Increase in the Advanced Glycation End Product Pentosidine in Bruch’s Membrane with Age

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PURPOSE. To determine whether there is an age-related increase of pentosidine in human Bruch’s membranes and to localize pentosidine and carboxymethyllysine (CML), two well-characterized, advanced glycation end products (AGEs) in aged human Bruch’s membranes and choroid in vivo.

METHODS. Human Bruch’s membrane samples were isolated from the retinal pigment epithelium (RPE) and choroid and subjected to reversed-phase high-performance liquid chromatography to determine pentosidine content. A polyclonal anti-pentosidine antibody and a monoclonal antibody specific for carboxymethyllysyline were used to localize AGEs in 20-month-old nondiabetic, 82-year-old nondiabetic, and 82-year-old diabetic globes.

RESULTS. Human Bruch’s membranes (n = 20) showed a linear age-dependent increase in pentosidine that reached approximately 0.17 millimoles pentosidine per mole hydroxyproline in late life (r = 0.896; P < 0.001). Immunohistochemical evaluation showed evidence of pentosidine in Bruch’s membrane, choroidal extracellular matrix, and vascular walls in the 82-year-old nondiabetic and diabetic globes. A similar staining pattern was found with the anti-CML antibody. Basal laminar deposits and drusen stained with both antibodies in the elderly nondiabetic eye. In contrast, neither antibody stained the 20-month-old tissue.

CONCLUSIONS. We provide biochemical and immunohistochemical evidence for the formation of pentosidine and CML structures in human Bruch’s membrane and choroid with age. These changes could promote aging of the RPE-Bruch’s membrane-choroid complex. (Invest Ophthal Vis Sci. 1999;40:775-779)

Age-related maculopathy is a disorder of the macula, often associated with visual impairment, that affects patients more than 50 years of age. The late stage of age-related maculopathy or age-related macular degeneration (AMD) is the most common cause of legal blindness among the elderly in Western countries. Although the exact pathophysiology of age-related maculopathy and AMD is unknown, the changes are thought to result from degeneration of the RPE-Bruch’s membrane-choroid complex. Factors that may be involved in this degenerative process include oxidative stress, hypoxia, light toxicity, nutritional deficiencies, and aging of the RPE-choroid.

Long-lived proteins in vivo are modified over time by nonenzymatic Maillard reactions between a protein’s primary amino groups and carbohydrate-derived aldehyde groups to yield advanced glycation end products (AGEs). AGEs have been linked not only to complications in diabetes, but also to age-related diseases such as cataract, Alzheimer’s disease, atherosclerosis, and pulmonary fibrosis. AGEs may contribute to the reduced compliance in the lamina cribrosa of the optic nerve, which could increase the susceptibility of axons to damage from elevated intraocular pressure in glaucoma.

The greatest accumulation of AGEs has been observed in uremia, regardless of the presence of hyperglycemia or diabetes, which suggests that other factors are involved in their formation. Considerable evidence shows that AGE formation is accelerated by oxidative stress. The formation of AGEs in the presence of oxygen has been termed glycoxidation, and occurs by a free-radical mechanism involving superoxide radicals and hydrogen peroxide. From a wide variety of possible AGE structures, only a few have been characterized. Two of these compounds are pentosidine, a lysine-arginine cross-link, and carboxymethyllysyline (CML). Both have been shown to accumulate with aging in vivo and are formed by glycoxidation.

Age-related changes in Bruch’s membrane have been documented, some of which could result from AGE formation. Bruch’s membrane is located in an area of high oxidative stress due to the high blood flow from the choroid and the significant exposure to light. We hypothesized that AGEs are formed in the RPE-Bruch’s membrane-choroid during aging. The purpose of this project was to determine whether the formation of pentosidine, a well-characterized AGE, was age related and to determine whether pentosidine and CML, another AGE, were localized in human RPE-Bruch’s membrane-choroid complexes during aging.

METHODS

Tissue Preparation

Intraocular contents from donor eyes were obtained from the Sierra Eye and Tissue Bank (Sacramento, CA) within 24 hours of death. Tissue was obtained after removal of the cornea and...
sclera. Eyes with a history of retinal disease were excluded. Eyes from three donors with a history of diabetes mellitus without diabetic retinopathy were included as positive control specimens for pentosidine analysis. The retina and vitreous were removed from the RPE-Bruch’s membrane-choroid. Using a dissecting microscope, the RPE was removed from Bruch’s membrane with a cotton swab. With fine forceps, the choroid was dissected from Bruch’s membrane in phosphate-buffered saline until the choroidal pigmentation was completely removed. A sample of tissue was prepared for histologic examination, and the remainder was lyophilized and prepared for reversed-phase high-performance liquid chromatography (HPLC) analysis.

A 20-month-old child with sickle cell disease who died of sepsis (Centralized Pathology Unit for Sickle Cell Disease, Birmingham, AL), an 82-year-old nondiabetic donor who died of pneumonia (National Disease Research Interchange, Philadelphia, PA), and an age-matched 82-year-old diabetic donor who died of end-stage cardiomyopathy (Medical Eye Bank of Maryland, Baltimore) were obtained within 24 hours of death for immunohistochemical evaluation. None of the eyes was known to be affected by AMD. Eyes were fixed in 2% paraformaldehyde and cryoprotected using graded sucrose infiltration.

**Quantification of Pentosidine by HPLC**

Lyophilized Bruch’s membrane samples (1–5 mg dry weight) were hydrolyzed in 4-ml glass vials fitted with polytetrafluoroethylene-lined screw caps, containing 750 μl 6 M HCl, at 110°C for 20 to 24 hours. After drying (Speed Vac SCI 10; Savant, Farmingdale, NY), samples were dissolved in water containing the internal standards pyridoxine (10 μM; Sigma, St. Louis, MO) and homoarginine (2.4 mM; Sigma); for every milligram of tissue (dry weight) 95 μl solution was used. For analysis of pentosidine and pyridinium cross-links (hydroxylysylpyridinoline and lysylpyridinoline), samples were diluted fivefold with 750 μl 6 M HCl, at 110°C for 20 to 24 hours. After drying (Speed Vac SCI 10; Savant, Farmingdale, NY), samples were dissolved in water containing the internal standards pyridoxine (10 μM; Sigma, St. Louis, MO) and homoarginine (2.4 mM; Sigma); for every milligram of tissue (dry weight) 95 μl solution was used. For analysis of pentosidine and pyridinium cross-links (hydroxylysylpyridinoline and lysylpyridinoline), samples were diluted fivefold with 0.5% (vol/vol) heptfluorobutyric acid in 10% (vol/vol) acetonitrile; 100 μl diluted samples was injected into the HPLC system, as described previously. Pyridoxine was used as an internal standard for cross-link analysis. Collagen content of the samples was determined by amino acid HPLC analysis, using homoarginine as an internal standard. Pentosidine was expressed as millimoles per mole hydroxyproline.

**Immunohistochemistry**

Ten-micrometer RPE-Bruch’s membrane-choroid cryostat sections were rinsed in phosphate-buffered saline and incubated in 0.3% H2O2–100% methanol for 30 minutes to block endogenous peroxidase. The tissue was blocked with 3% goat serum and 3% blotto for 30 minutes at room temperature and incubated overnight in a humidified chamber at 4°C with 5 μg/ml anti-pentosidine rabbit polyclonal antibody6 or 2 μg/ml anti-AGE mouse monoclonal IgG-1, which recognizes CML (6D12; Wako, Richmond, VA). Detection was performed using an ABC staining kit (Vector, Burlingame, CA). The sections were counterstained with nuclear-fast red (Vector). Control specimens included sections incubated with nonimmune rabbit IgG (Vector) or mouse IgG1 (Dako, Carpinteria, CA). Competitive experiments were performed by preincubating for 2 hours with 1:200 excess AGE-bovine serum albumin (BSA) for the monoclonal anti-AGE antibody, and pentosidine-modified BSA for the anti-pentosidine antibody.

**RESULTS**

**Quantification of the Pentosidine in Bruch’s Membrane**

Pentosidine levels were studied by HPLC in 20 samples of isolated Bruch’s membrane from nondiabetic donors ranging in age from 28 to 71 years. Our method of careful dissection provided complete removal of the RPE and choroid, leaving only Bruch’s membrane and remnants of the choriocapillaris (data not shown). The content of collagen, expressed as the percentage dry weight of Bruch’s membrane, showed no age-dependent change (data not shown).

**Detection of Pentosidine and Carboxymethyllysine in the RPE–Bruch’s Membrane–Choroid Complex**

To determine the localization of pentosidine in human RPE–Bruch’s membrane–choroid complexes, we performed immunohistochemical experiments using a polyclonal anti-pentosidine antibody. In an 82-year-old nondiabetic eye with no known history of AMD, Bruch’s membrane, the extracellular matrix of the choroid, and choroidal vasculature showed immunohistochemical evidence of pentosidine (Fig. 2). In the 82-year-old nondiabetic eye, basal laminar deposits (BLDs) and

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**FIGURE 1.** Age-related increase of pentosidine in human Bruch’s membrane. The pentosidine content of isolated Bruch’s membrane from nondiabetic eyes (n = 20; age range, 28–71 years) reached 0.17 millimoles pentosidine per mole hydroxyproline (Hyp) in late life. For comparison, Bruch’s membrane samples from eyes of elderly diabetic donors aged 67, 68, and 69 years showed levels of 0.15, 0.18, and 0.17 millimoles pentosidine per mole hydroxyproline, respectively. Pentosidine concentration was determined by: pentosidine (in millimoles per mole hydroxyproline) = 0.002(age) − 0.002 (r = 0.896; P < 0.001). (●) nondiabetic eyes; (○) diabetic eyes.
drusen were identified. These structures showed prominent staining with the anti-pentosidine antibody (Figs. 2A, 2B). Similar to the nondiabetic eye, the 82-year-old diabetic eye showed staining for pentosidine in Bruch's membrane and in the extracellular matrix and vasculature of the choroid (Fig. 2D). In contrast, the RPE-Bruch's membrane–choroid from a 20-month-old nondiabetic eye showed no immunohistochemical evidence of pentosidine (Fig. 2C). Minimal immunoreaction was observed in the old or diabetic eyes incubated with nonimmune rabbit IgG (Fig. 2E). No reaction was seen in the 82-year-old nondiabetic globe with the anti-pentosidine antibody preincubated with excess pentosidine-modified BSA (Fig. 2F).

Because pentosidine represents a small proportion of potential AGEs that can form, the localization of a prevalent glycoxidation product, CML, was determined using a monoclonal antibody. (A) An 82-year-old nondiabetic eye shows evidence of CML in Bruch's membrane, BLDs, and choroid. (B) Drusen (DRU) also stained with the monoclonal anti-AGE antibody.
nal anti-AGE antibody specific for this AGE. A staining pattern similar to that of the anti-pentosidine antibody was seen in Bruch’s membrane and choroidal extracellular matrix and vasculature in the 82-year-old nondiabetic and diabetic eyes (Fig. 3A). Basal laminar deposits and drusen stained prominently with the anti-CML antibody (Fig. 3B). The monoclonal anti-AGE antibody did not detect CML in the 20-month-old nondiabetic Bruch’s membrane and choroid. Nonimmune mouse IgG1 and preincubation of anti-CML antibody with excess AGE-BSA showed no reaction (data not shown).

**DISCUSSION**

In this study, we showed for the first time that AGE structures were present in the Bruch’s membrane-choroid complex using HPLC and immunohistochemical analyses. HPLC analysis of human Bruch’s membranes showed an age-related increase in pentosidine without a change in collagen content. These findings were complemented with immunohistochemical studies in which pentosidine was localized in aged but not in young Bruch’s membrane and in choroidal extracellular matrix and vasculature. Because pentosidine is thought to represent less than 1% of AGEs in tissue, further immunohistochemical analysis using a monoclonal anti-AGE specific for CML was performed to determine whether other AGEs could be identified in the RPE-Bruch’s membrane-choroid. These experiments identified CML in the same ocular structures. With both antibodies, AGEs were found in BLDs and drusen.

The Bruch’s membrane samples analyzed for pentosidine were carefully dissected from the RPE and choroid except for portions of the choriocapillaris. Enzymatic digestion was avoided to prevent digestion and potential loss of AGEs during tissue processing. Our analysis found no age-related change in the collagen content of Bruch’s membrane. We are not aware of any rigorous studies in the literature quantifying a change in Bruch’s membrane collagen content with age. It is of interest that the quantity of pentosidine in aged Bruch’s membrane samples was identical with that in aged Bruch’s membrane samples from diabetic eyes. We expected a higher level of pentosidine formation in diabetic samples because of the availability of glucose as a substrate. It is possible that the high oxidative stress of the RPE-Bruch’s membrane complex is similar to that occurring in uremia, wherein the formation of AGEs is enhanced. The formation of pentosidine may be tissue dependent. For example, similar levels of pentosidine were found in aged dura mater as in our Bruch’s membrane samples. In contrast, threefold lower levels of pentosidine were measured in cortical bone of elderly subjects.

Pentosidine and CML were identified immunohistochemically in Bruch’s membrane and the choroid. We hypothesize that the formation of these glycoxidation products in Bruch’s membrane and the choroid are the result of an abundance of AGE precursors and a high oxidative stress environment. The outer retina, which receives a significant amount of nutrients from the choroid, has a high metabolic demand. To satisfy this demand, the choroid has a high blood flow that exposes the RPE-Bruch’s membrane complex to substantial levels of sugars and oxygen. In addition, Bruch’s membrane is exposed to significant light, particularly the blue wavelengths, which augments the oxidative stress that would promote AGE formation. Pentosidine and CML can be formed from oxidized ascorbic acid, which suggests that the two AGEs can be formed in the absence of glucose. This finding may have particular relevance, because the vitreous and posterior fundus contain high levels of ascorbic acid. Pentosidine and CML have been considered markers of oxidative stress. We suggest that these AGEs are markers for oxidative stress in the aged Bruch’s membrane-choroid complex.

Not only are AGEs markers of oxidative stress, they are also potent stimulators of oxidative stress. Protein-containing AGEs produce significantly higher amounts of free radicals than normal protein. Binding of AGEs to an AGE-specific receptor, RAGE, an integral membrane protein in the immunoglobulin superfamily, is another pathway of inducing significant oxidative stress. It results in generation of thiobarbituric acid-reactive substances, upregulation of heme oxygenase, and activation of transcription factor NF-kB. In preliminary studies using reverse transcription-polymerase chain reaction, we have seen that RAGE is expressed in cultured human RPE cells (unpublished data). It is currently unknown whether RAGE is expressed in the RPE in vivo, or whether it plays a role in altered structure and function of the RPE-Bruch’s membrane complex. Regardless, the potentiation of oxidative stress by AGEs suggests that AGEs could play a role in aging of the RPE-Bruch’s membrane-choroid complex.

AGEs were identified in Bruch’s membrane and BLDs, which are composed in part of an abnormal accumulation of matrix proteins such as laminin, heparin sulfate proteoglycans, fibronectin, and collagen IV. These findings are of interest because the thickness of human Bruch’s membrane increases with age, and BLDs have been highly correlated with the development of AMD. The homeostasis of the basement membrane and development of BLDs is dependent on the formation and degradation of basement membrane components. AGE cross-links make proteins resistant to enzymatic proteolysis. Recently, AGEs were found to increase collagen IV, reduce matrix metalloproteinase 2 expression, and increase tissue inhibitor of metalloproteinase 1 expression in mesangial cells. These factors would promote matrix accumulation and, possibly, BLD formation.

We also found that drusen, which have been correlated with aging of the RPE and possibly AMD, stained for AGEs with both antibodies. The accumulation of lipofuscin, a component of drusen, has been correlated with aging of the central nervous system and Alzheimer’s disease. Recently, Horie et al. showed that pentosidine and CML colocalized with lipofuscin pigments in neuronal perikarya of brain tissue of aged subjects or of those with Alzheimer’s disease. They suggested that lipofuscin formation involves not only lipid peroxidation products, but also glycation products.

Our biochemical and immunohistochemical data show a quantitative age-related increase of the AGE pentosidine in Bruch’s membrane and localization of pentosidine and CML to the Bruch’s membrane-choroid including BLDs and drusen. Although these in vivo data do not establish a role for AGEs, they provide the rationale for determining whether AGEs have a role in disease of the RPE-Bruch’s membrane-choroid complex. To provide evidence that AGEs can alter the RPE-Bruch’s membrane-choroid, we recently showed that pentosidine at physiologically relevant levels induces platelet-derived growth factor-B chain mRNA and protein levels in cultured RPE cells. Further work showing changes in the RPE by AGEs is in progress.
Analysis of p16 (CDKN2/MTS-1/INK4A) Alterations in Primary Sporadic Uveal Melanoma

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Purpose. To define more clearly the role of the tumor suppressor gene p16 in uveal melanoma by determining the relative contribution of all known mechanisms of p16 inactivation in this tumor.

Methods. A comprehensive genetic analysis of the p16 gene was performed in 33 primary sporadic cilioretinal and choroidal melanomas. Fourteen highly polymorphic microsatellite markers surrounding the p16 locus on chromosome 9p21 were used for the microsatellite analysis. Sequence analysis of the p16 gene was performed on those tumors with 9p21 loss of heterozygosity. To investigate methylation as an alternative mechanism of inactivation of p16, methylation-specific polymerase chain reaction was performed on all tumor DNA samples.

Results. Loss of heterozygosity (LOH) was found in 8 of 33 (24%) uveal melanomas. No evidence of a second region of LOH that did not include the p16 locus was found. Four cases had hemizygous losses including markers both distal and proximal to p16. Homozygous deletion of the p16 gene was detected in the 4 remaining cases by microsatellite analysis. Sequence analysis revealed no p16 mutations in the tumors with hemizygous loss of p16. Methylation of the 5' CpG island of p16 was found in one tumor with 9p21 LOH and in another without LOH.

Conclusions. p16 inactivation by HD or methylation occurs in 27% of uveal melanomas, representing the most common molecular genetic alteration identified thus far in uveal melanoma. (Invest Ophthalmol Vis Sci. 1999;40:779-783)

Uveal melanoma is the most common primary intraocular malignancy in adults and the second most common site of origin of malignant melanoma. Although many of the clinical features and histopathologic indicators of uveal melanoma have been established, little is known about the genetic alterations that are involved in the development of uveal melanoma and the progression to metastatic melanoma. Malignant transformation during tumor progression is known to result from a series of genetic changes. Determining the nature and timing of these changes in uveal melanoma will aid in understanding the biology of this tumor type and could lead to the development of new diagnostic and therapeutic strategies.

In human cancer, the frequency of inactivation of the tumor suppressor gene p16, located on chromosome 9p21, is reported to be second only to inactivation of p53. Germine mutations of p16 are found in about half of patients with familial cutaneous melanoma, and p16 mutations have been found in many cutaneous melanoma cell lines. Although loss of heterozygosity (LOH; usually due to deletion of one allele at a given locus) of p21 is one of the most frequent genetic alterations identified in human cancer, intragenic mutations of p16 have been detected infrequently in many primary cancers with LOH at 9p21. Instead, very small homoyzgous deletions (HDs) of p16 have been detected in these cancers using mic-