, Focal 3	3	1, Focal 2	1, Focal 2	Focal 1	2	3	2
31	3	3	1	2	1	Focal 3	2	2	3	1, Focal 2	2–3	3	2
32	1	3	1	2	1	2, Focal 3	2	2	2, Focal 3	2, Focal 3	1, Focal 2–3	3	2
33	0	3	2	2	1	1, Focal 3	3	2	2	Focal 2	2, Focal 3	3	2
34	1	3	1	2	2	1, Focal 3	3	2	2	Focal 1	1	2, Focal 3	1

See the footnote to Table 3 for an explanation of the data and abbreviations that have not been used in the text.

kines that are associated with the pathogenesis of AMD. The serum level of both cytokines was increased significantly in subjects with AMD, and both were overexpressed in histologic sections of postmortem eyes with AMD.

In the early stage of AMD, IP-10 serum levels were increased significantly (Fig. 1). Histologic sections of eyes with early AMD exhibited increased immunoreactivity for IP-10 in the RPE (Fig. 4D), and some eyes had focal staining within the basal linear/laminar deposit. Of interest, the peak serum IP-10 concentration was detected at AREDS stage 3, remained high in GA, and decreased slightly in the subjects with CNV ($P < 0.03$ when compared to the peak level). Other inflammatory diseases, such as Graves' disease,[28] type 1 diabetes mellitus,[39] graft rejection,[40] and cerebral malaria,[23] exhibit high levels of IP-10 in the circulation and in local tissues during the initial and developing phases of the immune response. In some of these diseases, IP-10 has been implicated as a pathogenic factor that mediates Th-1 type inflammation by acting to recruit a subset of leukocytes and promote their subsequent migration into the tissue. In eyes, the potential role of IP-10 is intriguing since the eye is considered an area of immune privilege.[41] Elevated circulating levels of IP-10 may be derived from activated lymphocytes as well as by the resident cells.[28] In tissues of eyes with AMD, leukocytes have been identified in the choroid beneath the area where drusen accumulate, suggesting that there is trafficking of a specific subset of immune cells.[42] Double staining of ocular tissues with AMD indicates that $CD45^+$ cells resembling leukocytes in the choroid also express IP-10 (FMM, KL, unpublished observation, 2009), and thus may represent a source of the IP-10.

The immunoreactivity of IP-10 in the macular RPE of control eyes was absent or present focally and usually with a low staining intensity (Fig. 4B, Table 3). In eyes with early AMD, GA, and CNV there was increased expression of IP-10 in RPE cells. Eyes with GA and CNV had loss of RPE in the center of the lesions, but residual RPE cells expressed IP-10 with more intense staining than that in control eyes. RPE cells play a critical role in the blood–retinal barrier and in the maintenance of the photoreceptor.[1] As RPE cells age or are subjected to oxidative stress, their expression profile of cytokines is altered and they may upregulate proinflammatory or proangiogenic cytokines such as VEGF, basic fibroblast growth factor-2, and interleukin-8 for wound-healing or other pathologic functions.[43–46] The ability of IP-10 to antagonize the angiogenic effect of VEGF may be particularly important in the pathogenesis of CNV. RPE-derived soluble VEGF is essential in the maintenance of the choriocapillaris.[47] In vitro and in vivo studies show that VEGF can induce overexpression of IP-10 in endothelial cells,[48] but whether this may occur with the RPE is unknown. Endothelial cells within the choroidal neovascular membrane and the connective tissue matrix associated with the CNV had strong expression of IP-10 (Fig. 4H), which is intriguing, since IP-10 is well-known for its angiostatic and antifibrotic activity.[31,32] It inhibits endothelial cell proliferation by competing with the cells for the binding sites of heparan sulfate proteoglycans, resulting in attenuation of new vessel

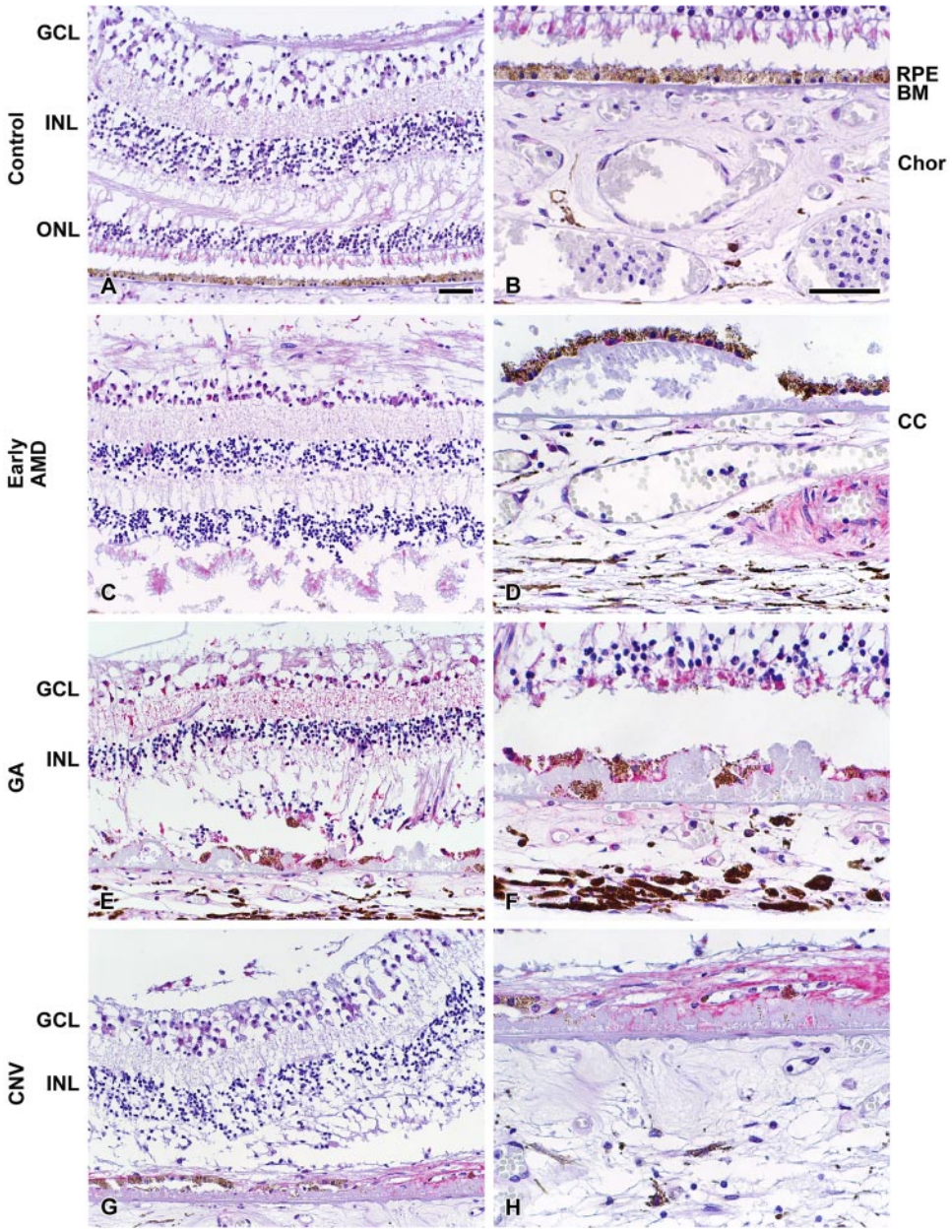

FIGURE 4. Immunohistochemical localization of IP-10 in postmortem eyes with various stages of AMD and in age-matched control eyes without AMD. IP-10 was detected using an alkaline phosphatase reagent. The tissue was counterstained with hematoxylin and the nuclei are blue. IP-10 was expressed in the neurosensory retina in the nerve fiber layer, GCL, INL, ONL, and photoreceptor outer segments; there was no consistent difference in staining among the eyes without (**A**, **B**) or with AMD (**C**, **E**, **G**). There was enhanced expression of IP-10 in the RPE of eyes with early AMD (**D**), GA (**F**), and CNV (**H**). IP-10 accumulated focally within the layer of basal linear/laminar deposit in GA (**F**) and CNV (**H**), and it was uniformly and strongly expressed by neovascular endothelial cells and within the connective tissue matrix associated with the CNV (**H**). Scale bar, 50 μm.

formation.[49,50] Based on these reports, we speculate that the IP-10 we observed within the neovascular membrane may be an important mechanism for dampening the effects of angiogenic and fibrotic cytokines released during development of CNV.

Eotaxin was reported recently to be expressed in surgically excised choroidal neovascular tissue, as was its receptor CCR3.[51] It was present in blood vessels and stromal cells in the excised neovascular tissues,[51] similar to our observations in donor eyes with CNV. In our study, eyes with early AMD and GA had increased expression of eotaxin by RPE cells and accumulation of eotaxin within basal linear/laminar deposits. We also observed constitutive expression of eotaxin in the neurosensory retina and stromal cells in the choroid. Eotaxin can be secreted by endothelial cells, smooth muscle cells,[52] fibroblasts,[53] bronchial epithelial cells, and inflammatory cells, such as macrophages and eosinophils.[54] Our study expands the list of tissue sources of eotaxin to include cells of the neurosensory retina and choroid. The angiogenic character of eotaxin was first proposed in 2001,[55] and a study by Takeda et al.[51] showed that CCR3 blockade was effective at reducing CNV in a mouse model. Takeda et al. concluded that eotaxin and its receptor are important contributors to neovascularization in association with AMD, and our results support this hypothesis.

In addition to searching for cytokines with potential pathogenic relevance in AMD, a second purpose of our study was to determine whether serum cytokine levels could serve as biomarkers to predict the early onset of AMD. Only serum IP-10 and eotaxin concentrations were significantly increased in subjects with AMD. Elevation of serum IP-10 has been proposed as a biomarker to predict the outcome in many human diseases such as severe acute respiratory distress syndrome,[56] coronary artery disease, especially in patients with restenosis,[57] and as a risk factor for renal allograft failure.[58] Serum eotaxin also increased in asthma,[59] in obesity,[60] and in a subgroup of hemorrhagic shock patients with multiple organ failure.[61] Eotaxin release may be stimulated in inflamed tissues, such as in allergic airway inflammation[33] and interstitial nephritis.[62] Interleu-

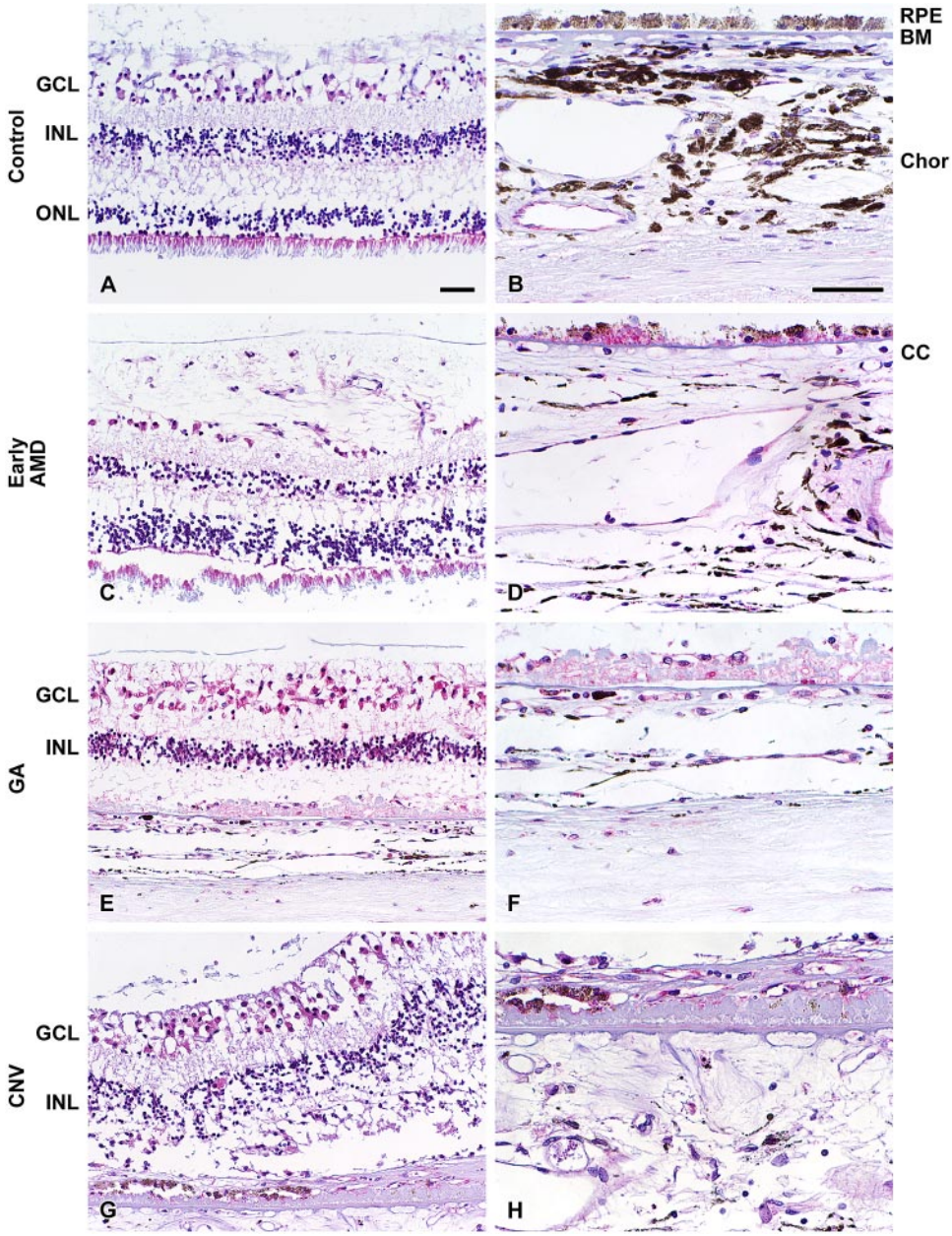

FIGURE 5. Immunohistochemical localization of eotaxin in postmortem eyes with various stages of AMD and in age-matched controls without AMD. Eotaxin was detected using an alkaline phosphatase reagent; tissue was counterstained with hematoxylin and the nuclei are blue. Eotaxin was expressed in the neurosensory retina in the nerve fiber layer, GCL, INL, ONL, and photoreceptor outer segments; there was no consistent difference in staining among the eyes without (**A**, **B**) or with AMD (**C**, **E**, and **G**). There was enhanced expression of eotaxin in the RPE of eyes with early AMD (**D**), GA (**F**), and CNV (**H**). Eotaxin accumulated within the basal linear/laminar deposit in all stages of AMD, though the staining was often patchy (focal). Eotaxin was expressed by neovascular endothelial cells and it was often present within the connective tissue matrix associated with CNV (**H**). Scale bars, 50 μm.

kin (IL)-6 was not elevated in our cohort. Seddon et al.[63] have reported elevated IL-6 levels in subjects with progression of AMD. Another large population cohort study by Klein et al.[64] found the elevation of IL-6 is in association only with geographic atrophy but not with early AMD or neovascular AMD. We cannot explain the reason for the difference. The disparity may be due to the different criteria in subject selection. We excluded all subjects who had cardiovascular disease other than hypertension. IL-6 has been proposed as a biomarker for cardiovascular and cerebrovascular disease[65] and in the study by Seddon et al.,[63] subjects with cardiovascular disease were included. Future controlled studies are needed to explore the association between IL-6 in AMD.

In our study, aging correlated significantly with progression of AMD (Fig. 3), and serum IP-10 level has been reported to increase in aging.[66] We therefore performed age-matched paired-comparison studies to exclude age as a factor in the elevation of IP-10 in the subjects with AMD. Both IP-10 and eotaxin were significantly elevated in subjects with AREDS stage 1 compared with the control group. Our results raise the possibility that serum levels of IP-10 and eotaxin may serve as biomarkers for early AMD, when vision is unaffected and before the appearance of any significant phenotypic changes in the retina. However, a larger study is needed to validate serum IP-10 and eotaxin as biomarkers for early AMD. Such a study will be useful since early diagnosis will become increasingly important as new agents are discovered that will retard the progression of dry AMD.

Acknowledgments

The authors thank Patricia D'Amore, PhD, for manuscript amendment, Andrius Kazlauskas, PhD, for providing helpful comments, Zuowei Su, PhD, for technical help with the immunohistochemical staining, Priscilla Chow for assisting with the immunohistochemical staining, and Susan Reeves for preparing the composite photomicroscopic figures.

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