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## SCIENTIFIC REPORT

# Expression of glutamine synthetase and cell proliferation in human idiopathic epiretinal membrane

S Kase, W Saito, M Yokoi, K Yoshida, N Furudate, M Muramatsu, A Saito, M Kase, S Ohno

*Br J Ophthalmol* 2006;90:96–98. doi: 10.1136/bjo.2005.078394

**Background/aim:** The mechanisms of the cellular origin and cell proliferation in the idiopathic epiretinal membrane (ERM) are unsolved. The aim of this study was to examine the expression of cell cycle related molecules and glutamine synthetase (GS), which is expressed in Müller cells and their processes, in ERM tissues.

**Methods:** The ERMs were surgically removed using pars plana vitrectomy. Formalin fixed, paraffin embedded ERM tissues were analysed by immunohistochemistry with anti-cyclin D1, p27 (KIP1), proliferating cell nuclear antigen (PCNA), and GS antibodies.

**Results:** The histopathological findings showed that all the ERMs consisted of oval or spindle mononuclear cells with thin collagen-like tissues. Immunoreactivity for GS was detected in collagen-like tissues of ERM, presenting a continuous, isodense pattern. GS immunopositive cells in all cases expressed PCNA in their nuclei. Nuclear immunoreactivity for cyclin D1 was noted in the ERM constituent cells, whereas p27 (KIP1) positive nuclei were not detected.

**Conclusion:** Cyclin D1 and PCNA were expressed in the idiopathic ERM, which was mainly derived from Müller cells and extensions of their processes.

The idiopathic epiretinal membrane (ERM) is characterised by the formation of a membrane covering the posterior pole of the fundus without any underlying retinal disease.<sup>1,2</sup> The ophthalmoscopic aspects of idiopathic ERM are variable, from a cellophane-like to a thick greyish membrane with retinal folds. Histopathological studies indicate that idiopathic ERM consists of collagen tissues and degenerated cells,<sup>3</sup> probably derived from glial cells.<sup>4,5</sup> However, it is unclear where the cells in the ERM originate or by what mechanisms they proliferate and extend.

Cell cycle progression is controlled by a series of kinase complexes that are composed of cyclins and cyclin dependent kinases (CDKs).<sup>6</sup> Cyclin D1 is rapidly induced upon the exposure of cells to mitogens, whereas p27 (KIP1) is extinguished during the late G1 phase.<sup>7</sup> Furthermore, proliferating cell nuclear antigen (PCNA) appears at the late G1 and reaches a peak level in the S-phase of the cell cycle.<sup>8</sup> The pathological condition in the retina also demonstrated an alteration in the cell cycle state in which p27 (KIP1) expressed in a normal state disappeared after murine retinal detachment, whereas cyclin D1 and PCNA were induced in the Müller cells.<sup>9,10</sup>

In this study, we examined the expression of cyclin D1, p27 (KIP1), and PCNA within the membranes picked up in human idiopathic ERM in order to clarify the cell cycle status of the membrane. Glutamine synthetase detected specifically in the Müller cells<sup>11</sup> was also analysed to investigate whether the Müller cells were responsible for the occurrence of the ERM.

## MATERIALS AND METHODS

Five patients with idiopathic ERM (two men and three women) underwent pars plana vitrectomy from October 2004 to April 2005 at Hokkaido University Hospital and Teine Keijin-kai Hospital. The specimens obtained from the patients with ERMs secondary to diabetic retinopathy, ocular sarcoidosis, retinal vein occlusion, and other ocular abnormalities were excluded from this study. The clinical data of the patients are summarised in table 1. Informed consent was obtained from all five patients. During the vitrectomy, phacoemulsification and intraocular lens implantation were simultaneously performed. Indocyanine green or trypan blue dye were not applied to the posterior vitreous cavity to visualise the inner limiting membrane (ILM).<sup>2</sup> Furthermore, the ILM was not peeled off. The posterior hyaloid was already detached in all patients. The membranes peeled and removed from the retina were fixed in 4% paraformaldehyde and paraffin embedded tissue sections were made for immunohistochemistry. All studies conformed to the tenets of the Declaration of Helsinki.

## Immunohistochemistry

The slides were dewaxed, rehydrated, and rinsed in phosphate buffered saline (PBS) twice, incubated with normal goat serum and then with anti-glutamine synthetase (GS) (1:200, Chemicon, Temecula, CA, USA), anti-proliferating cell nuclear antigen (PCNA) (1:100, Zymed, South San Francisco, CA), anti-p27(KIP1) (1:50, Santa Cruz Biotech, Santa Cruz, CA), and anti-cyclin D1 (1:50, Santa Cruz Biotech, Santa Cruz, CA, USA) antibodies. Primary antibodies were incubated at room temperature for 2 hours. Slides were washed three times in TRIS buffered saline (TBS) and the second antibody was added. Binding of the primary antisera was localised using FITC conjugated goat anti-mouse IgG, donkey anti-goat IgG, or goat anti-rabbit IgG (dilution, 1:200; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA). Nuclei were then stained with YO-pro-1 for 5 minutes. The slides were examined by laser scanning confocal microscopy (MRC-1024; Bio-Rad, Richmond, CA, USA; and LSM 510; Carl Zeiss, Oberkochen, Germany). For the negative control in each staining, serial sections without the primary antibody and with PBS were incubated with the FITC conjugated goat anti-mouse IgG. For positive control of p27(KIP1), we examined the expression in human retina of enucleated eyes immunohistochemically, and then confirmed a variety of the immunopositive cells in inner and outer nuclear layers, as recently described.<sup>12</sup>

**Abbreviations:** ERM, epiretinal membrane; GS, glutamine synthetase; ILM, inner limiting membrane; KIP1, cyclin D1, p27; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PVD, posterior vitreous detachment; TBS, TRIS buffered saline

**Table 1** Clinical data of patients with idiopathic epiretinal membranes

| Case | Age | Eye | Symptoms | VA (pre) | VA (post) | Follow up | PVD | Immunopositive cells (%) |           |
|------|-----|-----|----------|----------|-----------|-----------|-----|--------------------------|-----------|
|      |     |     |          |          |           |           |     | PCNA                     | cyclin D1 |
| 1    | 73  | R   | BV, MM   | 20/100   | 20/60     | 5M        | +   | 87.1                     | 97.6      |
| 2    | 68  | R   | BV       | 20/50    | 20/40     | 7M        | +   | 93.0                     | 82.4      |
| 3    | 65  | L   | BV, MM   | 20/100   | 20/40     | 2M        | +   | 93.8                     | 90.5      |
| 4    | 73  | L   | BV       | 20/200   | 20/120    | 1M        | +   | 93.8                     | 57.1      |
| 5    | 53  | L   | BV, MM   | 20/60    | 20/60     | 1M        | +   | 91.7                     | 75.0      |

BV, blurred vision; MM, metamorphopsia; VA, visual acuity; pre, preoperative; post, postoperative; M, month; PVD, posterior vitreous detachment; PCNA, proliferating cell nuclear antigen.

## RESULTS

The histological findings in this study showed that all the ERMs consisted of oval or spindle mononuclear cells with thin collagen-like tissues (fig 1A). Neither microvessels with red blood cells nor lymphoid cell infiltration were observed. Both cell rich and cell poor regions intermingled in the specimens. The immunohistochemical examinations revealed that immunoreactivity for GS was predominantly detected in collagen-like tissues of the ERMs, presenting a continuous, isodense pattern (fig 1B–D). This immunoreactivity and the configuration of GS positive tissues were identical in all ERMs obtained from five patients. On the other hand, GS negative tissues were sporadically observed (arrowhead in fig 1), which were found in the membranes of only three cases. More than 90% of the cell nuclei observed in serial sections of the specimens showed nuclear immunoreactivity for PCNA (fig 2A–C) in all cases. Cyclin D1 was also expressed in more than 80% of their nuclei (fig 3B–D), whereas a few cells showed slightly low nuclear immunoreactivity. In contrast with PCNA and cyclin D1, expression of p27 (KIP1) was not detected in any cells (data not shown). Details of cell count in PCNA and cyclin D1 immunoreactivity are shown in table 1.

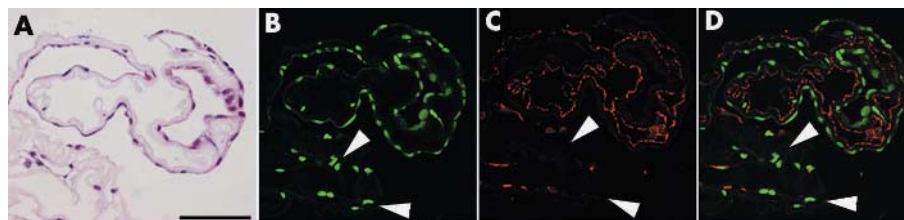
## DISCUSSION

The present study showed that the epiretinal membranes obtained from five patients consisted of oval or spindle

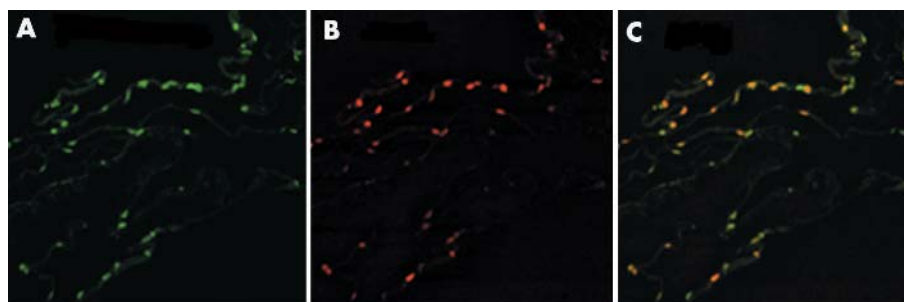
mononuclear cells with thin collagen-like tissues in haematoxylin and eosin staining. The histological findings were consistent with those of recent reports using transmission electron microscopy.<sup>3</sup> Furthermore, it was shown that these membranes had no microvessels or lymphoid cell infiltration, suggesting that angiogenesis and the inflammatory process were not responsible for the proliferation on idiopathic ERM.

Previous morphological investigations have shown that idiopathic ERM contains cells probably derived from glial cells. However, it has not yet been clarified whether the cells stem from Müller cells or astrocytes.<sup>4,5</sup> The prominent findings in this immunohistochemical study revealed that the majority of ERM components showed GS immunoreactivity, indicating that the idiopathic ERM constituent cells originated mainly from Müller cells because GS immunoreactivity is specifically expressed in Müller cells,<sup>11</sup> but not in astrocytes.<sup>13</sup> Furthermore, the continuous appearance of GS immunoreactivity in collagenous tissues of the ERM also implied that these collagenous tissues resulted from the extension of Müller cell processes through the ILM, since GS is also expressed in Müller cell processes.<sup>14</sup> It is, however, clear that a minor part of the ERM is composed of non-glial cells with no GS immunoreactivity, which are probably fibrocytes, retinal pigment epithelial cells, or myofibrocytes.<sup>15</sup>

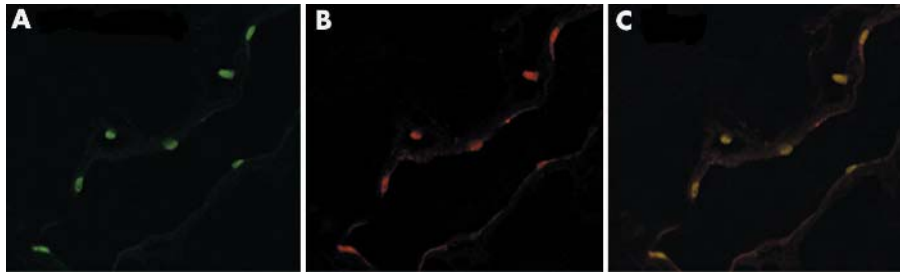
The present study with immunodetection of cyclin D1 and PCNA demonstrated that the Müller derived cells in the



**Figure 1** Haematoxylin and eosin staining (A), YO pro-1 nuclear staining (B, D, green) and immunodetection of glutamate synthetase (GS) (C, D, red) in the serial sections. The ERM consists of monotonous mononuclear cells with oval or spindle nuclei and thin collagen-like tissues in case 1 (A). Linear immunoreactivity for GS is detected in collagen-like tissues of the ERM (C, D). In contrast, GS negative parts are partially observed (B–D, arrowheads). Bar equals 50  $\mu$ m.



**Figure 2** YO pro-1 nuclear staining (A, C, green) and immunodetection of proliferating cell nuclear antigen (PCNA) (B, C, red) in the human idiopathic epiretinal membrane (ERM). A variety of ERM constituting cells express PCNA in the nuclei.



**Figure 3** YO pro-1 nuclear staining (A, C, green), and expression of cyclin D1 (B, C, red) in the human idiopathic epiretinal membrane (ERM). Nuclear immunoreactivity for cyclin D1 is observed in the cells of the ERM (B, C).

ERMs showed nuclear immunoreactivity for cyclin D1 and PCNA, while presenting no p27 (KIP1) positive reaction in any of the cases. In the developing retina, S-phase cells are present, and p27 (KIP1) appears to inhibit proliferation of Müller glia in cell cycle withdrawal in the postnatal retina.<sup>9 10 16</sup> Furthermore, it is known that cyclin D1 and PCNA are induced in Müller cells while p27 (KIP1) is degraded after iatrogenic retinal detachment in mice, suggesting that cell cycle related molecules exert significant actions on the development of proliferative vitreoretinopathy following detachment.<sup>10</sup> Thus, the alteration in expression among cyclin D1, PCNA, and p27 (KIP1) within all the ERMs observed in this study indicates that cell cycle related molecules have an important role in glial cell proliferation of ERMs.

Almost all patients with idiopathic ERM, including those in the present study, showed complete posterior vitreous detachment (PVD). Although it has been reported that PVD is a significant factor modulating epiretinal membrane formation in Terson syndrome<sup>17</sup> and also predisposes to epiretinal gliosis,<sup>18</sup> the mechanism of the gliosis remains unknown. Recent studies demonstrated that extracellular signal regulated kinase (ERK) functions as an upstream activator of transcription from the cyclin D1 promoter containing the AP-1 binding site.<sup>19</sup> Immunohistochemical studies with experimental retinal detachment revealed that the ERK and AP-1 transcription factor were activated, and cyclin D was subsequently induced, after iatrogenic detachment.<sup>10 20 21</sup> These findings suggest that the activation of the ERK pathway may lead to the induction of cyclin D1 in epiretinal gliosis.

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