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Age-Related Decline in VIP-Positive Parasympathetic Nerve Fibers in the Human Submacular Choroid

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Abstract

Purpose—An age-related decline in macular choroidal blood flow (ChBF) occurs in humans. Vasodilatory nerve fibers containing vasoactive intestinal polypeptide (VIP) innervate choroidal blood vessels. The current study was conducted to examine the possibility that an age-related loss of these fibers might occur in the submacular choroid in humans, and thus contribute to a decline in ChBF.

Methods—Macular choroid punches were collected from 35 healthy human donors ranging from 21 to 93 years of age. Choroidal samples were immunolabeled using anti-VIP and the peroxidase–antiperoxidase method. VIP-positive nerve fiber abundance was quantified in up to 12 fields per punch. Fifty macular punches were analyzed, and results for eye pairs were averaged. Choroidal vessel diameter (ChVD) was measured for these same fields. The relationship between age and vessel diameter or VIP-positive fiber abundance was analyzed. Multivariate statistical models were generated correcting for gender, variables related to the tissue specimens, and potential procedural sources of variability.

Results—The fully adjusted multivariate models showed a significant age-related reduction in both the VIP-positive fiber abundance ($P = 0.0003$, adjusted $R^2 = 0.51$) and ChVD ($P < 0.0001$, adjusted $R^2 = 0.63$), with slopes of -0.45 and -0.19 , respectively. Adjusting for the same variables, VIP-positive fiber abundance showed a significant direct correlation with ChVD.

Conclusions—The results indicate a significant age-related decline in VIP-positive nerve fibers and vessel diameter in the submacular choroid in disease-free human donor eyes. These findings suggest that a decline in the neural control of ChBF and vessel diameter may explain the reductions in ChBF and its adaptive control observed clinically with aging.

The choroid is the vascular supply to the outer retina and is responsible for supplying nutrients and removing waste from the outer retina. The 15 to 20 ciliary arteries that run parallel to the

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sclera are the conduits for the blood to enter the choroid.¹ Choroidal circulation accounts for approximately 85% of total ocular blood flow² and 65% of the ocular oxygen supply.³ Photoreceptors have high metabolic rates,⁴ and an adaptively regulated high rate of choroidal blood flow (ChBF) is likely to be important in maintaining the health of the retina.^{5,6} Normal ChBF levels have been shown to be vital in the maintenance of the overlying outer retina, as evidenced by the retinal pigment epithelium (RPE) and photoreceptor dysfunction and loss concomitant with outer retinal hypoxia⁷⁻⁹ or diminished ChBF.¹⁰⁻¹² Studies in pigeons have specifically demonstrated that diminished basal ChBF, loss of choroidal vessels, and impaired parasympathetic regulation of the ChBF occur during the aging process before degenerative changes in the outer retina and most likely contribute to subsequent losses of photoreceptors.^{13,14} These findings suggest that abnormalities in choroidal morphology and ChBF may precede and contribute to age-related decline in vision.

Similar reductions in ChBF occur in the macula of humans as they age.^{15,16} Moreover, reductions in ChBF in excess of what aging alone would predict have been observed beginning in the early stages of age-related macular degeneration (AMD),¹⁷⁻²² the leading cause of blindness in humans older than 65 years.²³ Adaptive regulation of ChBF also is impaired in aged humans, since compensatory responses of ChBF to fluctuations in systemic blood pressure are abnormal in elderly humans.²⁴ These various findings raise the possibility that abnormalities in macular ChBF and/or in its adaptive regulation play a role in the pathogenesis of normal age-related declines in visual ability and in the sequence of events leading to the catastrophic losses observed in AMD.

The mechanisms responsible for the reduced ChBF and impaired adaptive regulation are uncertain. Loss and narrowing of submacular choroidal vessels in normal aged eyes and in atrophic AMD eyes has been reported.^{21,25,26} Such vascular changes may contribute to the reduction in basal ChBF seen with aging and in early AMD. Like in birds and other mammals, ChBF in humans is regulated by vasodilatory parasympathetic and vasoconstrictory sympathetic nerve fibers.^{4,27} The major parasympathetic innervation of the choroid arises from the pterygopalatine ganglion (PPG), which receives its preganglionic input via the seventh cranial nerve.^{4,28,29} This parasympathetic innervation utilizes vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) as potent vasodilators of choroidal vessels.³⁰⁻³³ In addition, the submacular choroid in humans contains intrinsic ganglion cells that synthesize VIP and NO, and contribute to the vasodilatory innervation of the choroid.^{30,34} A diminution in vasodilatory innervation from either source would be expected to both reduce ChBF and impair its adaptive regulation. Consistent with this possibility, diminished ChBF and adaptive choroidal vasodilation in aged pigeons has been attributed to impaired parasympathetic control of the choroid.^{13,14} Thus, diminished ChBF and its adaptive control in humans could, at least in part, stem from impaired parasympathetic control of the choroid. In the present study, we used immunolabeling for VIP, a robust marker of these parasympathetic nerves and a major vasodilator, to examine the possibility that age-related loss of VIP-containing fibers occurs in the submacular choroid in humans.

Methods

Tissue Preparation

This study adhered to the tenets of the Declaration of Helsinki and received IRB approval. A total of 35 healthy human subjects (17 men and 18 women) ranging from 21 to 93 years of age (mean, 62.8 ± 19.7 years [SD]) were included in the study. Of these, both eyes were available for study from 15 donors, for a total of 50 macular punches. Data from eye pairs were averaged. The eyes were obtained from the MidSouth Eye Bank (Memphis, TN) and the Lions Eye Bank (Portland, OR) and were stored in 10% formalin until they were used in the study. Some pairs of eyes were excluded from use because of associated factors that might have led to skewed

or inaccurate conclusions. Among these factors was diagnosis of diabetic retinopathy or the presence of diabetes mellitus for ≥ 5 years, retinal detachment with macular involvement, glaucoma, AMD, and inflammatory disease of the uveal tract. Eyes with long periods of time between death and enucleation (longer than ~8 hours) and/or preserved in glutaraldehyde were also not used.

The anterior segment and vitreous gel of each eye were removed after a circumferential cut, approximately 4 mm from the limbus. A dissecting microscope (SZ-PT; Olympus, Center Valley, PA) with a mounted digital camera (CoolSnap; Photometrics, Tucson, AZ) was used to capture high-resolution images (MetaVue software; Universal Imaging Corp. division of Photometrics, ver. 6.2r6; West Chester, PA) for detailed fundus evaluation. The images were placed in a database and objectively evaluated in a blinded fashion. After examination of the macular region and confirmation of no ocular disease, a 6.0-mm diameter dermal biopsy punch (Miltex, York, PA) was centered about the fovea and used to isolate the entire macular region from each posterior segment (Fig. 1). Examples of the posterior pole of donor eyes are shown in Figures 2A and 2C. The macular choroids were then removed from the scleral and retinal layers with the aid of the dissecting microscope.

Immunohistochemistry

To remove endogenous pigmentation, we bleached the macular choroid samples in 0.25% potassium permanganate. The reaction was stopped with 1% oxalic acid, and then the choroid punches were rinsed three times with 0.1 M sodium phosphate buffer (PB; pH 7.4).³⁵ Our previously published protocols were used for immunohistochemical staining.³⁶ Briefly, nonspecific binding sites were blocked with 5% dry milk, 1% goat serum, and 0.3% Triton X-100, in 0.1 M PB for 2 hours at room temperature. Rabbit anti-VIP antibody (1:1000; Immunostar, Hudson, WI) was diluted in 1% dry milk, 1% goat serum, 0.3% Triton X-100, and 0.1 M PB before incubation for 96 hours at 4°C. Choroid punches were then washed three times with 0.1 M PB before incubation donkey anti-rabbit antibody secondary antibody (1:50, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. After three rinses with 0.1 M PB, choroid samples were incubated in peroxidase-rabbit antiperoxidase (PAP) for 1 hour at room temperature. After three additional rinses with 0.1 M PB, immunolabeling in the macular choroids was visualized with diaminobenzidine (DAB). Samples were incubated in DAB for 10 minutes, followed by an additional 10-minute incubation after addition of hydrogen peroxide. After three additional 5 minute rinses with 0.1 M PB, samples were mounted on glass slides, dried, cleared, and coverslipped for analysis.

Imaging and Analysis of Choroid Samples

In the human macula, VIP-positive fibers have been reported to innervate blood vessels of the outer choroid,^{37,38} which include precapillary arteries and arterioles and perhaps postcapillary venules and veins.³⁹ Our analysis thus focused on the blood vessels of the outer choroid, with no reliable distinctions possible between arteries and veins. A microscope (Olympus), equipped with differential interference contrast optics was used for viewing each immunostained choroid punch. Each choroid was divided into four quadrants, and up to three representative images of blood vessels within each quadrant were captured (by observers blind to the age or other traits of the donor) with a video camera (Newvicon; JVC, Aurora, IL), and NIH Image (version 1.63, available by ftp at zippy.nimh.nih.gov/ or at <http://rsb.info.nih.gov/nih-image/>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD), using standard lighting and image capture settings. Images were evaluated by a trained grader in a blinded fashion. To correct for any slight variations in background staining intensity and lighting during image capture, the backgrounds of all images were standardized to an intensity of 100 on the 0 to 255 gray scale in NIH Image. The vessel boundaries were outlined to determine the area of each vessel, whereas the area within the vessel boundaries occupied by VIP-positive fibers was

determined using the thresholding capabilities of NIH Image. The percentage coverage of VIP-positive fibers along blood vessels in each tissue sample was determined by computer (Excel; Microsoft, Inc., Redmond, WA), as the percentage of total vessel area occupied by VIP-positive fibers for that sample. Examples of captured images of choroidal vessels with VIP-labeled fibers are shown in Figures 2B and 2D. Vessel diameters were measured manually in the captured images. The most prominent vessel was selected and measured at three locations along its length in each image. The mean vessel diameter was calculated for each image and then for each macular choroid sample (Excel; Microsoft, Inc.).

Statistical Analyses

The relationship with age and of the average VIP-positive fiber abundance (expressed as the percentage of VIP-positive fiber coverage) and of the average vascular diameter (in micrometers) for each of the blood vessels in the 12 fields analyzed per choroidal punch was analyzed with a general linear model (SAS Statistical Software, ver. 8.1; SAS Institute, Inc., Cary, NC). The results for eye pairs were averaged, such that only one value for the variables of interest was entered in our analyses for each donor ($N = 35$). Multivariate (multiple regression) models were generated correcting for gender, time elapsed between death and fixation of the tissue, and time spent by the tissue in fixative. Note that the latter two variables related to the tissue specimens could affect the antigenicity of the VIP-positive fibers. In addition, since three separate batches of tissue specimens were processed by three different experimenters and two distinct laboratory staff members captured the digital images to be analyzed, we also controlled for these two variables in the multiple regression model. Last, we also controlled for the possible need, in some experiments, for multiple tissue sample bleaches, as this too could affect antigenicity. Before analysis, all continuous variables included in the study were first evaluated for normality of distribution by the Kolmogorov-Smirnov test. Non-normally distributed variables were log transformed.

RESULTS

The distribution of most variables did not meet the criteria for normal distribution and were therefore normalized by conversion to natural log (ln) values. Using the converted data in simple regression (univariate) analyses, we observed that the natural log of VIP-positive fiber density on blood vessels tended to decline as a function of the natural log of age, but the correlation was not significant ($P = 0.0785$; Fig. 3). Similarly, only a modest age-related decline in the natural log of vessel diameter as a function of the natural log of age was observed, and this correlation too was not significant ($P = 0.7324$; Fig. 4). However, multiple regression (multivariate) analyses revealed that the relationship of age with both VIP-positive fiber density and vessel diameter was markedly confounded by both variables related to the tissue specimens: time elapsed between death and fixation of the tissue, and time spent by the tissue in fixative. Once these variables were controlled for, inclusive also of gender and experimenter (see the Methods section), the fully adjusted multivariate models revealed a strong correlation of the natural log of age with both the log of blood vessel VIP-positive fiber abundance ($F = 5.99$, $P = 0.0003$, adjusted $R^2 = 0.51$) and the natural log of choroidal vessel diameter ($F = 9.35$, $P < 0.0001$, adjusted $R^2 = 0.63$). By the multivariate analysis, ln VIP staining was estimated to decline with the natural log of age more strongly than did ln vessel diameter. Specifically, the slope (β) estimate for ln VIP staining was -0.45 ± 0.17 (SE; $P = 0.017$), whereas that for ln-vessel diameter was -0.19 ± 0.06 , SE; $P = 0.005$). After adjustment for the same variables (gender, death-to-fixation time, time in fixative, and the experimental variables), multiple regression analysis showed that the natural log of VIP-positive fiber density correlated positively with choroidal vessel diameter ($F = 5.40$, $P = 0.0006$, and adjusted $R^2 = 0.48$ for the fully adjusted multivariate model). The slope for this relationship was

estimated at 0.99 ± 0.47 (SE; $P = 0.044$). With few exceptions, the predicted values generated by the fully adjusted model corresponded fairly well to the observed values.

To relate our findings back to actual age, percent of VIPergic fiber vascular coverage, and vessel diameters, the study sample was divided in age quartiles, and the mean adjusted percent coverage of the VIP-positive fibers and vascular diameters were calculated for the upper (age, >81 years; $n = 9$) and lower (age, <49 years; $n = 9$) age quartiles. The adjusted ln-transformed percentage of the VIP-positive coverage was 2.36 ± 0.36 (SD) for the lowest age quartile and 1.93 ± 0.36 (SD) for the highest. In percentage terms, these figures were equal to $11.70\% \pm 3.27\%$ (SD) and $7.64\% \pm 2.25\%$ (SD) for the two quartiles, respectively, which corresponds to a 35% lower amount of VIPergic fibers in the older group compared with the younger group ($P = 0.018$). The adjusted ln-transformed choroidal vessel diameter was 4.66 ± 0.15 (SD) for the lowest age quartile and 4.50 ± 0.15 (SD) for the highest. These values corresponded to actual vessel diameters of 106.99 ± 13.41 and 90.65 ± 13.29 μm (SD), respectively, which are indicative of 16% smaller vascular diameters on average in the older age stratum compared with the younger one ($P = 0.020$).

Last, we evaluated other variables that collected in the course of the investigation to assess the possibility that additional biologically plausible systemic factors may modify significantly the observed relationship between vascular VIP-positive fiber abundance or choroidal vessel diameter and age. History of smoking, history of hypertension, cardiovascular disease (CVD), and use of nitric oxide donors (as antihypertensive and/or CVD-related medications) were all expressed as a yes/no variable and added individually to our multivariate statistical model. All subjects who were active smokers or had quit smoking within 1 year of death were classified as positive for smoking history. The adjusted ln-transformed average VIP levels and diameters derived from our multivariate analyses after stratification by the criteria just described are provided in Table 1. None of these variables significantly affected either VIP-positive fiber abundance or choroidal vessel diameter in our analyses.

DISCUSSION

Choroidal and retinal vessels are the main vascular supply to the outer and inner retina, respectively. The choroidal arteries branch to form the choriocapillaris, which supplies nutrients to the RPE and photoreceptors. Although the oxygen and nutrient exchange occurs at the level of the choriocapillaris, these vessels lack innervation and thus active control of blood flow through the choroid occurs at the level of the choroidal arteries and arterioles via a dense network of parasympathetic and sympathetic nerve fibers.¹ These nerve fibers contribute to the high flow rate of the choroidal vessels and to the ability of the ChBF to increase adaptively in response to retinal needs. As such, they provide the choroid with a neural link between systemic cardiovascular changes and the minute vascular adjustments needed to maintain a relatively constant perfusion pressure to supply the overlying retina, especially in response to periods of low systemic blood pressure.⁴⁰ Deviation of ChBF outside the bounds of the retina's metabolic demands has the potential to cause hypoxic or hyperoxic damage and could eventually affect retinal function. Parasympathetic fibers thus are likely to play a key role in safeguarding the retina from dangerous downward fluctuations in its supply of oxygen and nutrients from the choroid.

Our investigation showed a decline in the VIP-positive nerve fiber coverage of macular choroidal blood vessels and a reduced diameter of choroidal arteries in the normal human donor eyes as a function of age. In addition, we have demonstrated that the estimated biological effect of age was greater on the VIP-positive fibers than on choroidal vessel diameter. It is uncertain whether the diminished vasodilatory innervation contributes to the reduced vessel diameter, or whether the reduced vessel diameters occur by a separate age-related process. Despite this

element of uncertainty, the observed declines in parasympathetic innervation of macular blood vessels and their diminished diameter represents a plausible explanation for the reduction in subfoveal ChBF observed in humans.²⁶ In turn, reduced subfoveal blood flow in the choroid may contribute to the declines in retinal function observed with age in humans^{41–45}—a hypothesis that is corroborated by findings in aging pigeons.^{13,14}

The lack of association of VIP-positive fibers with any of the systemic factors that we examined in our analyses does not support the notion that any of these factors had a significant effect on the observed age-related decline in the VIP-positive subfoveal choroidal nerve fibers and vascular diameters in humans, which is consistent with the *in vivo* finding that the ChBF of treated hypertensive subjects does not differ significantly from that of normotensive subjects.⁴⁶ In addition, although ChBF response to exercise and carbogen in smokers has been reported to be abnormal,^{47,48} basal ChBF in smokers is not different from normal.^{49,50} In our study, normotensive subjects tended to have slightly higher VIPergic fiber percentage coverage and slightly larger vascular diameters. It is therefore possible that, with a larger sample size, a significant difference between hypertensive and normotensive subjects may be found. However, our findings allow us to conclude that the effect of this variable is not likely to be large.

Recently, the suspected biological role of inflammation in AMD^{22,51} has been confirmed by genetic studies, which have revealed how factors linked to the modulation of inflammatory phenomena are key determinants of the likelihood of having AMD.^{52–63} Chronic, low-grade inflammation is already an established contributor to systemic atherosclerosis,^{64–66} in which progressive damage accumulates to the expense of the vascular endothelium. In the vascular system, an age-related decline of nitric oxide-dependent mechanisms is also known to occur in humans.^{30,34} It is therefore conceivable that the progressive decline in the VIP-positive fibers and vascular caliber of the submacular choroids that occurs with aging may exacerbate the local role of genetically determined inflammatory phenomena in the pathogenesis of AMD. These choroidal changes could act as an independent precipitating factor (e.g., ischemia of the photoreceptors and RPE and reduced delivery of nutrients to the retina, such as carotenoids). This hypothesis has already been proposed by Grunwald et al.⁶⁷

An alternative but not mutually exclusive possibility is that the choroidal changes may play a more direct role in the inflammatory causal route, via decreased clearance of postinflammatory debris and other related compounds in the sub-macular region. Such a hypothetical vicious circle could contribute to AMD pathogenesis by facilitating the accumulation of drusen, RPE dropout, and geographic atrophy in the macula, a portion of the human eye where thinning and disruption of the Bruch's membrane is known to occur in eyes with AMD and to correlate with the localization of AMD-associated lesions.⁶⁰ Based on this hypothesis and on the evidence that AMD eyes display ChBF reductions that are greater than age-matched healthy subjects and proportional to AMD severity,^{22,67} one would predict that AMD eyes would display a greater decline in VIP-positive fibers than predicted by aging alone. Preliminary studies are under way to test this hypothesis.

In conclusion, our present findings provide evidence for an age-related decline in VIP-positive submacular choroidal nerve fibers and choroidal vascular caliber, providing a rationale for considering a therapeutic intervention that will improve sub-macular ChBF as a means of preventing, mitigating, or perhaps even removing what could be a fundamental precipitating factor in retinal aging and in the pathogenesis of AMD.

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Figure 1.

Images showing the trephine that was used to collect the retinochoroidal punches from donor eyes (A) and the locations of the punches themselves (B). The 6-mm punch (radius from the fovea: 3 mm), as shown in (B), was centered around the fovea centralis. This location allowed us to collect specimens from nearly the entire macular region (the limits of the punch are outlined), which is typically affected by AMD. The fovea is situated approximately 4 mm temporally from the optic disc margin. A small notch was placed in the superior aspect of the punch for orientation purposes.

Figure 2.

Examples of fundus images (**A, C**) of human donor eyes with no macular disease and VIP immunolabeling (**B, D**) of choroidal vessels. Fundus image (**A**) and VIP immunohistochemical results (**B**) from the eye of a 33-year-old donor. The view of the choroidal specimen shows a representative sub-macular choroidal vessel, surrounded by stroma. The abundant beaded striae running across the vessel lumen are the VIP-positive fibers of the choroidal vessel. Analysis of 12 representative fields from this punch revealed that $21.22\% \pm 6.2\%$ (SD) of the vessel surface was covered by VIP-positive fibers. Fundus image (**C**) and immunohistochemical results (**D**) of a representative submacular choroidal vessel from a 75-year-old donor eye. Note the conspicuously reduced diameter of the vessel and the lesser amount of VIP-positive fibers in this elderly donor compared with the young donor. Analysis of 12 fields in this eye showed that $6.49\% \pm 2.5\%$ (SD) of the vessel was covered by VIP-positive fibers. Approximate vessel borders and VIP-positive fibers are indicated by *lines* and *arrows*, respectively. Magnification bars: (**A, C**) 2 mm; (**B, D**) 20 μ m.

Figure 3.

Plot of the natural log of choroidal vessel area covered by VIP-positive fibers versus the natural log of age for the samples of donors. By univariate analysis, the natural log of VIP-positive fiber density tended to decline as a function of the natural log of age, but not significantly so ($F = 3.30$, $P = 0.0785$, $R^2 = 0.0633$). Utilization of multivariate analysis to adjust for the confounding factors of such variables as gender, time elapsed between death and fixation of the tissue, time spent in fixative, and experimenter revealed a highly significant reduction in VIP-positive labeling with age ($F = 5.99$, $P = 0.0003$, adjusted $R^2 = 0.5069$).

Figure 4.

Plot of the natural log of choroidal vessel diameter versus the natural log of age for the donor samples. By univariate analyses, the natural log of the choroidal vessel diameter declined slightly as a function of the natural log of age, but not significantly so ($F = 0.12$, $P = 0.7324$, $R^2 = 0.0036$). In a multivariate analysis to adjust for confounding from gender, time elapsed between death and fixation of the tissue, time spent in fixative, and experimenter, a highly significant reduction in vessel diameter with age became apparent ($F = 9.35$, $P < 0.0001$, adjusted $R^2 = 0.6321$).

Table 1

Adjusted In-Transformed Average VIP and Diameter

Variable	Sample Size [*]	In-VIP Labeling		In-Vessel Diameter	
		No	Yes	No	Yes
History of hypertension	20/15	2.13 ± 0.36	2.06 ± 0.39	4.58 ± 0.13	4.54 ± 0.15
History of smoking	25/10	2.10 ± 0.35	2.09 ± 0.38	4.55 ± 0.15	4.59 ± 0.13
History of CVD	13/22	2.11 ± 0.40	2.09 ± 0.38	4.56 ± 0.14	4.56 ± 0.14
Death by AMI	22/13	2.10 ± 0.33	2.09 ± 0.36	4.56 ± 0.14	4.57 ± 0.14
Use of nitroderivatives	25/6 [†]	2.07 ± 0.30	2.10 ± 0.32	4.57 ± 0.10	4.54 ± 0.12

n = 35. Data are expressed as the mean ± SD. AMI acute myocardial infarction; CVD, cardiovascular disease.

^{*} Data indicate absence (no)/presence (yes) of specified characteristic.

[†] The use of nitroderivatives could not be ascertained with certainty in four subjects.