**Objective:** To investigate the localization of \(\text{N}^\text{e}-(\text{carboxymethyl})\text{lysine} (\text{CML})\), a component and major immunologic epitope of advanced glycation end products, in aged eyes and choroidal neovascular membranes (CNVMs) surgically excised from eyes with age-related macular degeneration.

**Methods:** Immunohistochemistry for CML was performed using 8 snap-frozen, surgically excised CNVMs. Twelve eyes from patients aged 69 to 82 years and 2 donor eyes, 1 each from a 23-week-old fetus and 21-year-old patient, without age-related macular degeneration or diabetic retinopathy were also examined. To determine if retinal pigment epithelial cells in CNVMs accumulate advanced glycation end products, cytokeratin and CML were stained in paired serial sections.

**Results:** Soft, macular drusen and/or basal laminar and basal linear deposits were observed in 8 of 12 aged eyes. Each case showed CML accumulation, while overlying retinal pigment epithelial cells showed no accumulation in all 12 eyes. In CNVMs, however, retinal pigment epithelial cells showed CML accumulation in their cytoplasm.

**Conclusion:** The additional accumulation of advanced glycation end products in soft, macular drusen and/or retinal pigment epithelial cells may play a role in the pathogenesis of CNVM formation in age-related macular degeneration.

**Clinical Relevance:** Recently, advanced glycation end products have been found to play a role both in aging changes and neovascularization. Localization of advanced glycation end products in the above-mentioned tissue may lead to a better understanding of the pathogenesis of age-related macular degeneration.


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**AGE-RELATED** macular degeneration (AMD) is the leading cause of blindness in elderly patients. The choroidal neovascularization (CNV) that occurs in the exudative form of this disease is the major risk factor for blindness. Recent investigations have revealed that advanced glycation end products (AGEs) may play a role in aging changes, and neovascularization, so we hypothesized that they might also be involved in the pathogenesis of AMD. Glucose reacts nonenzymatically with proteins to form Schiff base and Amadori products. Further incubation of Amadori products lead to the formation of AGEs that includes \(\text{N}^\text{e}-(\text{carboxymethyl})\text{lysine} (\text{CML})\). There is a spontaneous and age-dependent increase in AGE accumulation in several different cells and tissues. Diffuse thickening of the inner aspect of Bruch membrane, associated with abnormal deposits, is a commonly observed aging change in the eye. The deposits have been designated as basal laminar or basal linear deposits, or soft, macular drusen depending on their site or manner of accumulation, and are an important associated and predisposing finding of AMD.

The substance CML is a dominant antigen for anti-AGE antibodies. To determine whether the accumulation of CML is associated with deposits such as soft, macular drusen, basal laminar, and basal linear and/or retinal pigment epithelial (RPE) cells, we performed immunohistochemistry for CML in surgically excised choroidal neovascular membranes (CNVMs) and in donor eyes without AMD or diabetic retinopathy at various ages.

**RESULTS**

**CML ACCUMULATION**

The tissue sections of 8 eyes from 12 aged donor eyes had soft, macular drusen and
MATERIALS AND METHODS

CNVMs AND DONOR EYES

Surgical excision of subfoveal CNVMs was performed on 8 eyes from patients with AMD who had not undergone foveal laser photocoagulation. Tenets of the Declaration of Helsinki were followed, informed consent was obtained, and institutional human experimentation committee approval was granted for this study. The surgical indications and the techniques used have been described. Each of the fresh, surgically excised CNVM specimens was immediately placed in a balanced salt solution at 4°C, then snap frozen in ornithine carbamyl transferase compound (Tissue Tek OCT Compound, Ames Co, Division of Miles Laboratories, Elkhart, Ind) in liquid nitrogen-cooled isopentane within 1 hour of surgical extraction. Chorioretinal specimens from 14 fresh, postmortem donor eyes (12 from patients aged 69-82 years, as well as 1 each from a 23-week-old fetus and a 21-year-old patient), without evidence of chorioretinal disease, were similarly processed. The donor eyes were obtained from the Lions Doheny Eye and Tissue Transplant Bank, Los Angeles, Calif.

IMMUNOHISTOCHEMISTRY FOR CML

Thawed tissue sections were incubated for 30 minutes with 1% bovine serum albumin and 0.3% hydrogen peroxide. The specimens were incubated for 1 hour with anti-CML antibody (6D12, Wako Bio-Products, Richmond, Va), then washed for 15 minutes with phosphate-buffered saline solution. A recent study revealed that 6D12 monoclonal antibody specifically recognizes CML-protein adduct. Immunoperoxidase detection was performed using avidin-biotin-complex (ABC Elite kit, Vector Laboratories, Burlingame, Calif) with aminoethylcarbazole as the red chromogen. Negative controls included substituting mouse nonimmune IgG for the primary antibody; omitting the primary antibody in the staining protocol; and using anti-CML antibody (2 µg/mL) adsorbed with an excess of CML-bovine serum albumin (100 µg/mL).

IMMUNOHISTOCHEMISTRY FOR CYTOKERATIN

To investigate if RPE cells accumulate CML in CNVMs, immunohistochemistry for cytokeratin (Dako Inc, Carpinteria, Calif) was performed in the serial section adjacent to that used for CML staining. Expression of cytokeratin is limited to RPE cells in retinchoroidal tissue.

basal laminar and basal linear deposits. In these 8 eyes, CML accumulation was observed in most soft, macular drusen and in basal laminar and basal linear deposits, although Bruch membrane also revealed focal CML deposition (Figure, A). This accumulation was not detected in the RPE layer of the 12 aged donor eyes examined. Sections stained with anti-CML antibody (2 µg/mL) adsorbed with an excess of CML-bovine serum albumin (100 µg/mL) did not show any positive reaction (Figure, B). The 2 control eyes from young donors did not show any CML accumulation. Findings from histologic examination of the 8 surgically excised, AMD-related CNVMs revealed a spectrum of changes, that ranged from moderately cellular membranes with prominent neovascularization to paucicellular fibrotic membranes with no demonstrable vascular channels. In all cellular CNVMs, CML-positive cells were observed in the membrane, focally forming a nestlike arrangement that suggested an origin from RPE cells. Some melanin-laden cells were strongly positive for CML (Figure, C). In CNVMs, no apparent drusen could be identified, probably because they were either destroyed in the process of the fibrous membrane formation or lost during surgical removal. In the CNVM section, serial to that stained with CML (Figure, C), cytokeratin-positive cells were seen in a distribution pattern similar to that seen in the AGE-positive cells (Figure, D). In the paucicellular fibrotic membrane, AGE-positive cells were mainly observed in the partially intact RPE layer (Figure, E). Negative controls did not show any positive reaction.

N°-carboxymethyl)lysine is one of the AGEs detected in vivo and is a major immunologic epitope for anti-AGE antibodies. Handa et al reported pentosidine deposition, another component of AGEs, in the Bruch membrane in aged eyes (patients aged 82 and 92 years), as well as in the RPE cells, and in the choroid, but not in a young eye (patient aged 20 months). The young eyes in our study (23-week-old fetus and 21-year-old patient) showed no evidence of CML accumulation in the retinchoroidal tissue (data not shown). In 8 of 12 control eyes (patients aged 69-82 years), CML deposition was detected in Bruch membrane and accentuated in deposits such as basal laminar and basal linear ones and in soft, macular drusen. As opposed to pentosidine, no apparent CML deposition was observed in the intact RPE layer or the choroid.

In contrast, CML accumulation in RPE cells was suggested in CNVMs, a feature not found in any of the control eyes not even in those RPE cells adjacent to the CML-positive soft, macular drusen. The cells positive for CML showed a distribution pattern similar to that of the cells positive for cytokeratin in CNVMs. Since cytokeratin is expressed only by RPE cells in the retinchoroidal tissue, this result suggests that RPE cells in CNVM have CML accumulation in their cytoplasm.

We and other investigators have reported the localization of vascular endothelial growth factor (VEGF) in CNVMs. In our previous study, smooth muscle actin-positive or fibroblastic (transdifferentiated) RPE cells were commonly found in the highly vascularized regions in CNVMs obtained from patients with AMD. These transdifferentiated RPE cells expressed VEGF, suggesting an angiogenic role of these cells and this growth factor in AMD-related CNV. The factors that trigger increased VEGF expression in RPE cells of CNVMs are unknown. In diabetic retinopathy, hypoxia is the major stimulus that induces retinal cells to express VEGF. Relative ischemia

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of the outer retina that may be caused by atherosclerosis and atrophy of choriocapillaris has been suggested to be involved in the development of exudative AMD. Other than ischemia, various agents including AGEs have been reported to have a promoting effect on VEGF expression in various cell types. Advanced glycation end products are of particular interest since they stimulate VEGF expression in both cultured RPE cells and the in vivo RPE layer. They also stimulate endothelial cells to secrete VEGF and have an in vitro angiogenic effect. This suggests that the additional accumulation of AGE in RPE cells may lead to VEGF overexpression in older patients’ eyes, resulting in the initiation and development of CNV.

In addition, CML could play a role in the fibrous membrane formation that accompanies CNV. The association between AGE accumulation and fibrosis has been reported. Advanced glycation end products induce overexpression of growth factors such as transforming growth factor β and platelet-derived growth factor in RPE cells, that may promote fibrosis.

The formation of CML has been shown to occur at sites of oxidant stress with hydroxyl radical formation. The finding of CML accumulation in soft, macular drusen, basal laminar and basal linear deposits, and RPE cells suggests that similar oxidant stress may also show up at these sites, where it could result in damage to Bruch membrane and surrounding tissue. This damage may contribute to disruption in Bruch membrane. This disruption causes CNV to extend from the choroid into the subretinal space and is another critical process for formation of CNV. Advanced glycation end products induce the increased expression of cytokines known to occur in CNV and in surrounding fibrous membrane, where these end products could play a pathologic role. Our study provides a basis for the hypothesis that abnormal accumulation of AGE in soft, macular drusen, basal laminar and linear deposits, and RPE cells may be involved in the pathogenesis of AMD.

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