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Müller cells in detached human retina express glial fibrillary acidic protein and vimentin *

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Abstract. We investigated the expression of vimentin and glial fibrillary acidic protein (GFAP) within Müller cells in normal human retinas and in detached human retinas of proliferative vitreoretinopathy (PVR) cases using the immunogold method. Müller cells in normal retinas showed vimentin immunoreactivity and faint GFAP immunoreactivity; however, in detached retinas they showed distinct GFAP immunoreactivity as well as vimentin immunoreactivity. Immunoelectron microscopic observation revealed that intermediate filaments (IF) within Müller cells in normal retinas showed vimentin immunoreactivity and that those within Müller cells in detached retinas showed both vimentin and GFAP immunoreactivity. Double staining for vimentin and GFAP showed that in detached retinas, these two protein immunoreactivities were observed in the same filaments. These results indicate that IF of human Müller cells consist of vimentin under normal conditions and that Müller cells in detached retinas contain different IF, which consist of vimentin and GFAP.

Introduction

Glial fibrillary acidic protein (GFAP) is a glial-cell-specific protein that was initially found to be the intermediate filament (IF) protein in reactive astrocytes [5]. Müller cells, a major component of non-neural cells in the retina, are classified as one of the glial cell types. It has been reported that in rat retina Müller cells normally contain the other IF protein, vimentin [2, 26]. Accumulation of GFAP in Müller cells has been observed in some animal models in ocular injury and retinal degeneration [1, 2, 4, 26]. Retinal detachment also causes a series of degenerative changes in the retina; the longer the duration of the detachment, the more the degenerative changes occur [6, 8, 10, 13, 15, 21]. Müller cells show proliferation and hypertrophy in response to degeneration after a long period of retinal detachment [6]. Questions as to whether human Müller cells also express vimentin and/or GFAP under normal conditions and after a long period of retinal detachment and how these two IF proteins are distributed within the Müller cells remain open. In this immunohistological study, we showed that IF of human Müller cells mainly express vimentin immunoreactivity under normal conditions and exhibit both GFAP and vimentin immunoreactivity in the same IF after a long period of retinal detachment.

Materials and methods

Antisera

Rabbit anti-GFAP antibody, mouse monoclonal anti-vimentin antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig) antibody, HRP-conjugated anti-mouse Ig antibody and non-immune rabbit serum were purchased from DAKO-PATTS (Glostrup, Denmark). Non-immune mouse IgG was purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Secondary antibody-coated colloidal gold and a silver enhancement kit (Inten SE) were purchased from Janssen Life Science Products (Olen, Belgium). The antibodies were used at the following dilutions: anti-GFAP antibody, 1:500; anti-vimentin antibody, 1.6 μg/ml; HRP-conjugated anti-mouse Ig antibody and HRP-conjugated anti-rabbit Ig antibody, 1:100; secondary antibody-coated colloidal gold, 1:80.

Characterization of the primary antibodies

The specificities of primary antibodies were tested using the immunoblotting technique. Normal human retina was solubilized in a denaturing buffer containing 1% sodium dodecyl sulfate (SDS), 20% glycerol, 1 m*M* phenylmethylsulfonyl fluoride, 100 m*M* dithiothreitol and 20 m*M* TRIS buffer (pH 8.6). The sample was applied to the well of a 10% polyacrylamide slab gel and SDS-gel electrophoresis was carried out using the buffer system of Laemmli

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[12]. Then, the gel was subjected to electrophoretic transfer onto nitrocellulose paper using Towbin's buffer system [27]. The transfer papers were quenched in a buffer (150 m*M* sodium chloride and 20 m*M* TRIS buffer, pH 7.5; TBS) containing 3% bovine serum albumin (BSA) and were subsequently incubated with anti-GFAP or anti-vimentin antibody for 1 h. The papers were washed three times in TBS containing 0.05% Tween 20 (Tween-TBS) and then treated with HRP-conjugated secondary antiserum for 1 h. After being washed with Tween-TBS, the papers were incubated with TBS containing 0.5 mg/ml 4-chloro-1-naphthol and 0.015% (w/v) hydrogen peroxide for 5 min. Finally, they were washed with distilled water, dried and photographed.

Tissue preparation

Specimens of detached retina were obtained through a localized retinectomy carried out to reattach retina in proliferative vitreoretinopathy (PVR) cases. Specimens from three cases of PVR that lasted > 3 months were used. Normal human retinas were obtained from eye-bank eyes (ages: 18, 38, and 80 years old) within 24 h postmortem. The neural retinas were fixed in a mixture containing 3% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline (PBS) at pH 7.2 for 2 h at room temperature. After fixation, the tissue fragments were dehydrated in serial ethanol gradient and embedded in Lowicryl K4M (Polaron Equipment Ltd., Watford, England). Semithin sections for light microscopy and ultrathin sections for electron microscopy were prepared in serial sections from the same block.

Immunogold silver staining for light microscopy

Semithin sections were treated with 1% BSA in PBS (BSA-PBS) for 10 min to minimize non-specific reactions. Then, sections were incubated with the primary antibody (anti-GFAP or anti-vimentin antibody) for 1 h at room temperature. After being washed three times with PBS, the sections were incubated with secondary antibody-coated colloidal gold for 1 h. The sections were rinsed again, followed by fixation with 2.5% glutaraldehyde to prevent the dissociation of the antibody-antigen complex during subsequent procedures. After being washed with distilled water, the sections were treated with silver enhancer (77 m*M* hydroquinone and 5.5 m*M* silver lactate in 200 m*M* citrate buffer, pH 3.85) and the reaction was then stopped with the fixing solution (Janssen Life Science Products). Finally, sections were counterstained with 0.5% toluidine blue. As negative controls, normal rabbit serum was used instead of anti-GFAP antibody and non-immune mouse IgG replaced anti-vimentin antibody.

Immunogold staining for electron microscopy

Ultrathin sections were picked up on nickel grids and incubated on drops of BSA-PBS for 10 min to block non-specific reactions. The grids were then incubated with the primary antibody for 1 h at room temperature. After three washes with PBS, the grids were incubated with secondary antibody-coated colloidal gold (diameter, 15 nm) for 1 h. Then the grids were rinsed three times with PBS and three times with distilled water. After being stained with uranyl acetate, sections were viewed with a JEOL 100CX transmission electron microscope (JEOL Inc., Tokyo, Japan). Instead of the primary antibodies, non-immune rabbit serum and non-immune mouse IgG were used as negative controls.

Double staining for electron microscopy

For double staining, the immunogold staining procedure was duplicated. Anti-vimentin antibody and anti-mouse IgG-coated colloidal gold (diameter, 5 nm) were used in the first step, and anti-GFAP antibody and anti-rabbit IgG-coated colloidal gold (diameter, 15 nm) were used in the second step. After immunostaining had been performed, the grids were stained with uranyl acetate and viewed with the transmission electron microscope.

Results

Immunoblotting

Solubilized proteins from normal retina produced many bands on the amido black-stained nitrocellulose paper (Fig. 1, lane B). The anti-GFAP antibody depicted a single protein (mol. wt., 49,000 daltons), which had the same molecular weight as GFAP (lane C); this indicated that the anti-GFAP antibody specifically recognizes GFAP. The anti-vimentin antibody stained the main-band protein (mol. wt., 58,000 daltons) and produced a few other faint bands (lane D). As the molecular weight of vimentin corresponds to the main band and vimentin is easily degraded by protease(s) in the tissue, these bands are considered to represent vimentin and its degradation products [16, 17]. No cross-reactivity was observed between the antibodies.

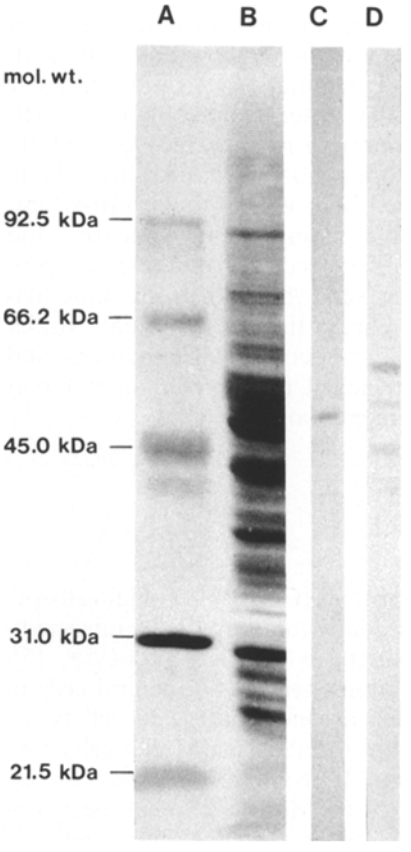

Fig. 1. Immunoblotting analysis of the primary antibody. Amido black staining of the nitrocellulose strips, showing the molecular weight standard (*lane A*) and the retinal homogenate (*lane B*). The anti-GFAP antibody reacted to a single protein (49,000 daltons) of the retina (*lane C*). The anti-vimentin antibody reacted to the main-band protein (58,000 daltons) and produced a few other faint bands (*lane D*). No cross-reactivity was observed between the antibodies

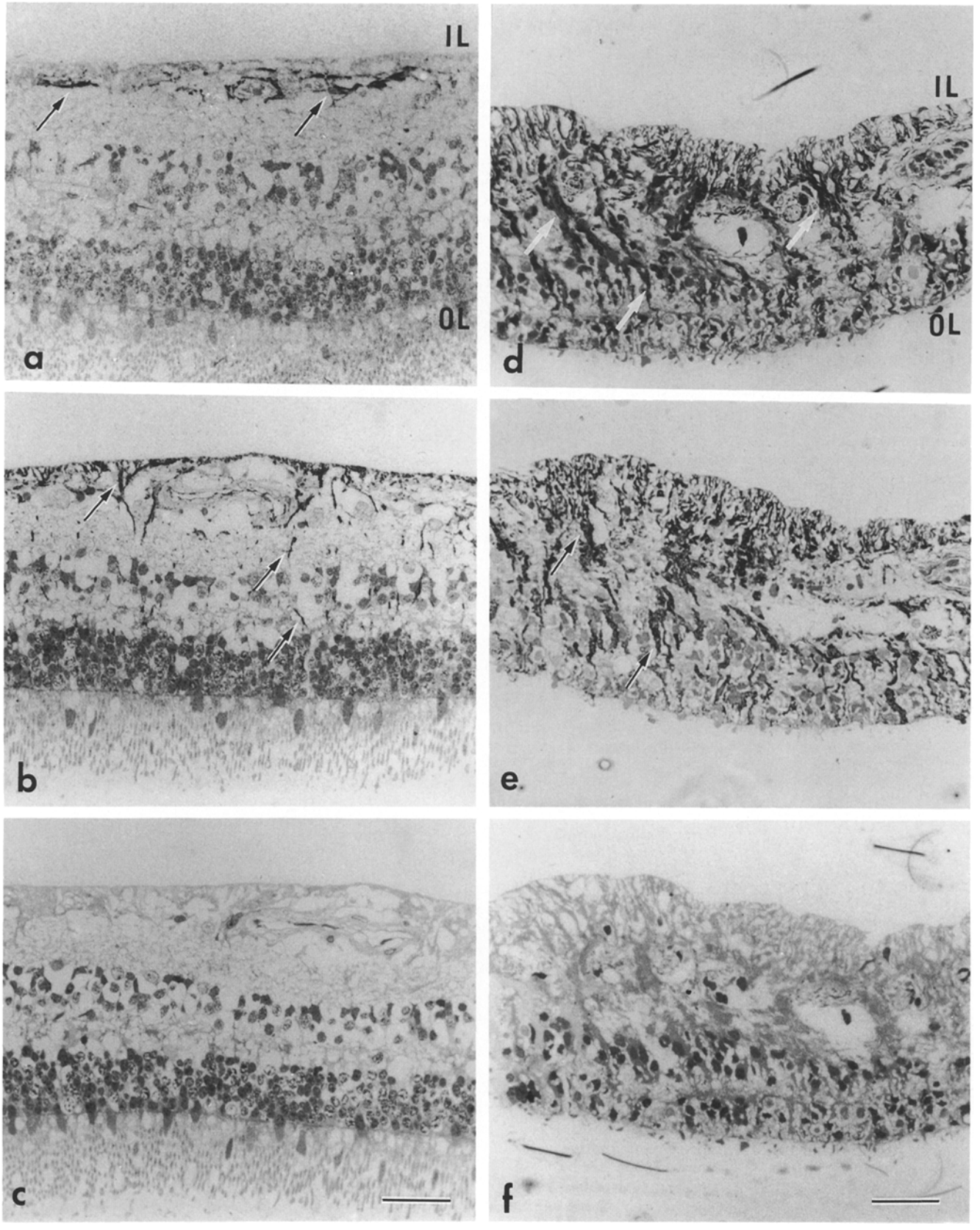

Fig. 2a–f. Light micrographs of the immunogold silver staining of normal and detached retinas in PVR. A positive reaction is represented by dark-colored silver precipitates. **a** In normal retinas, retinal astrocytes showed GFAP immunoreactivity (*arrows*) but Müller cells did not. **b** Müller cells in normal retinas expressed anti-vimentin immunoreactivity (*arrows*). Müller cells in detached retina show both **d** GFAP (*arrows*) and **e** vimentin (*arrows*) immunoreactivity. The immunostaining of the negative controls for **c** GFAP and **f** vimentin showed no immunoreactivity. *IL*, internal limiting membrane; *OL*, outer limiting membrane. Bar = 50 μm

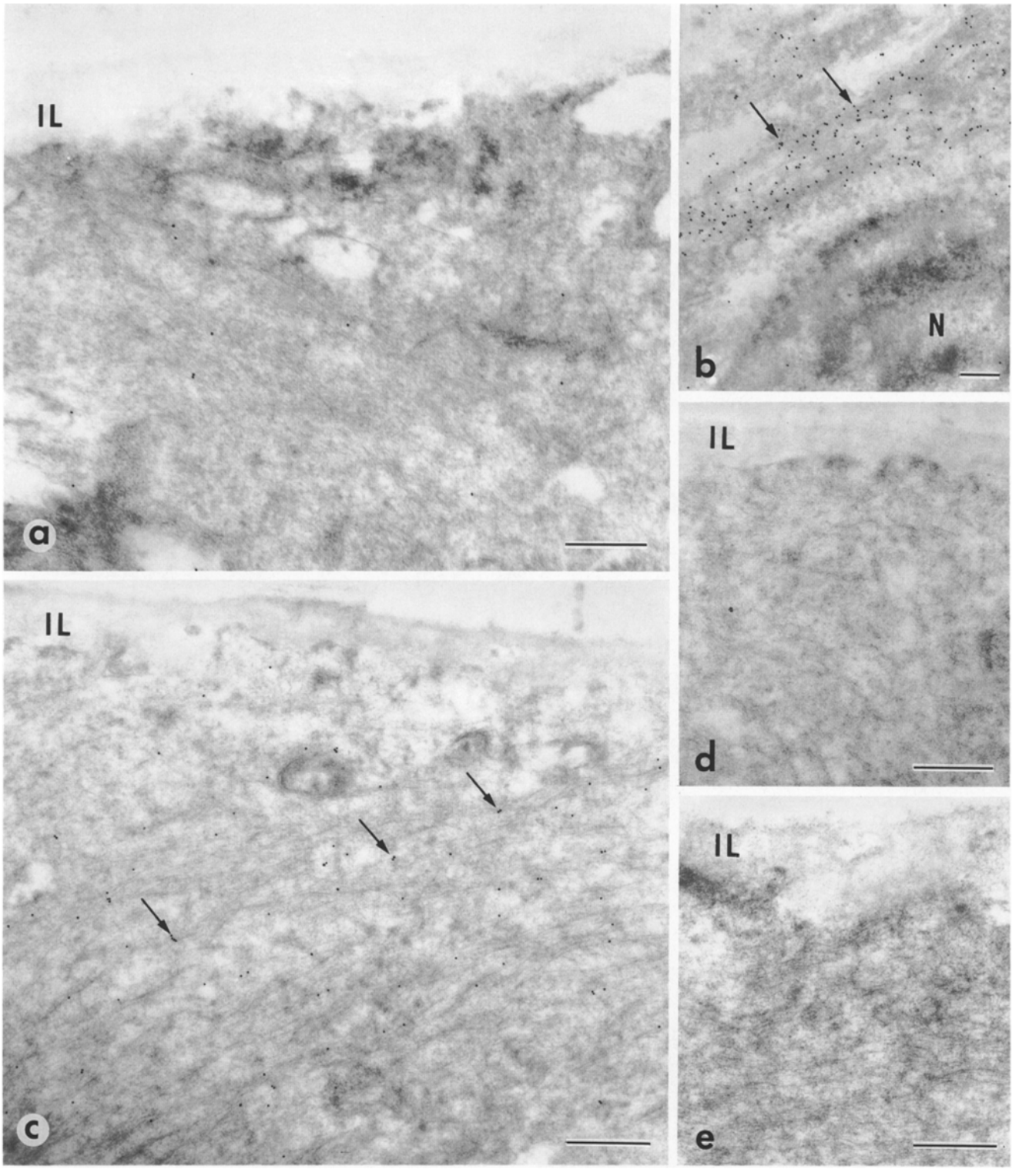

Fig. 3a–e. Immunoelectron micrographs of normal retina. **a** Faint GFAP immunoreactivity was observed on the intermediate filaments within the Müller cell. **b** The retinal astrocyte has intermediate filaments on which marked GFAP immunolabelling was observed (*arrows*). **c** Vimentin immunolabelling was observed on the intermediate filaments within the Müller cell (*arrows*). Negative controls for **d** GFAP and **e** vimentin immunostaining showed no immunolabelling within the Müller cell. *IL*, internal limiting membrane; *N*, nucleus. Bar = 0.25 μm

Light microscopy

In normal human retinas, GFAP immunolabelling was observed in the innermost retina, including the nerve-fiber and ganglion-cell layers (Fig. 2a). From their location and morphology, the GFAP-positive cells were interpreted as being retinal astrocytes. Müller cells, however, did not express GFAP immunoreactivity. Vimentin immunolabelling in normal retina was observed as radially oriented processes that extended from the inner

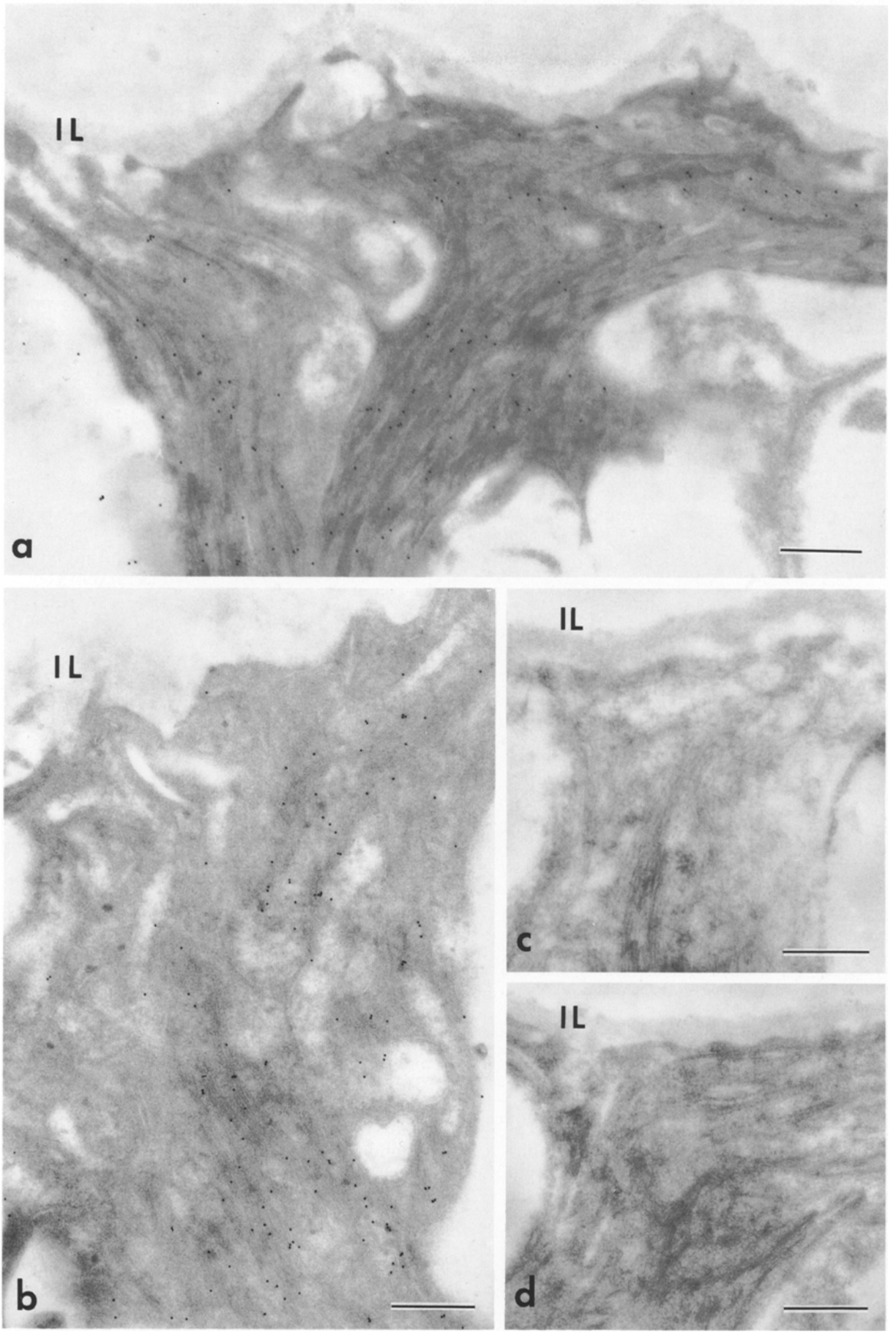

Fig. 4a–d. Immunoelectron micrographs of detached retinas. Müller cells in detached retinas expressed **a** GFAP and **b** vimentin immunoreactivity. Negative controls for **c** GFAP and **d** vimentin immunostaining showed no immunolabelling within the Müller cell. *IL*, internal limiting membrane. Bar = 0.25 μm

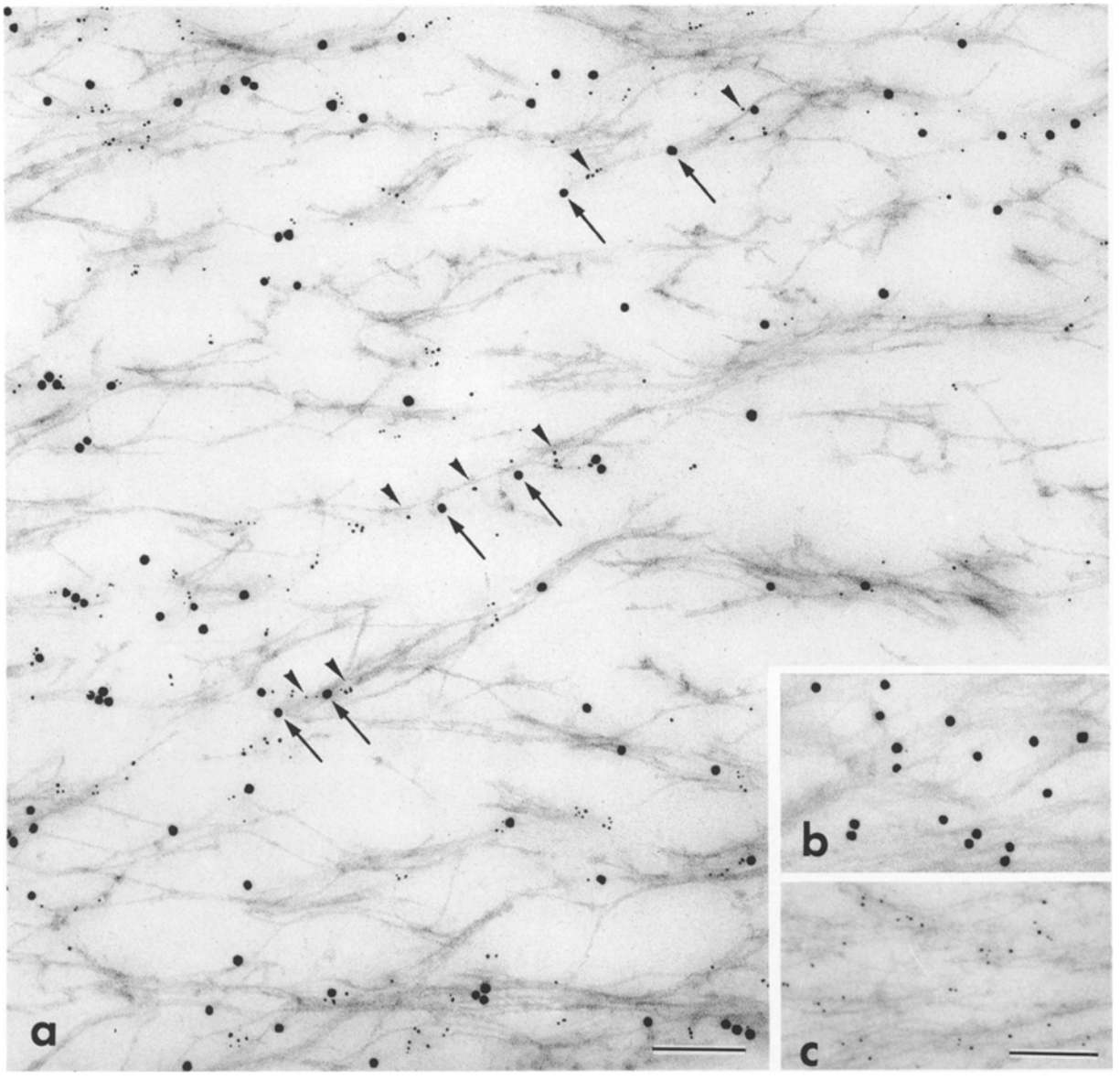

Fig. 5a–c. Electron micrographs of double staining in a detached retina. **a** Large gold particles (*arrows*) showed GFAP immunoreactivity and small gold particles (*arrowheads*) exhibited vimentin immunoreactivity. GFAP and vimentin immunoreactivity were observed on the same intermediate filaments. **b** On the section from which anti-vimentin antibody was omitted, only GFAP immunoreactivity was observed. **c** The section from which anti-GFAP antibody was omitted showed only vimentin immunoreactivity. Bar = 0.12 μm

limiting membrane through the inner nuclear layer into the outer limiting membrane (Fig. 2b). This staining pattern represented vimentin immunoreactivity in Müller cells.

In detached retinas, degeneration of the photoreceptor layer was marked. Most photoreceptor cells had disappeared from the outer nuclear layer. Both GFAP and vimentin immunolabelling was observed within the Müller cells, extending radially from the internal limiting membrane to the outer limiting membrane (Fig. 2d, e). In control sections (Fig. 2c, f) no immunoreactivity was observed.

Immunoelectron microscopy

In normal retinas, faint GFAP immunoreactivity was observed on the IF of the Müller cells (Fig. 3a). The retinal astrocytes, however, contained bundles of IF on which marked GFAP immunolabelling was observed (Fig. 3b). IF in normal Müller cells showed distinct vimentin immunoreactivity (Fig. 3c). In detached retinas, IF within Müller cells exhibited both GFAP and vimentin immunoreactivity (Fig. 4a, b). None of the control sections showed significant immunoreactivity (Figs. 3d, e and 4c, d).

Double staining for electron microscopy

GFAP and vimentin immunoreactivities were very closely related and were seen on the same IF in Müller cells of detached retinas (Fig. 5a). The control section from which the anti-vimentin antibody was omitted showed only GFAP reactivity (Fig. 5b); that from which the

anti-GFAP antibody was omitted showed only vimentin immunoreactivity (Fig. 5c).

Discussion

Müller cells in various species express GFAP in response to retinal injury and degeneration [1, 2, 4, 26]. Shaw and Weber [26] and Bignami and Dahl [1] have demonstrated that normal Müller cells express the other IF protein, vimentin. To investigate the relationship between these proteins, it is necessary that antibodies capable of discriminating between GFAP and vimentin be used. The immunoblotting analysis demonstrated that these antibodies do not cross-react.

Light and electron microscopic findings of the present study showed that Müller cells in normal human retinas showed mainly vimentin immunoreactivity. In detached retinas, however, human Müller cells showed both GFAP and vimentin immunoreactivity. Very few immunohistological studies on human Müller cells have been reported. Molnar et al. [14] and Hiscott et al. [11] demonstrated that human Müller cells do not normally show GFAP immunoreactivity. O'Dowd and Eng [18] and Ohira et al. [20] reported positive GFAP immunoreactivity for Müller cells in some, but not all, human retinas obtained at autopsy. The discrepancy between the present results and those of previous studies may be attributable to substantial differences in the time between death and the fixation of the tissue. All of these reports, however, also demonstrated GFAP immunoreactivity for Müller cells in degenerated retina. Recently, Erickson et al. [7] showed GFAP accumulation in Müller cells of cat retina after retinal detachment. Hiscott et al. [11] demonstrated GFAP immunoreactivity for human Müller cells after a long period of retinal detachment. These studies and the present results indicate that human Müller cells express GFAP immunoreactivity in response to degenerative changes in the retina, including retinal detachment.

We showed that human Müller cells mainly express vimentin immunoreactivity under normal conditions. It is noteworthy that Müller cells in detached retina showed both vimentin and GFAP immunoreactivity. This may suggest that GFAP accumulation in detached retina is not simply the result of a switch in the synthesis of IF protein from vimentin to GFAP. Co-expression of different IF proteins occurs in some cell types [9, 24, 25]. Keratin and vimentin form the two distinct IF systems in the epithelial cell line [9]. Another case of co-existence of different IF proteins involves some glial cells [24, 25], which have GFAP and vimentin that form co-polymers.

Through double staining in immunoelectron microscopy, we found that vimentin and GFAP immunoreactivities are very closely related and that some IF show both GFAP and vimentin immunoreactivity in Müller cells of detached retina. As we used the post-embedding immunostaining method, antibody access may have been limited; unlabelled areas of the IF are thought to be attributable to limited antibody access. Although we should admit the limitations of the method we used, our results do indicate that some of the IF within Müller cells of detached retinas are co-polymers of GFAP and vimentin. A similar transition in IF proteins from vimentin to vimentin plus GFAP has been reported in the development of retinal astrocytes and radial glia in rats [23, 26]. This transition has been considered to be the marker of glial differentiation.

It has been reported that GFAP-positive cells were found in preretinal proliferative tissue in PVR [19, 22]. Our results suggest the possibility that GFAP-positive Müller cells are involved in the proliferative process.

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