Normal Aging Involves Modulation of Specific Inflammatory Markers in the Rat Retina and Choroid

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Recent work has suggested that inflammation is a common component of a number of age-related diseases. The hypothesis of the present study was that normal aging of the retina and choroid would increase levels of inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2), and tumor necrosis factor alpha (TNF-α). To investigate this hypothesis, gene expression and protein analyses were completed on retinal and choroidal samples from Fischer 344 × Brown Norway F1 hybrid rats at 8, 22, and 32 months of age. Aging of the choroid produced significant increases in PGE2, with decreased TNF-α protein. Protein levels and messenger RNA of iNOS and TNF-α protein levels were significantly decreased in the aging retina in contrast to PGE2 protein activity, which was increased with age in the retina. These results suggest that PGE2 is likely involved in the aging process in both the retina and choroid, whereas iNOS plays a role predominantly in the retina.

Key Words: Inflammatory markers—Retina—Choroid—Aging.

AGE-RELATED macular degeneration is the leading cause of blindness in people older than 65 years (1). With the largest segment of the population entering into this age range, this will only become a more prevalent problem in the years to come. There are two forms of macular degeneration, wet and dry. The dry form of macular degeneration is associated with the formation of drusen, whereas the wet form presents with vascular growth of the choroid and retina. In addition to both forms of macular degeneration, there are a large number of age-associated diseases that involve degeneration of one or more target organs, including Alzheimer’s disease, Parkinson’s disease, and rheumatoid arthritis (2). A common factor in these diseases is a dominant inflammatory component (2,3). Although recently there has been interest in potential inflammatory components involved in both forms of macular degeneration (4,5), less is known about inflammatory cytokines involved in the normal aging process of the choroid and retina.

A recent review has presented an account of how morphological changes in normal aging may occur and how these are altered in macular degeneration (6). Investigations into the disease process of macular degeneration have lead to a better understanding of normal morphological changes in the choroid, Bruch’s membrane, and the retinal pigmented epithelium (RPE). Normal aging of the choroid involves substantial thickening of Bruch’s membrane, which separates the choroid from the neural retina (7). Some of this thickening in aging is likely due to changes in extracellular matrix turnover (8). In addition to the thickening of Bruch’s membrane, there is increased oxidative injury due to normal aging (9–11). This oxidative stress may contribute to the formation of drusen, protein aggregates found along Bruch’s membrane (12). Drusen are believed to form during aging due to dysfunction of the RPE cells (6). Besides oxidative stress, normal aging of the choroid is also associated with increased formation of advanced glycation end products (AGEs) (13). AGEs can bind their receptors and induce cell injury and destruction, which is observed in both diabetic retinopathy (14,15) and in macular degeneration (16). Therefore, although a great deal is known about morphological changes in the choroid with age, less is known about alterations in markers of inflammation that may occur throughout the life span.

The normal aging process of the retina is less clear than that of the choroid, because an age-related disease is not associated. Normal aging of the retina is associated with the loss of rod photoreceptors with age (17,18). In addition, hyperglycemia can induce formation of AGEs, which can damage the retina. It is unclear whether normal aging of the retina involves modulation of glucose levels or inflammatory markers altered in other aged organs.

Changes in inflammatory markers are increased in other organs with aging (19,20). Viral or bacterial infections can lead to the production of inducible nitric oxide synthase (iNOS) and other cytokines, which are then released after several hours into many different regions of the brain (21). This may be relevant to the retina, as the retina is derived from the same progenitor cells as brain regions. Specific to the eye, Anderson and coworkers (5) investigated drusen obtained from both normal donors and donors with macular degeneration, which suggested that local inflammation in the choroid/RPE region may lead to the formation of drusen. Because some have suggested that inflammatory markers may be involved in changes associated with normal aging of
the choroid and retina, we chose to investigate which particular factors may be involved in each target. The goal of the present study was to investigate whether iNOS, prostaglandin E2 (PGE2) and its receptor subtype EP2, and tumor necrosis factor alpha (TNF-α), factors observed to be involved in inflammation in a variety of diseases (22,23), are altered during the normal aging process of the retina and choroid in an aging rat model, the Fischer 344 × Brown Norway F1 hybrid (F344 × BN F1). Investigations of messenger (mRNA) levels and protein analysis were done on rats at 8, 22, and 32 months of age.

**Materials and Methods**

**Animals**

Male F344 × BN F1 hybrid rats aged 8 months (n = 6, ~20 years of age in human years), 22 months (n = 6, ~50 to 60 years of age in human years), and 32 months (n = 6, ~80 to 90 years of age in human years), purchased from the National Institute on Aging through Harlan, were used to determine changes in inflammatory marker expression in the retina and choroid with age. This rat strain was used because they show fewer age-related diseases and biological variability (24). Rats were anesthetized using pentobarbital (150 mg/kg), and the eyes were removed. The cornea was cut, and the lens and vitreous were removed. The retina and choroid were placed into separate tubes for RNA isolation and protein analysis. All procedures were approved by the Institutional Animal Care and Use Committee at the Southern Illinois University-Carbondale.

**RNA Isolation and Reverse Transcription**

RNA was isolated from retinal and choroidal samples at each age using chloroform and isopropanol. Once isolated, the RNA samples were assessed by spectrophotometry and run on a gel to verify quantity and quality of the sample. One microgram of RNA was used for reverse transcription following the instructions for the Improm II Reverse Transcription Kit (Promega Madison, WI). RNA isolation and reverse transcription were completed as published previously (25).

Primers used in real-time PCR are in Table 1. To correct for change in both quality and quantity of RNA, samples were normalized by using the ratio of the concentration of the desired complementary DNA to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for retinal samples (26). Choroid samples were normalized using 18s ribosomal RNA (rRNA) because we had previously found that GAPDH levels change with age in the choroid (26). Therefore, all choroid samples were normalized using 18s rRNA. To calculate the variation in steady-state RNA levels, the change in cycle threshold (ΔCt) for the 8-month rat retina and choroid expression of iNOS and TNF-α was calculated by subtracting the corresponding GAPDH/18s rRNA levels (internal control) from their respected threshold (Ct) primer level. Then, the ΔCt was calculated by subtracting the average of the ΔCt in the 8-month rat retina/choroid from the ΔCt of each animal for the gene of interest. Changes in steady-state gene expression are reported as x-fold increases (2-ΔΔCt), relative to the 8-month rat retina or choroid. Significance in the 2-ΔΔCt was accepted at p < .05, determined by computer analysis (Prism Software; GraphPad, San Diego, CA). The statistical analysis of steady-state RNA is based on work from other groups (27).

**Protein Expression**

Western Blot analysis was conducted to determine whether markers of inflammation were altered with increasing age. Western blot procedures were similar to those done previously (23). Total protein content of retinal and choroidal lysates was assessed using the bicinchoninic acid assay (Pierce, Rockford, IL). Once protein content was known, 50 μg of protein was loaded onto a 4%-12% gradient gel and transferred to nitrocellulose membrane. Primary antibodies to iNOS (1:500 dilution; Chemicon, Temecula, CA) and PGE2-EP2 receptor (1:500; Chemicon) were applied overnight at 4°C. Secondary antibodies conjugated to hors eradish peroxidase were applied the following day, followed by detection using enhanced chemiluminescence (Amer sham Biosciences, Little Chalfort, UK). Chemiluminescence images were viewed on a Kodak 2000r Carestream Health Imaging, New Haven, CT. Densitometry was conducted by using the data acquisition program from Kodak ID. Upon completion of chemiluminescence, equal lane loading was checked by Ponceau S Solution (Sigma, St. Louis, MO). Statistical analysis of results from 22- and 32-month-old animals was compared with those from the 8-month control.

**Enzyme-linked immunosorbent assays.**—An enzyme-linked immunosorbent assay (ELISA) to measure PGE2 (Endogen; Pierce Biotechnology, Rockford, IL) and TNF-α (Fisher Scientific, Pittsburgh, PA) content in samples from the aged retina and choroid was used according to manufacturer’s instructions, except that equal amounts of protein were loaded for each well. Fifty micrograms of protein from each sample at each age was loaded into each well of the ELISA kit. Analysis was done to compare the optical density values obtained for the 22- and 32-month-old animals with those of the 8-month retina or choroid.

**Results**

**Retina**

Both gene expression and protein levels of iNOS are significantly decreased with age in the retina (p < .05 vs 8 months, Figure 1A–C, Table 2), suggesting that inducible nitric oxide production is decreased in the aged retina. This
is in contrast to the diabetic retina, where iNOS levels are observed to increase due to hyperglycemia (28).

ELISAs on samples from the aged retina indicate that aging is associated with a significant increase in PGE2 levels $(p < .05$ vs 8 months, Figure 2A, Table 2) at both the 22- and 32-month ages. Although PGE2 activity was increased, no changes were observed in protein levels of the PGE2-EP2 receptor subtype (Figure 2B and C, Table 2).

Conflicting results were obtained for TNF-α expression as the gene expression is significantly increased with age, whereas protein levels of TNF-α were slightly, but significantly, decreased with age at 32 months relative to 8 months $(p < .05$ vs 8 months, Figure 3A and B, Table 2), perhaps suggesting that the mRNA is not stable or not effectively translated into protein. We have found previously that aging results in a discrepancy between mRNA expression and protein levels (26). These changes are biological, because using different housekeeping genes/proteins does not affect the outcome.

### Choroid

Unlike the retina, slight increases in both gene expression and protein levels of iNOS were observed in the choroid, but none reached statistical significance (Figure 4A–C, Table 2).

PGE2 levels were significantly increased in the aged choroid $(p < .05$ vs 8 months, Figure 5A, Table 2), which is similar to those results obtained in the aged retina. These results suggest that a common mechanism for increased PGE2 expression may be at play in both the aged choroid and retina. Associated with the increased PGE2 levels in the aged choroid, there are also increased levels of the PGE2-EP2 receptor subtype $(p < .05$ vs 8 months, Figure 5B and C, Table 2). Because the protein levels of PGE2-EP2 are increased in the

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**Table 1. Primers Used in Real-Time PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>iNOS Forward:</td>
<td>TGAGAGAGCAGAGGAAAATGAAC</td>
</tr>
<tr>
<td>Reverse:</td>
<td>CAAGGAATTATACACGGAAGGG</td>
</tr>
<tr>
<td>TNF-α Forward:</td>
<td>CTTTATCTACTCCCAAGGTTCTC</td>
</tr>
<tr>
<td>Reverse:</td>
<td>TTACTCCCTGTTATGAAATGCG</td>
</tr>
<tr>
<td>18s rRNA Forward:</td>
<td>TCAAGAAGAAAGTCCGAGGTT</td>
</tr>
<tr>
<td>Reverse:</td>
<td>GGACATCTAAGGCGGATACAG</td>
</tr>
<tr>
<td>GAPDH Forward:</td>
<td>TCCACCACCTGTGCTGTA</td>
</tr>
<tr>
<td>Reverse:</td>
<td>ACCACAGTCCATGCCCATCAC</td>
</tr>
</tbody>
</table>

*Note: iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor alpha. Used for the real-time polymerase chain reaction experiments. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for the retina and 18s ribosomal RNA (rRNA) for the choroid, because we have observed changes in GAPDH with age in the choroid.*

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**Table 2. Changes in mRNA Expression and Protein Levels in the Aging Retina and Choroid**

<table>
<thead>
<tr>
<th>Retina</th>
<th>Choroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA</td>
<td>Protein</td>
</tr>
<tr>
<td>iNOS</td>
<td>Decrease at 32 mo</td>
</tr>
<tr>
<td>PGE2</td>
<td>Decrease at 22 and 32 mo</td>
</tr>
<tr>
<td>PGE2-EP2</td>
<td>N/C</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Increase at 32 mo</td>
</tr>
</tbody>
</table>

*Note: iNOS = inducible nitric oxide synthase; mRNA = messenger RNA; N/C = not changed; PGE2 = prostaglandin E2; TNF-α = tumor necrosis factor alpha. Observed changes in mRNA gene expression and protein levels of the inflammatory markers investigated in the retina and choroid of Fischer 344 × Brown Norway F1 hybrid rats at 8, 22, and 32 months of age.*
aged choroid, but not the retina, this may represent different inflammatory markers used between these tissues.

In the aged choroid, TNF-α gene expression was significantly increased at 22 months of age (p < .05 vs 8 months), but returned to normal by 32 months of age (Figure 6A, Table 2). Protein levels of TNF-α at 22 months were significantly decreased as compared with 8 months (Figure 6B, Table 2). In the aged retina, a similar trend for protein and mRNA was observed at 32 months, rather than at 22 months in the choroid, again suggesting that the mRNA for TNF-α is not efficiently converted into protein.

**DISCUSSION**

The goal of the present study was to characterize changes in common markers of inflammation during the normal aging process in the retina and choroid in rats. Investigations of iNOS, PGE2 and PGE2-EP2 receptor, and TNF-α suggest that normal aging involves both tissue-specific and perhaps inflammatory marker-specific changes. In the choroid, no significant changes were observed in iNOS protein or mRNA, whereas significant changes were observed at 32 months in PGE2 and PGE2-EP2 receptor. TNF-α, on the other hand, had increased mRNA expression and decreased protein expression at 22 months of age. The increase in TNF-α gene expression may lead to the upregulation of other inflammatory cytokines, such as PGE2. Others have reported that increased TNF-α expression can increase transcription of a number of other growth factors (29), as well as inflammatory markers (30). Klein and coworkers (30) found that no significant changes were observed in systemic inflammatory cytokines, suggesting that a local inflammation is mediating the changes observed. To address this idea, Anderson and coworkers (5) had investigated drusen obtained from both normal donors and donors with macular
Inflammation in the choroid/RPE region may lead to the formation of drusen, which is a strong indicator of future vision problems (5). Little work has focused on changes in prostaglandins in the choroid. Work in other targets, such as the periodontal bones, suggests that PGE2 levels can be increased with age (31). Others have shown that cyclooxygenase 1 and 2 are upregulated in cremaster arterioles with age (32), which could lead to increased PGE2 activity. Some work on corneal endothelial cells has suggested that aging may increase cellular migration that is mediated by PGE2 (33). These findings would agree with the data observed for the aged rat choroid, where both PGE2 and its receptor PGE2-EP2 subtype were significantly increased with age.

In contrast to the finding of limited changes in iNOS levels in the choroid, significant decreases in iNOS expression and protein levels were observed in the aged retina. The changes do not seem to occur in the retina until the 32-month time point. Although less work has focused on iNOS in the retina during normal aging, much work has been done on the role of iNOS in the brain. A theory has been proposed that exposure to infections over the life span may lead to cell death through overexposure of specific brain regions to iNOS. Viral or bacterial infections can lead to the production of iNOS and other cytokines, which are then released after several hours into many different regions of the brain (21). These cytokines can lead to apoptosis of both neurons and glial cells in specific regions, such as the pituitary and pineal gland, which may ultimately lead to the loss of antioxidant factors and increase the aging process (21). In a similar manner, Sennlaub and coworkers (34)
The amount of protein is loaded into all wells, the results are presented as mean absolute values. The enzyme-linked immunosorbent assay (ELISA) analysis. Because the same messenger RNA expression for TNF-α (TNF-α) in the aged choroid. The bar graph of the steady-state messenger RNA expression for TNF-α in the aging choroid. The results from the enzyme-linked immunosorbent assay (ELISA) analysis. Because the same amount of protein is loaded into all wells, the results are presented as mean absorbance as a percentage of the 8-month values. *p < .05 versus 8 months, N = 5 for real-time polymerase chain reaction and N = 4 for ELISA analysis.

Figure 6. Gene expression and protein activity of tumor necrosis factor alpha (TNF-α) in the aged choroid. (A) The bar graph of the steady-state messenger RNA expression for TNF-α in the aging choroid. (B) The results from the enzyme-linked immunosorbent assay (ELISA) analysis. Because the same amount of protein is loaded into all wells, the results are presented as mean absorbance as a percentage of the 8-month values. *p < .05 versus 8 months, N = 5 for real-time polymerase chain reaction and N = 4 for ELISA analysis.

have shown that iNOS is a critical mediator of retinal neuron apoptosis in ischemic proliferative retinopathies, such as diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity. The present results of declining iNOS levels with age may suggest that normal aging of the retina does not involve a specific disease, such as those investigated by Sennlaub and coworkers (34). It may be that either a chronic infection or severe trauma is required to increase iNOS levels in the retina. Results for PGE2-EP2 receptor subtype in the aged retina are opposite of the observations in the choroid and findings for iNOS in the retina. Nonetheless, the finding of increased PGE2 with age in the retina follows from results from the choroid (in the present study) and the results from the periodontal bone (31) and cremaster arterioles (32). Similar increases in PGE2 were also observed in the retina following surgical denervation of young rats (23). These results likely suggest that the normal aging process in the retina involves some local inflammation.

As was observed for TNF-α levels in the choroid at 22 months, gene expression for TNF-α was increased in the retina at 32 months, whereas protein levels were decreased. Although the exact mechanism resulting in the lack of protein translation from increased mRNA expression is not known (26,35), something within the TNF-α mRNA product is not stable. The observation of decreased protein levels of TNF-α in the aged retina further supports a hypothesis that any inflammation occurring in the aged retina is due to PGE2 activity.

Further investigations into the discrepancy between gene expression and protein levels of TNF-α in both the choroid and retina with age should be conducted to better understand the reason that mRNA is not being effectively translated into protein. It is unclear whether the altered mRNA expression cannot be translated to protein or is degraded. Alternatively, it may be something in the cellular milieu that leads to decreased protein levels in spite of increased mRNA expression.

In conclusion, results suggest that specific markers of inflammation are modulated in both the retina and choroid in an inflammatory marker-specific fashion. Even though many traits of the retina and choroid are similar, age-related changes in iNOS and PGE2-EP2 are very different. However, both the choroid and retina respond to the aging process by increased PGE2 activity and decreased TNF-α activity. Future studies should investigate potential mechanisms for these changes in inflammatory cytokines in the various cell types of each tissue. Overall, these results further support the suggestion that normal aging does involve a local inflammatory component and indicates which factors are involved in each of the retina and choroid, as well as the time course of activation of specific markers over the life span of the rat.

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References

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