Identification of ganglion cell neurites in human subretinal and epiretinal membranes

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Aim: To determine whether neural elements are present in subretinal and epiretinal proliferative vitreoretinopathy (PVR) membranes as well as in diabetic, fibrovascular membranes removed from patients during vitrectomy surgery.

Methods: Human subretinal and epiretinal membranes of varying durations were immunolabelled with different combinations of antibodies to glial fibrillary acidic protein, vimentin, neurofilament protein and laminin.

Results: Anti-neurofilament-labelled neurites from presumptive ganglion cells were frequently found in epiretinal membranes and occasionally found in subretinal membranes. In addition, the neurites were only observed in regions that also contained glial processes.

Conclusions: These data demonstrate that neuronal processes are commonly found in human peri-retinal cellular membranes similar to that demonstrated in animal models. These data also suggest that glial cells growing out of the neural retina form a permissive substrate for neurite growth and thus may hold clues to factors that support this growth.

Injury to central nervous system (CNS) tissue often stimulates the activation of glial cells, ultimately resulting in their proliferation and hypertrophy throughout the damaged region. One consequence of this gliosis can be impaired regeneration of neurones as their processes are unable to migrate through the damaged area to re-establish synaptic connections. The newly formed glial “scar” acts as a physical barrier to regeneration and contains molecules inhibitory for neuronal growth. Retinal detachment, which separates the neural retina from the retinal pigment epithelium (RPE), initiates a similar reaction from the radial glial cells of the retina, the Müller cells. Within 3 days after detachment, many Müller cells undergo cell division and hypertrophy within the retina. Also at this time, a subpopulation of these cells begin to grow out of the retina into the newly created “subretinal space,” resulting in subretinal fibrosis. Ultimately these cells form large subretinal glial “membranes” or scars on the surface of photoreceptors that can impede the regeneration of photoreceptor outer segments after retinal reattachment.

Methods

Tissue preparation

Human subretinal (n = 5) and epiretinal (n = 12) membranes with PVR, as well as epiretinal fibrovascular membranes from patients with diabetes (n = 3) were obtained at the time of vitrectomy surgery and immediately placed in fixative (4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4; Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) and stored at 4°C until used. The membranes used in this study were from both men and women, and the length of time between diagnosis and epiretinal membrane removal ranged from 2 months to 1 year. Detailed notes of the status of all the eyes were unavailable, although it is known that all the eyes had tractional retinal detachments. Surgery in humans was conducted at Moorfields Eye Hospital, London, UK, the Academic Medical Center, Amsterdam, The Netherlands, and Cottage Hospital in Santa Barbara, California, USA. All procedures had institutional research ethics committee approval and adhered to the tenets of the Declaration of Helsinki.

Immunocytochemistry

The subretinal and epiretinal membranes were processed whole, without embedding or sectioning. After fixation, tissue

Abbreviations: CNS, central nervous system; GFAP, glial fibrillary acidic protein; PVR, proliferative vitreoretinopathy; RPE, retinal pigment epithelium

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samples were rinsed in phosphate-buffered saline and incubated in normal donkey serum (1:20) in phosphate-buffered saline, 0.5% bovine serum albumin, 0.1% Triton X-100 and 0.1% azide (PBTA) overnight at 4°C on a rotator. The following day the primary antibodies were added overnight at 4°C on a rotator in PBTA. Anti-neurofilament (1:500; mouse monoclonal; Biomeda, Hayward, CA, USA; binds to the 70 and 210 kDa subunits) or biotinylated anti-neurofilament (Biomeda antibody, biotinylated by Vector Labs, Burlingame, California, USA) was used to label ganglion cell processes. (The biotinylated neurofilament antibody allowed pairing with other mouse monoclonal antibodies.) This antibody does not label horizontal cells in the human retina as it does in some species and thus it specifically identifies ganglion cells. The neurofilament antibody was used in combination with glial fibrillary acidic protein (GFAP; rabbit polyclonal) and vimentin (mouse monoclonal; both used at 1:400; both from Dako, Carpinteria, California, USA) to follow the possible association of the neurite outgrowth with glial cells. After rinsing of the primary antibodies in PBTA, the secondary antibodies (donkey anti-mouse CY3, donkey anti-rabbit CY2; Jackson ImmunoResearch, West Grove, Pennsylvania, USA) were added together, each at 1:200 in PBTA, overnight at 4°C on a rotator. The biotinylated anti-neurofilament was added to the sections after the primary and secondary antibodies to the other proteins were completed.

Streptavidin CY3 was used to bind to the biotinylated antibody. On the final day, the sections were rinsed in PBTA, mounted on glass slides using 5% n-propyl gallate in glycerol and viewed on a BioRad 1024 laser scanning confocal microscope (Hercules, California, USA) or an Olympus FluoView 500 (New York, New York City, USA). Each image was a projection of 5–10 images taken at 0.5 μm intervals.

RESULTS

All the PVR epiretinal membranes contained areas rich in anti-neurofilament-labelled processes (fig 1A–F, examples from three different cases; fig 2A–F; examples from one case). This labelling, however, was limited to regions that also had anti-GFAP labelled glial processes. In some cases the anti-neurofilament labelling appeared throughout the “sheets” of glial-labelled tissue (A–D) while in other cases the anti-neurofilament labelling occurred directly adjacent to fine glial processes (E, F). Note: the punctate labeling in (C) and (D) represents fine neurite processes arranged at right angles to the longer processes. Bar: 20 μm (A, C,D,E); 100 μm B; 10 μm (F).

Figure 1 Neurites in PVR epiretinal membranes. A–F: Laser scanning confocal images of epiretinal membranes labeled with anti-neurofilament (red) and anti-glial fibrillary acidic protein (blue or green). The membranes were removed from 3 different patients (A, B), patient 1 (C, D), patient 2 (E, F), patient 3). Anti-neurofilament labelling was observed only in regions of the membranes also containing anti-GFAP labelled glial processes. In some cases the anti-neurofilament labeling appeared throughout the “sheets” of glial-labelled tissue (A–D) while in other cases the anti-neurofilament labelling occurred directly adjacent to fine glial processes (E, F). Note: the punctate labeling in (C) and (D) represents fine neurite processes arranged at right angles to the longer processes. Bar: 20 μm (A, C,D,E); 100 μm B; 10 μm (F).
processes. In these cases, the neurofilament-labelled processes were observed directly adjacent to the glial processes (fig 1E,F) or to wrap around them (fig 2F, arrows). In addition to neuronal processes, a few membranes also appeared to contain small cell bodies with neurites radiating from them (fig. 2C,D, arrowheads), although further study is needed to determine whether these are indeed cell bodies.

All the fibrovascular epiretinal membranes taken from patients with diabetes contained anti-neurofilament-labelled neurites, which were located in regions that contained anti-vimentin-labelled glial cells (fig 3A–C). The membranes also contained numerous anti-laminin-labelled vessels (figs 3A,B), as well as an anti-laminin-labelled inner limiting (fig 3C,D), which was intentionally removed at the time of surgery. No obvious differences in labelling patterns were observed between the diabetic and PVR membranes (besides the presence of vessels).

Only two of the five subretinal PVR membranes sampled contained anti-neurofilament-labelled processes, but as in the case with the epiretinal membranes, they were always observed in association with intermediate filament-containing glial cells (fig 4A,B). All the subretinal membranes contained anti-GFAP and anti-vimentin-labelled glial cells, along with non-glial areas containing macrophages, presumptive pigmented RPE cells, and rod outer segments and rod cell bodies scattered throughout (data not shown). As with the epiretinal membranes, the neurites were restricted to regions with glial processes.

**DISCUSSION**

Previously, we have shown the presence of neurites in subretinal and epiretinal membranes in the feline retina in an animal model of retinal detachment and reattachment. Data from this study indicate that human peri-retinal membranes removed at the time of vitreoretinal surgery also contain neurites and these processes, as in the feline model, appear to prefer a glial substrate over other available surfaces on which they might grow. These neurites could be identified in both PVR and diabetic fibrovascular epiretinal samples. Although both horizontal and ganglion cell neurites are routinely observed in the subretinal and epiretinal membranes from the feline retina, those observed in the human membranes almost certainly originate only from ganglion cells since the neurofilament antibody is specific to this cell type in human retina. Neurite growth into epiretinal membranes appears to be common since 100% of the samples contained neurofilament-labelled processes. It is less common, however, in the subretinal membranes. The reason for this difference is unclear, but may relate to the fact that ganglion cells are more closely situated to epiretinal membranes than to subretinal membranes. Alternatively, the reduced occurrence of neurites in subretinal membranes could simply reflect our limited sample size.
Nevertheless, the observation of subretinal neurites in two specimens indicates that growth into this space can occur in the human eye and in the animal models.

We have previously shown that Müller cell hypertrophy and neurite growth occurs within a few days after detachment in the feline model of detachment. When the neurite sprouting occurred in the human samples is unknown; however, the length of time between diagnosis and epiretinal membrane removal ranged from 2 months to 1 year, and there was no obvious correlation between the duration of membrane growth and the extent of neurite growth. Although detailed patient histories are unavailable, it is known that all the eyes had tractional retinal detachments. It can be assumed, therefore, that these were aggressive, contractile membranes when removed from the eye. Whether this contributes to neurite growth is not known. Indeed, it would be interesting to know whether the more stable, slow-growing membranes in eyes without detachments contained neurites.

As elsewhere in the CNS, injury to the retina initiates an activation of glial cells, ultimately resulting in the formation of glial scars. After retinal detachment, we observed that Müller cells hypertrophy and grow into the space between the retina and the now distant, underlying epithelial layer, where they form the equivalent of a glial scar. Interestingly, the growth of neurites through glial scars in the retina seems to be in contrast with other regions in the CNS where gliosis, in general, impedes the growth of neurites. For example, after injury to the spinal cord, specific molecules such as NoGo are present on glial cells, establishing a molecular, as well as a physical barrier to the growth of neurones. Although the molecules involved in the guidance of newly formed neurites in the retina are not known, our data seem to indicate that retinal glial cells form a permissive substrate for neurite growth. Indeed, we never see neurites growing alone in the subretinal space; they are always associated with Müller cell processes. They also grow out of the neural retina only at sites where Müller cells grow beyond the outer limiting membrane to form a glial scar. Thus, the Müller cells may hold clues to molecules that support this growth and potentially aid in the development of treatments to stimulate neuronal growth in other CNS injuries. In addition, since we have observed the same phenomena in feline and, recently, mouse retina, animal models are now in place to search for the molecules or genes involved in both glial cell hypertrophy and neurite sprouting that occur in many different retinal injuries and diseases.

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**Figure 3** Neurites in diabetic fibrovascular epiretinal membranes. Laser scanning confocal images of 2 fibrovascular epiretinal membranes labeled with anti-vimentin (blue), anti-laminin (green) and biotinylated anti-neurofilament (red). (A): The complex cluster of neurites (red) appears in a region containing glial processes (blue) and does not associate with the numerous laminin labeled blood vessels (green). (B): The same image shown in “A” with the blue channel turned off. (C): A small group of neurites (red) are shown adjacent to lightly labeled glial processes (blue) embedded in a wrinkled “sheet” of anti-laminin labeled inner limiting membrane (green). (D): The same image shown in (C) with the blue channel turned off. Bar: 50 μm (A, B); 200 μm (C, D).
Figure 4 Neurites in a PVR subretinal membrane. (A): Laser scanning confocal images of a subretinal membrane labeled with anti-vimentin (blue), anti-glial fibrillary acidic protein (green) and biotinylated anti-neurofilament (red). Numerous neurofilament labeled neurites are present among the GFAP and vimentin labeled glial processes. B: Monochrome (blue), anti-glial fibrillary acidic protein (green) and biotinylated anti-neurofilament (red) confocal images of a subretinal membrane labeled with anti-vimentin (green).

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