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Phenotypic Variation of Retinal Pigment Epithelium in Age-Related Macular Degeneration

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Abstract

PURPOSE. To determine whether retinal pigment epithelium (RPE) in eyes with age-related macular degeneration (ARMD) express vimentin and α smooth muscle actin (αSMA), two cytoskeletal proteins associated with phenotypic variation in culture.

METHODS. Six eyes with late ARMD and three age-matched control eyes were preserved in buffered 4% paraformaldehyde and cryosectioned at 10μ m. Stages of RPE morphology and pigmentation were assessed by the Alabama Age-Related Macular Degeneration Grading System. Vimentin,α SMA, and glial fibrillary acidic protein (GFAP) expression was detected by indirect immunofluorescence. These results were compared with regional variations in disease severity.

RESULTS. RPE changes in ARMD included acquired expression of vimentin, butα SMA-positive cells were rare. GFAP expression increased in Müller cells in the neural retina in association with RPE changes and photoreceptor degeneration.

CONCLUSIONS. The initial stages of RPE changes in eyes with ARMD mimic those reported for cultured RPE cells. The absence of αSMA-positive cells in regions of RPE atrophy suggests that RPE are lost rather than persist in a dedifferentiated

state.

Age-related macular degeneration (ARMD) is the leading cause of untreatable new vision loss among older adults in the United States and other industrialized countries. [1] [2] [3] The causes of ARMD are poorly understood, but it is agreed that its most prominent clinical and histopathologic features are lesions involving the retinal pigment epithelium (RPE) and Bruch's membrane. [4] [5] Early ARMD is characterized by minor to moderate vision loss associated with focal or diffuse sub-RPE debris (drusen and basal deposits, respectively) and changes in RPE pigmentation. Late ARMD is characterized by severe vision loss associated with geographic RPE atrophy, with or without choroidal neovascularization (CNV) and its sequelae.

A pivotal role of the RPE in ARMD pathogenesis has been postulated for almost three decades. [6] Clinically visible hyper- and hypopigmentation of macular RPE increase with age and are predictors of late ARMD ranking in quantitative importance with drusen. [1] [2] [3] [7] [8] [9] [10] The functional status of RPE cells in these states is incompletely understood. Pigmentary abnormalities are transient with a time course of several years. [11] [12] [13] Histopathologic studies reveal pigment clumping, loss of pigment, heterogeneity of cell sizes and shapes, formation of multiple layers, and presence of pigmented cells in the subretinal space. [5] [14] Cells that are thought to be RPE in CNV membranes surgically excised from patients with exudative ARMD [15] [16] [17] [18] [19] [20] express the myofibroblast marker α smooth muscle actin (αSMA), the intermediate filament protein cytokeratin, and the angiogenic agent vascular endothelial growth factor.

Changes in RPE morphology associated with ARMD have been formalized by the Alabama Age-related Macular Degeneration Grading System (ALARMDGS [21]), summarized in Table 1 . RPE atrophy or absence of a pigmented layer (grade 4) is the logical end point of cellular degeneration, whether in large areas (geographic atrophy) or small (nongeographic atrophy). Heaping and sloughing of cells into the subretinal space (grade 2) and anterior migration of cells into the retina (grade 3) are logical intermediate stages for classifying disease-related RPE changes on a per-eye basis. However, it is not yet clear that these stages represent all the possible steps between stages 1 and 4 for individual

RPE cells. Not only can RPE cells migrate anteriorly, but they may also die in situ or dedifferentiate into another phenotype unrecognizable as RPE.

A growing body of evidence suggests that RPE cells are programmed to react to environmental changes with alterations in cell phenotype and function. [22] [23] After isolation and maintenance in culture, porcine RPE cells change from an epithelial to myofibroblast-like cell type, as defined by changes in cell morphology, loss of pigment granules, cytokeratin 18 expression, and acquired expression of vimentin and αSMA. [24] [25] Coincident with these phenotype changes are the increased ability of the cells to adhere to certain extracellular matrix molecules, generate tractional forces on three-dimensional collagen matrices, and secrete growth factors, such as insulin-like growth factor I and platelet-derived growth factor. Although the functional significance of these RPE changes in fibrocontractive diseases such as proliferative vitreoretinopathy is apparent, their function in ARMD is much less clear. It is possible that the increased risk of late ARMD signaled by a hyperpigmented RPE may be indirect, through its association with diffuse deposits in Bruch's membrane, [26] [27] [28] or direct, through secretion of growth factors or cytokines from RPE cells in an activated state.

We hypothesized that ARMD-associated aberrations in RPE morphology represent definable stages of phenotypic modulation. We tested this hypothesis by examining RPE in the eyes of donors with ARMD and describing changes in cell morphology, pigmentation, and expression of the same cytoskeletal proteins known to vary in culture.

Methods

Human Eyes and Clinical Records

Human eyes were obtained within 3 hours of death from nondiabetic donors. All eyes were inspected internally and photographed to document grossly visible chorioretinal disease in the macula. [21] Ophthalmic histories were obtained through contact with donor families and follow-up with ophthalmologists. Histories were reviewed by a retina specialist (NEM) to exclude eyes with a history of non-ARMD chorioretinal disease in the macula. Use of human tissues and clinical records was approved by institutional review at the University of Alabama at Birmingham.

Tissue Preparation

Globes used for immunohistochemistry were preserved by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 6 hours after removal of the anterior segment. Samples approximately 8 × 5 mm from the macular retina-RPE-choroid, which contained the fovea and temporal half of the optic nerve head, were cut from each eye with a razor blade. All samples were cryoprotected with successive solutions of 10%, 20%, and 30% sucrose in PB, 4:1 30% sucrose-mounting compound solution (Histo-Prep; Fisher Scientific, Fairlawn NJ), and 2:1 30% sucrose-mounting compound solution for at least 30 minutes each and then frozen in −70°C isopentane. [29] Specimens were sectioned at 10 μm on a cryostat (CM3000; Leica, Deerfield, IL), collected on gelatinized slides, dried on a hot plate at 40°C to 60°C, and stored at −20°C. Before immunohistochemical experiments, the slides were warmed to room temperature, postfixed with acetone for 3 minutes, and heated to 50°C for 60 minutes. Some sections were stained with periodic acid-Schiff and hematoxylin for histopathologic evaluation.

Indirect Immunofluorescence Microscopy

Slides with cryosections were blocked with 20% nonimmune serum in PBS from the same species as the secondary antibody for 60 minutes at room temperature. Sections were then probed with primary and secondary antibodies in 2% nonimmune serum in PBS for 1 hour each with three 5-minute washes between probes. Photomicrographs were taken (Optiphot; Nikon, Inc., Melville, NY) equipped with epifluorescence illumination and phase-contrast optics using a 35-mm camera (Ilford Delta 100 and SFX 200 film; Ilford Photograph Corp., Paramus, NJ). Images were scanned from negatives (SprintScan 4000; Polaroid Corp, Cambridge, MA) and assembled into composite photomicrographs with image management software (Adobe Photodeluxe; Adobe Systems, Inc., San Jose, CA).

Reagents

Primary antibodies used in this study included mouse anti-smooth muscle actin (clone 1A4; Sigma Chemical Co., St. Louis, MO), mouse anti-vimentin (clone V9; Dako A/S, Glostrup, Denmark), mouse anti-cytokeratin 18 (clone CY-90; Sigma), mouse anti-glial fibrillary acidic protein (GFAP; clone GA5; Sigma) and rabbit anti-GFAP (Dako). The specificity of these antibodies has been previously verified by Western blot analysis. [25] [30]

Secondary antibodies included allophycocyanin-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR), rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA),and fluorescein-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch). Normal goat and donkey sera were obtained from The Binding Site, Ltd. (Birmingham, UK).

Analysis of Immunoreactivity in Cryosections

For each eye, we analyzed one slide containing two cryosections for each primary antibody and one control slide. Each slide set was examined in a masked fashion by two independent graders. The degree of RPE change was assessed by bright-field microscopy using the five grades of RPE morphology designated by the ALARMDGS [21] (Table 1) . The entire 8-mm length of each section was divided into different zones of RPE morphology that ranged in length from less than 100 μm for clumps of cells in ARMD eyes to several millimeters for normal RPE in non-ARMD eyes. The mean number of graded zones in the two sections was 15.6 in each ARMD eye. All RPE grades (0–4) were found in all ARMD eyes. Sections were then viewed with epifluorescence illumination to make binary judgments about immunoreactivity (“labeled” or “unlabeled”) as a function of RPE grade. Data from each grade were pooled across eyes. Choroidal fibroblasts and vascular smooth muscle cells served as an internal positive control for vimentin and αSMA labeling, respectively.

The relationship of GFAP immunoreactivity to RPE and photoreceptor changes was assessed using monoclonal anti-GFAP. Areas of positive and negative immunoreactivity were identified, and a grade for RPE and photoreceptor morphology assigned. For this analysis, the RPE grades provided by the ALARMDGS were collapsed into three categories: normal (0, 1), reactive (2, 3) and atrophic (4). Photoreceptors were assessed as normal, degenerating (shortened outer segments, broadened inner segments, and/or thinned outer nuclear layer), or absent.

Results

Characterization of ARMD Eyes

To characterize changes in RPE antigen expression associated with ARMD, cryosections of

ARMD eyes were probed with the same antibodies previously used to characterize porcine RPE in culture. [24] To obtain RPE grades ranging from normal to complete atrophy in single specimens, we analyzed six eyes from patients with late ARMD. Clinical and histopathologic information from eyes with late ARMD are summarized in Table 2 . Patients were 77 to 94 years old (mean, 86.2) and included three women and three men. Five of the six eyes with available histories had clinical diagnoses of ARMD. Four eyes had clinical history of CNV. Two eyes had laser photocoagulation, one of which was scotoma limiting. Histopathologic evaluation showed that all six study eyes had evidence of CNV that progressed to fibrovascular scar, a continuous layer of basal deposits, and a large area of geographic RPE atrophy (grade 4) bounded by a transitional zone of reactive RPE [14] (grades 2–3). Two eyes had drusen.

ARMD-Associated Changes in Vimentin and αSMA Expression

To compare culture-associated changes in RPE antigen expression with those associated with ARMD, cryosections of the late-ARMD eyes and three age-matched control eyes were probed with antibodies to vimentin and αSMA (Table 3) . Each section from ARMD eyes analyzed for immunoreactivity typically had two to three areas of each RPE grade, with the less-involved tissues flanking more severe lesions. Staining results between the two sections from each eye agreed closely, and the results across eyes were consistent. Grade 0 RPE cells were uniformly negative for vimentin content (Fig. 1A , compared with 1B for autofluorescence and 1C for pigmentation), but a variable percentage of cells at grades 1 and higher contained vimentin immunoreactivity (Table 3) . Cells at grade 1 contained a rim of positive immunoreactivity at the basal margin (Figs. 1D 1E 1F). At grade 2, the proportion of cells with vimentin expression increased, and the extent of labeling within individual cells increased to include the entire cell margin with faint fibrillar elements in the cytoplasm (Fig. 1G , compared with 1H and 1I). Of note, in regions of RPE atrophy (grade 4), the vimentin content of the layer immediately above Bruch's membrane appeared to increase markedly (Fig. 1J , compared with 1K and 1L). However, because this labeling coincided with complete loss of pigment and autofluorescent granules, it was not clear whether it corresponded to the labeling of RPE cells undergoing phenotypic modulation or to the encroachment of vimentin-positive Müller cells from the overlying retina.

In contrast to the abundance of vimentin-immunoreactive cells, relatively few cells near Bruch's membrane were positive for αSMA. When present, αSMA-positive cells were typically fibroblast-like in morphology and had no autofluorescence and pigment granules (Figs. 2A 2B 2C 2G 2H 2I , arrows). To distinguish between phenotypically modulated RPE cells and Müller cells, we examined αSMA expression in cryosections that were dual-labeled with a mouse monoclonal antibody against αSMA and a rabbit polyclonal antibody against GFAP. This analysis indicated that the αSMA-positive cells (Figs. 2A 2G) also had no GFAP immunoreactivity (Figs. 2B 2H) and therefore were of nonglial origin. The paucity of αSMA-positive cells (Figs. 2D 2E 2F) persisted throughout the range of RPE changes through grade 4. From this result we concluded that the αSMA-positive RPE cells of the type observed in culture are rare in eyes with late ARMD (Table 3) .

ARMD-Associated Changes in GFAP Expression

In the course of the double-label experiments performed with polyclonal anti-GFAP, we also observed regional changes in Müller cell GFAP expression (Figs. 2J 2K 2L) that appeared to be associated with reactive RPE and photoreceptor degeneration. We therefore probed sections from ARMD eyes with monoclonal anti-GFAP (clone GA5), the more common probe for reactive changes in Müller cells. [31] [32] [33] In control eyes and in uninvolved areas of ARMD eyes, little or no GFAP immunoreactivity was detectable in Müller cells (Fig. 3A) . However, in involved areas of the ARMD eyes, GFAP expression was observed in Müller cells in neurosensory retina overlying areas of diseased RPE (Figs. 3B 3C 3D) . To examine the relationship between ARMD-associated disturbances and GFAP expression more closely, the GFAP-positive and GFAP-negative regions of ARMD eyes were graded for changes in RPE and photoreceptor morphology. This analysis (Table 4) showed that GFAP-positive regions were preferentially associated with areas of reactive or atrophic RPE and areas where photoreceptors were absent.

Discussion

Understanding the pathogenesis of ARMD would be served by greater knowledge about disease-related changes in the cells involved. It is agreed that atrophy (i.e., the loss of a pigmented layer) is the end stage of RPE cells in ARMD. The status of RPE cells before atrophy is less clear, and we offer three potential pathogenetic mechanisms for RPE

atrophy: death in situ, modulation to a phenotypic unrecognizable as RPE, or migration. The answer to this question is important, because it provides insight into the nature of the signal received by RPE cells during disease progression. Based on published evidence from porcine RPE [24] [25] and similar observations in human RPE (Guidry C, unpublished data, 1998), we tested the hypothesis that RPE cells modulate their phenotype in ARMD. Our results support this hypothesis in the early stages of ARMD-associated RPE change: Large hyperpigmented RPE cells preferentially expressed the cytoskeletal protein vimentin. In contrast, grade 0 (morphologically normal) cells were uniformly negative for vimentin content.

Other investigators using the same antibody have reported that cells overlying choroidal melanomas also upregulate vimentin expression. [22] [34] In that study, vimentin-positive cells were considered hyperplastic RPE cells by morphologic criteria and the fact that other sections showed abundant cytokeratin 18–positive cells of the same morphology. [22] The appearance of RPE vimentin immunoreactivity also occurs in retinoblastoma [34] and experimental retinal detachment in monkey. [35] In all these conditions, including ARMD, the basal and basolateral aspects of RPE are labeled.

In contrast to vimentin, αSMA immunoreactivity was detectable in only a few cells, even at the late, atrophic stage of ARMD-associated RPE change. Others have found that αSMA expression is undetectable in morphologically normal or atrophic RPE overlying choroidal tumors. [22] Further, αSMA-positive cells that are presumed to be RPE by virtue of being labeled with a pancytokeratin antibody are present in surgically excised CNV membranes. [20] CNV is hypothesized to be a process with dynamic initiation, maintenance, and involutional stages. [36] Because of the long survival intervals between last clinical examination and donor death (Table 2) , CNV membranes in our donor eyes with late ARMD were at postinvolutional stages of disease, considerably later than the surgically excised CNV membranes examined by others. [20] Thus, it is likely that the expression of αSMA exhibits several different phases, including transient expression during early stage of CNV maturation. This idea is strengthened by studies showing thatα SMA-positive cells undergo apoptosis in CNV membranes. [37] Furthermore, these changes are not specific to ARMD, because αSMA-positive, morphologically hyperplastic cells also occur in subretinal membranes overlying choroidal tumors. [22]

The functional consequences of increased vimentin immunoreactivity for RPE are currently unclear. Vimentin expression has long been associated with the onset of mitosis [38] and may signal increased proliferative potential. However, recent studies indicate that vimentin expression and architecture are dynamic during other cellular activities. For example, during active cytoskeletal remodeling associated with cell spreading in culture, vimentin is initially found in nonfilamentous forms that are replaced first by short fibrous structures and then by extensive filamentous networks connected to other cytoskeletal systems. [39] Further, a role for vimentin in cell motility associated with wound healing is implicated by studies in mammary epithelial cells and vimentin-deficient fibroblasts. [40] [41] Thus, it is possible that the reaction to local perturbation reflected by increased vimentin immunoreactivity in stage 2 RPE in ARMD includes enhanced potential for cell migration. This notion is consistent with the position of stage 3 RPE cells within the neurosensory retina.

Although involvement of Müller cells in ARMD-associated fibrovascular lesions has been shown, [20] [42] our observation of increased GFAP expression by Müller cells in eyes with late ARMD is novel and also warrants discussion. Dramatic changes in Müller cell GFAP expression occur in response to numerous retinal insults, including laser- or light-induced damage, diabetic retinopathy, retinal detachment, and inherited retinal degeneration. [31] [32] [43] [44] [45] In the case of light or laser damage and diabetic retinopathy, increased GFAP expression may reflect a response to generally perturbed retinal physiology. [31] [46] However, in the case of retinal detachment, inherited degeneration, and ARMD, increased GFAP expression may reflect a secondary response to a primary insult to another cell type, [43] [44] although our results do not allow exclusion of the possibility of a primary role for Müller cells in this process. It is interesting that ARMD shares with retinal detachment not only upregulation of vimentin in RPE, but also the presence of Müller cell processes separating photoreceptors from RPE. [42] [47] In ARMD the most likely inductive cells are photoreceptors and/or RPE. These effects may be mediated by direct contacts between Müller cells and photoreceptors, [44] Müller cell responses to humoral factors secreted by the RPE, [25] or both. Understanding the relative contribution of these mechanisms to retinal degeneration requires a more detailed analysis of photoreceptor, RPE, and Müller cell changes at earlier stages of ARMD than was covered in this study.

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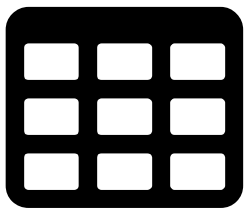
TABLE 1.



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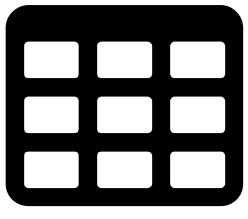
Grades of ARMD-Associated RPE Change According to the ALARMDGS

TABLE 2.



View Table

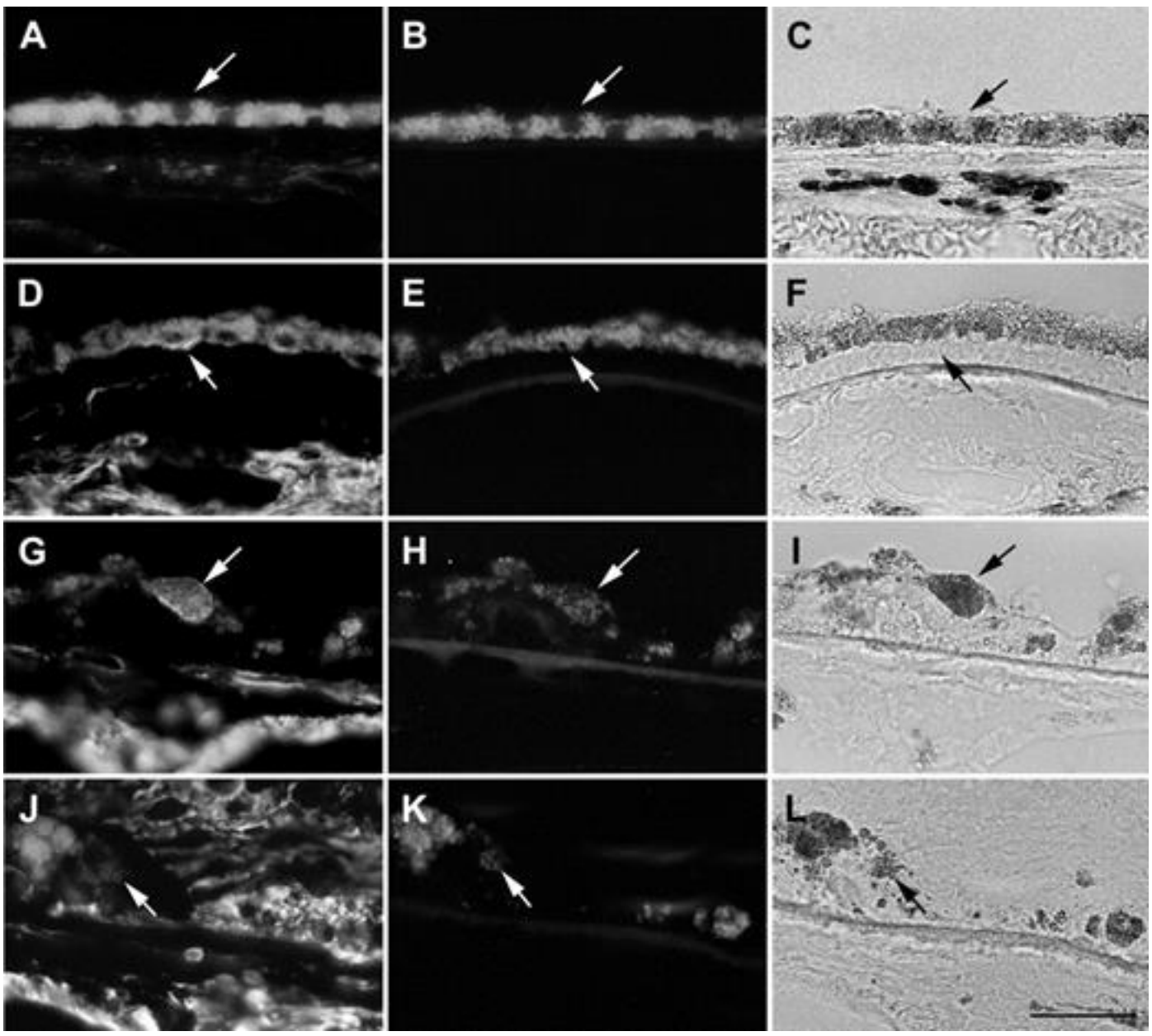
ARMD Eyes Studied

TABLE 3.

View Table

RPE Immunoreactivity as a Function of RPE Grade

FIGURE 1.



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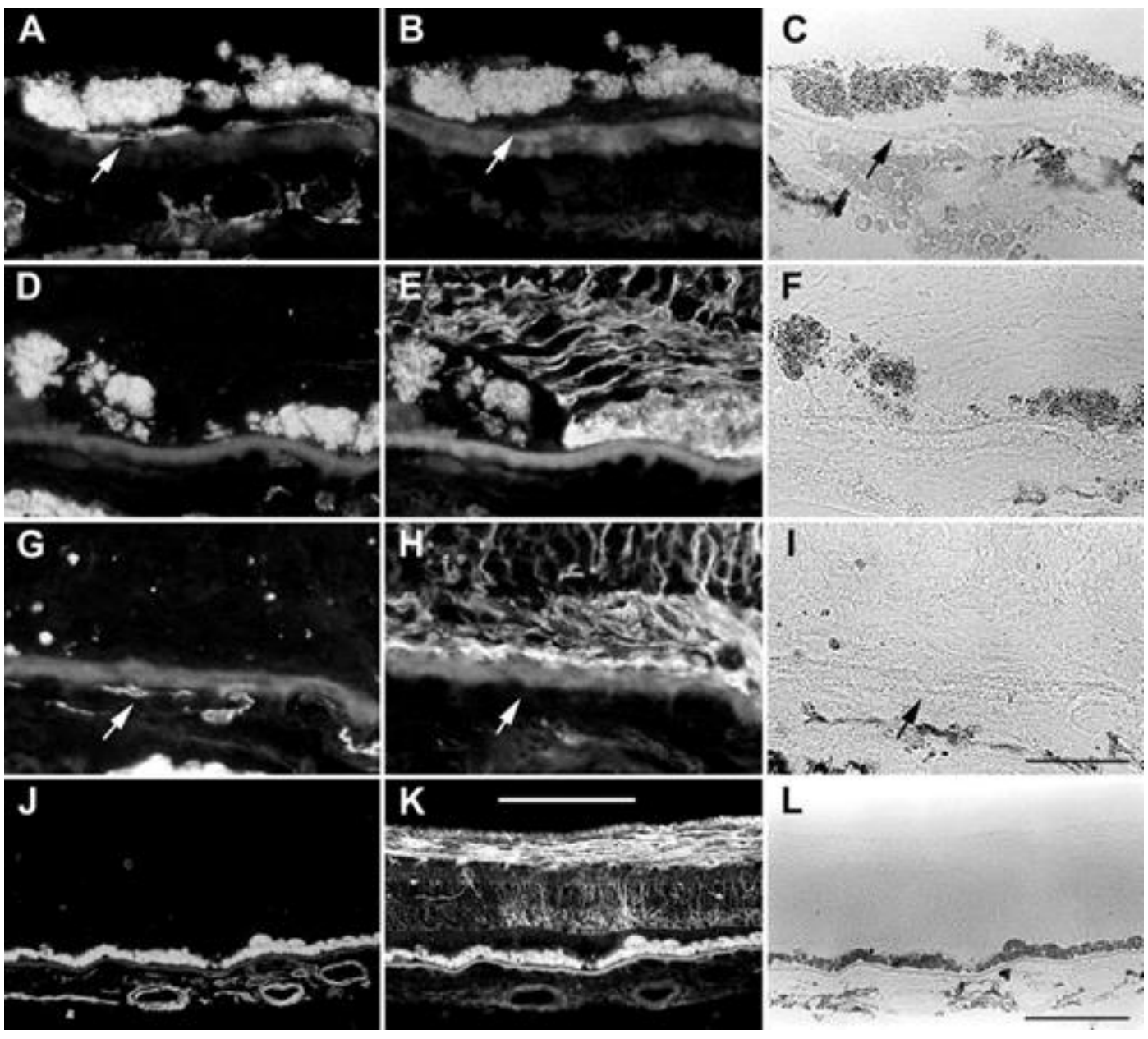
ARMD-associated changes in RPE cell vimentin immunoreactivity, autofluorescence, and pigmentation. Cryosections of human retina and choroid from patients with varying degrees of RPE cell change were probed with monoclonal anti-vimentin and allophycocyanin-conjugated secondary antibody. The same microscopic fields were examined sequentially by epifluorescence microscopy using allophycocyanin-specific excitation–emission filters (**A**, **D**, **G**, **J**) or fluorescein excitation–emission filters as a measure of cell autofluorescence (**B**, **E**, **H**, **K**) and bright-field microscopy for cell pigment granule content (**C**, **F**, **I**, **L**). (**A**–**C**) Grade 0 RPE change; (**D**–**F**) grade 1 RPE change; (**G**–**I**) grade 2 RPE change, and (**J**–**L**) grade 4, RPE atrophy. *Arrows*: same feature in each photomicrograph, as a point of reference. Scale bar, 50 μm.

Figure 2.



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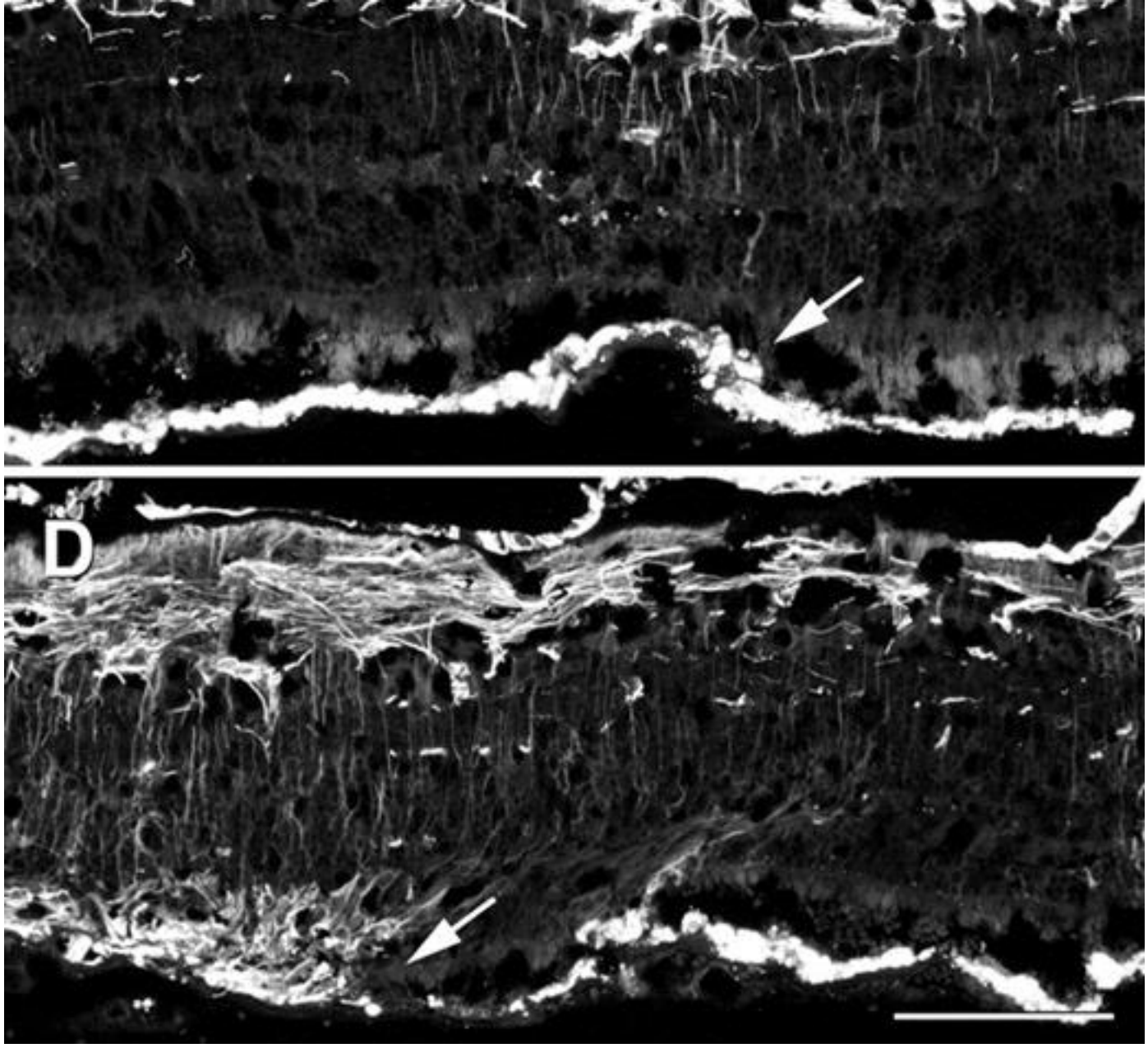
ARMD-associated changes in RPE cell αSMA and GFAP immunoreactivity. Cryosections of retina and choroid of patients with varying degrees of RPE cell change, including grades 2 (**A–C**, **D–F**, **J–L**) and 4 (**G–I**) were dual labeled with mouse monoclonal anti-αSMA and rabbit polyclonal anti-GFAP, followed by rhodamine- and fluorescein-conjugated secondary antibodies, respectively. The same microscopic fields were examined sequentially by epifluorescence microscopy, using rhodamine excitation–emission filters for αSMA (**A**, **D**, **G**, **J**) or fluorescein excitation–emission filters for GFAP (**B**, **E**, **H**, **K**) and by bright-field microscopy for pigment content (**C**, **F**, **I**, **L**). (**K**, *white line*) Area of enhanced GFAP immunoreactivity. Scale bars, in (**I**) for (**A–I**) 50 μm; in (**L**) for (**J–L**) 200 μm.

Figure 3.



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RPE cell-associated changes in retinal-GFAP expression in eyes with ARMD. Cryosections of human retina and choroid were probed with monoclonal anti-GFAP followed by rhodamine-conjugated secondary antibodies. (**A**) GFAP immunoreactivity in the uninvolved area of the ARMD eye was confined to astrocytes in the nerve fiber layer. (**B**, **C**) Focal areas of enhanced immunoreactivity in Müller cells associated with sub-RPE deposits (*arrows*). (**D**) Larger area of enhanced immunoreactivity associated with RPE atrophy (*arrow*). Scale bar, 200 μm.

TABLE 4.

View Table

Changes in Retinal GFAP Immunoreactivity as a Function of RPE and Photoreceptor Change

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