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# Ganglion cell neurites in human idiopathic epiretinal membranes

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## ABSTRACT

**Aim:** To identify and confirm the presence of neural elements in idiopathic epiretinal membranes removed from patients' eyes during vitrectomy with epiretinal membrane peeling.

**Methods:** Human epiretinal membranes from patients with no other known eye disease and of varying durations were labelled immunohistochemically with antibodies for neurofilament protein, laminin and either vimentin or GFAP; proteins expressed in ganglion cells, the inner limiting membrane (ILM), and Muller cells, respectively.

**Results:** Anti-neurofilament labelled neurites, presumed to originate from ganglion cells, were found in all 32 idiopathic epiretinal membranes examined. The neurites were only observed in regions of anti-vimentin or -GFAP labelled glial cells, both of which were observed embedded in anti-laminin labelled material assumed to originate from the ILM.

**Conclusions:** We show that neurofilamentous processes, presumed to originate from retinal ganglion cells, are found universally in idiopathic epiretinal membranes, suggesting that the presence of these membranes is sufficient to stimulate neurite growth in the absence of trauma or disease. In addition, since neurites were invariably found in association with glial cells, the glia may play a permissive role in neurite growth both within the retina and into extra-retinal glial membranes.

Idiopathic epiretinal membranes (ERMs) are composed of non-vascularised tissue growth along the inner limiting membrane (ILM) on the retinal surface. There is no underlying or preceding pathology in the ocular history associated with these membranes. However, the development of an idiopathic ERM is usually preceded by a posterior vitreous detachment.<sup>1</sup> The prevalence of ERMs in the general population over 70 years of age is around 11.6%.<sup>2</sup> These membranes can be asymptomatic but may cause metamorphopsia or micro- and macropsia of varying degrees. If they are symptomatic, surgical removal of the membrane by vitrectomy and peeling is an option.

Many different cellular components of the epiretinal membranes have been identified in extensive ultrastructural studies. Within these fibrocellular sheets, hyalocytes from the vitreous, glial cells in the form of both Muller cells and astrocytes,<sup>3,4</sup> and fibroblast-like cells might all be responsible for the contractile properties associated with ERMs.<sup>5,6</sup> Retinal pigment epithelial cells have not been found to be part of the cellular population in idiopathic ERMs.<sup>5</sup> The extracellular matrix components that have been identified in ERMs include actin, tenascin, fibronectin, laminin and collagen type I, III and IV in variable quantities.<sup>7-9</sup>

It has been shown previously in feline retina that neurites from ganglion cells and horizontal cells can be found after experimental retinal detachment next to reactive Muller cells in both the vitreous and subretinal space.<sup>10-12</sup> However, this was considered to be a secondary reaction to the severe insult of retinal detachment. Similarly, we have shown that ganglion cell neurites could be found in ERMs in human patients with reactive epiretinal and subretinal membranes after retinal detachment with proliferative vitreoretinopathy or secondary to severe proliferative diabetic retinopathy.<sup>13</sup> Interestingly, these neurites were only observed in regions of glial growth, suggesting that the neurites are using the glia as a scaffold for growth. We show here that in idiopathic ERMs, where there is presumably no injury to the retina, ganglion cell neurites also extend outside the neural retina in association with glial cells.

## METHODS

Membranes were collected at the time of vitrectomy surgery from 32 patients with idiopathic ERMs. None of the patients had a prior history of ocular disease, inflammation, trauma or laser treatment. No patient had diabetes or other systemic disease with potential ocular manifestations.

After peeling, the membranes were immediately placed in fixative (4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4; Electron Microscopy Sciences, Fort Washington, PA) and stored at 4°C until used. The membranes used in this study were from both males and females, and the length of time between diagnosis and epiretinal membrane removal was variable from 1 to 9 months). The human surgery was conducted at the Academic Medical Center, Amsterdam, The Netherlands. All procedures had institutional research ethics committee approval and adhered to the tenets of the Declaration of Helsinki.

## IMMUNOCYTOCHEMISTRY

The ERMs were processed whole, without embedding or sectioning. Following fixation, the tissue samples were rinsed in phosphate buffer (PBS) and incubated in normal donkey serum (1:20) in PBS, 0.5% BSA, 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 protein (mouse monoclonal, 1:500, Biomed, Hayward, CA) and a biotinylated neurofilament antibody (Biomed) antibody, biotinylated by Vector Labs, Burlingame, CA) was used to label ganglion cell processes. The use of 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.<sup>14</sup> The neurofilament antibody was used in various combinations with antibodies to vimentin (mouse monoclonal; 1:400; Dako, Carpinteria, CA), laminin (rabbit polyclonal; 1:50; Sigma, St. Louis, MO) and GFAP (rabbit polyclonal, 1:400; DAKO, Carpinteria, CA). Following rinsing of the primary antibodies in PBTA, the secondary antibodies (donkey anti-mouse conjugated to CY3 for neurofilament, donkey anti-mouse CY2 for vimentin, donkey anti-rabbit CY5 for laminin and GFAP; Jackson ImmunoResearch, West Grove, PA) were added together, each at 1:200 in PBTA, overnight at 4°C on a rotator. The biotinylated anti-neurofilament was added to the six samples that were being stained with a different mouse monoclonal antibody, 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 galate in glycerol and viewed on an Olympus FluoView 500 laser scanning confocal microscope (Olympus, New York). Final images were collected as projections of five to 10 images taken at depths of 0.5 µm intervals.

## RESULTS

All 32 of the idiopathic ERMs contained areas labelled with anti-neurofilament or biotinylated anti-neurofilament, although results from only five patients are shown here: Patient 1: fig 1A,B; Patient 2: fig 1C,D; Patient 3: fig 2A,B; Patient 4: fig 2C,D; Patient 5: fig 3. The neurofilament staining occurred as long, thin, tortuous processes of uniform calibre sometimes appearing as single "fibres" but most frequently appearing as clusters of branching filaments (figs 1–3, red). These processes were consistently observed in association with anti-GFAP or -vimentin glial staining, never extending away from the glia onto other surfaces of the membrane (fig 1A–D, blue). Since retinal astrocytes in general do not label with vimentin antibodies, and Muller cells label with both vimentin and GFAP antibodies,<sup>14 15</sup> it is assumed that the glia labelled in the membranes are primarily Muller cells. Like the neurites, the glia also appeared as tortuous processes, some thin and some thick, but overall there was always more glia present than neurites. The membranes also contained many areas that were not labelled with any of the antibodies used in this study. Presumably these areas contain mostly extracellular matrix components,<sup>7–9</sup> but the neurites were not observed growing on these surfaces. Since the ILM often adheres to the ERMs when they are peeled from the retina, we stained with anti-laminin to see if the ILM could act as a substrate for neurite growth. In fact, many of the membranes did contain ILM, and neurites were consistently observed coursing within the ILM as well as on its surface (fig 2A–D; ILM, green; neurites, red). Labelling for glial cells, however, was not performed on these particular samples, so it was unknown if the neurites were actually extending onto the ILM. When the three antibodies to glia, ILM and neurites were combined simultaneously, it became clear that the neurites are present only in regions containing glial cells and never grew onto ILM without glia (fig 3, glia: anti-vimentin, blue; ILM: anti-laminin, green; neurites: anti-neurofilament, red). The extent of neurite growth into the different samples was variable, and the number of neurites ranged from one to many, with no obvious correlation to the known duration of the ERM or age of the patient.

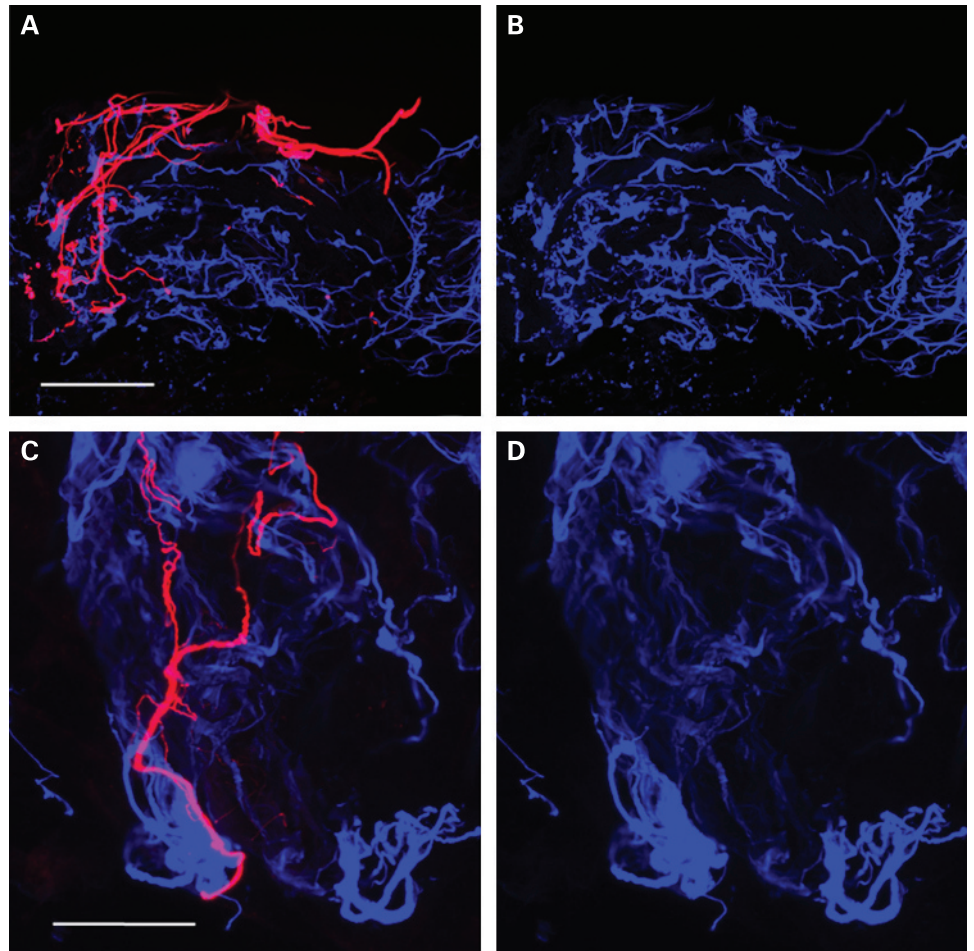
## DISCUSSION

Neurite growth following retinal detachment appears to be a relatively common event. Indeed, neurofilament labelled processes extending from ganglion cells have been found in the feline retina to occur within just a few days after experimentally induced retinal detachments<sup>11</sup> in human retinas with detachments of varying durations<sup>14</sup> and in human sub- and epiretinal membranes after severe ocular insult such as retinal detachment with proliferative vitreoretinopathy (PVR) and proliferative diabetic vitreoretinopathy (PDR).<sup>15</sup> The growth of these neurites was considered to be induced by the severe retinal trauma of retinal detachment or by proliferative (diabetic) retinopathy, where there are many inflammatory and potential (neural) growth factors present.<sup>16 17</sup> In this study, we show that idiopathic epiretinal membranes, removed from eyes with no previous ocular insult, also show the growth of neurites into epiretinal membranes indicating that trauma is not necessarily needed to induce their growth.

The neurites found in these idiopathic ERMs are similar in structure to those found in the feline detached retinas and in human PVR and PDR retinas, and they behave similarly in that they only grow on a glial substrate. This is in contrast to neurite growth elsewhere in the central nervous system where glial cells are generally thought to be inhibitory to neurite growth.<sup>18</sup> This may be due to differences in the specific cell types in different regions of the central nervous system and hence the neural growth factors produced by these cell populations. Retinal glial cells comprise astrocytes in the nerve fibre layer and Muller cells, radial glia generally considered modified astrocytes. Even though astrocytes as well as oligodendrocytes are thought to inhibit axon growth elsewhere in the CNS,<sup>18</sup> the glia in the retina may contain factors that are conducive to neurite growth. For example, after injury to the spinal cord, axon growth inhibitory ligands, mainly in the form of myelin associated protein, NoGo-A and oligodendrocyte-myelin glycoprotein, are produced by oligodendrocytes and reactive astrocytes.<sup>19</sup> These growth-inhibitory molecules appear to alter growth cone morphology and growth cone collapse in injured CNS tissue.<sup>18 20</sup> Oligodendrocytes and myelin do not occur in the retina, so the absence of this cell type and their axon growth inhibitory ligands may explain the ability of the neurites to sprout in the retina. Once they sprout, the Muller cells then appear to create a permissive substrate facilitating the growth of the neurites which can grow to great lengths within the retina, eventually extending into extracellular membranes on both sub- and epiretinal surfaces.

The fact that neurites have been identified growing on the retinal surfaces in a variety of conditions raises some important issues. Why do they grow in the first place? The data from the detachment experiments suggest that neuronal sprouting occurs secondary to the degeneration of photoreceptors. This makes sense in the case of bipolar and horizontal cells which are synaptically connected to photoreceptors. Indeed, neurite sprouting from these two cell types has been shown to occur directly adjacent to retracted rod synaptic terminals.<sup>10</sup> Ganglion cells, of course, are not connected to photoreceptors, raising the issue of what induces them to grow neurites. Moreover, in the case of idiopathic ERMs, there is no underlying photoreceptor degeneration. What all the conditions have in common, however, appears to be Muller cell reactivity. In the case of retinal detachment and reattachment, Muller cells grow into both the subretinal space and the vitreous. Neurites from horizontal cells and ganglion cells appear to use these reactive glia as a scaffold along which they grow great distances both

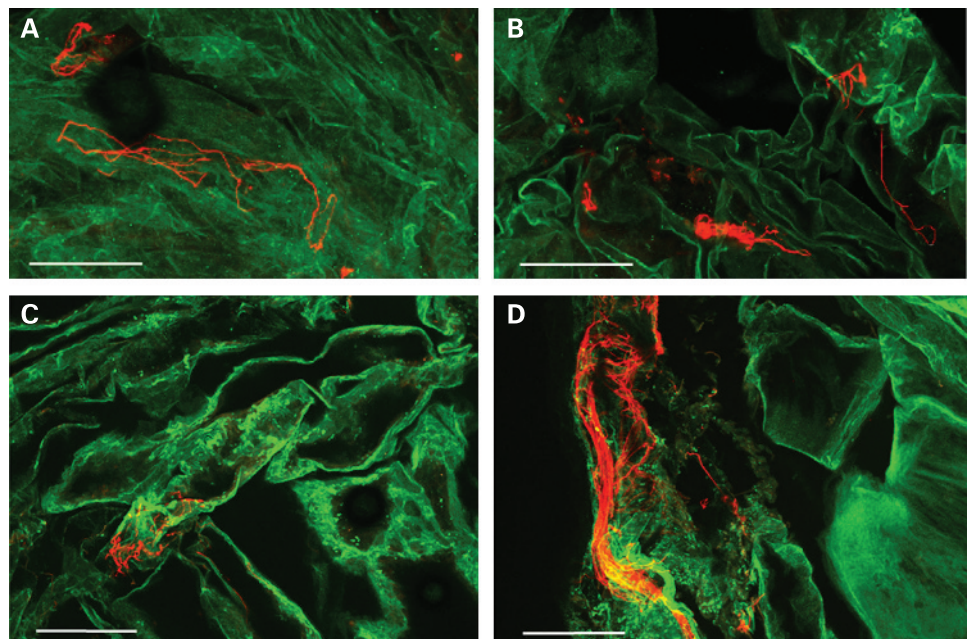
**Figure 1** Laser scanning confocal images of idiopathic epiretinal membranes showing neurites growing adjacent to glia. (A,B) Patient 14; (C,D) patient 3. The tissue was labelled with antibodies to glial acidic fibrillar protein (GFAP, blue) to show the presence of glial cells and neurofilament protein (red) to identify neurites. The neurofilamentous processes appear to grow only adjacent to the glia. B and D show the GFAP stained Muller cells without the anti-neurofilament staining.



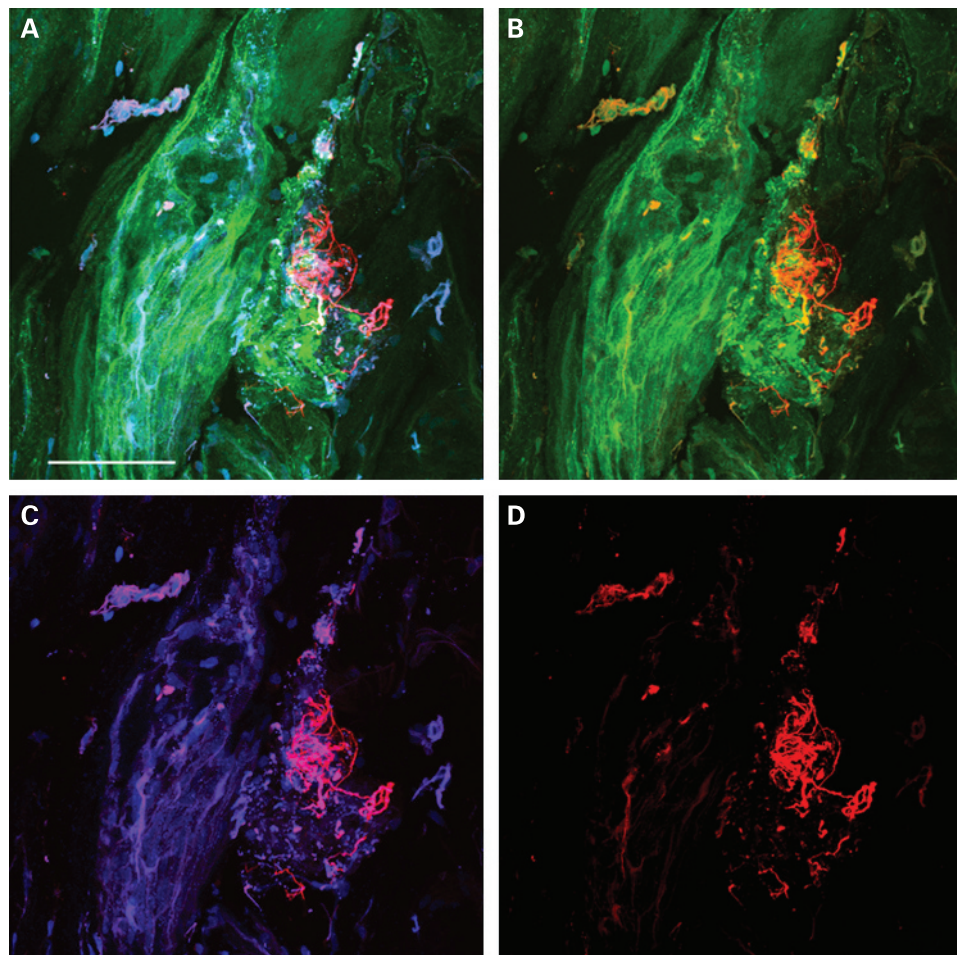
within the retina and onto glial scars in the subretinal space.<sup>10</sup> Ganglion cell neurites in the vitreal membranes also occur in association with the processes of glial cells.<sup>15</sup> While the signal from glia to stimulate neurite growth is unknown, it has been

suggested that cytokines released from reactive glia may activate the ganglion cells.<sup>16</sup> Also, following injury to the retina and subsequent activation of glial cells, the ganglion cells begin expressing GAP 43,<sup>11</sup> a developmentally regulated molecule

**Figure 2** Laser scanning confocal images of idiopathic epiretinal membranes showing the association of neurites with the inner limiting membrane (ILM). (A,B) Patient 23; (C,D) patient 4. Anti-neurofilament labelled processes (red) can be seen embedded in the wrinkled appearing anti-laminin labelled inner limiting membrane (green).



**Figure 3** Laser scanning confocal images of an idiopathic epiretinal membrane illustrating the association of neurites with glia and the inner limiting membrane. The membrane is triple-labelled with anti-laminin (green), anti-*vimentin* (blue) and biotinylated anti-neurofilament (red). A–D are all the same image from patient 5 with different combinations of proteins (colours) to more readily see the specific labelling patterns. A shows all the antibodies combined; B is the same image without the blue GFAP stain; C is without the green laminin stain; and D shows only the neurofilament staining.



expressed at high levels during development that induces neurons to sprout. In the adult, however, it virtually disappears after axons have matured. It is possible then that some signal from the activated glia stimulates upregulation of GAP 43, which in turn signals the ganglion cells to sprout neurites.

A second question regarding the presence of newly formed neurites is whether they perform any function and whether their removal has any consequences. Interestingly, horizontal cells and ganglion cells appear to grow a different class of neurites—long, uniform, sparsely branched processes that are more characteristic of axons than dendrites. If they are indeed axons, it may be difficult for the ganglion cells to establish any functional connection. The neurites and the Müller cell processes that occur in the ERMs, however, were clearly torn away from their parent cell at the time of surgery. Since this can be relatively traumatic, it may be ideal to find some method for stopping the growth of ERMs as soon as they are discovered or a method for making them regress, thus avoiding any potential cellular damage caused by surgical procedures. Indeed, future studies are aimed at elucidating the molecule(s) expressed by Müller cells that are involved in neurite guidance as well as molecules that may control Müller cell growth.

**Competing interests:** None.

**Ethics approval:** All procedures had institutional research ethics committee approval and adhered to the tenets of the Declaration of Helsinki.

**Patient consent:** Patient consent was obtained.

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