Immunocytochemical Characteristics of Müller Cells Cultured From Adult Rabbit Retina

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Müller cells are the predominant glial cells in the retina. They have not only a structural but also a nutritional role.1 Several studies have suggested that Müller cells have a regulatory role in relation to neuronal function.2-4 In pathological situations Müller cell processes may partially replace neuronal tissue in the degenerating retina.5 Recent studies6-8 have indicated that epiretinal membranes obtained by vitrectomy contain GFAP-positive glial or Müller cells. Although Müller cells play an obviously important role in the normal and pathological retina, there have been few direct studies5 because of the difficulty of isolating a sufficient number of Müller cells from the retina. Previous immunohistological studies of the intact mammalian retina have revealed that vimentin9-11 and S-100 protein12-15 are good identification markers for Müller cells, although there is some doubt about the presence of S-100 protein in human Müller cells.16-17 An isozyme C of carbonic anhydrase (CAC) has been demonstrated in Müller cells of chick,18 rodent19 and human,8,20 and there is agreement that this enzyme is present in Müller cells but not in astrocytes.8,19,20 Since carbonic anhydrase activity has been found in the rabbit retina by earlier histochemical studies21,22 the isozyme C might be also a specific marker of Müller cells in rabbits.

Recently a technique to culture Müller cells from the adult mammalian retina has become available,23-25 and this technique has been used by some workers to study the immunocytochemical characteristics of such cells cultured from rat retina.24,25 As yet, however, little is known about the antigenic expression of glial markers in cultured Müller cells and before using such cells as representative of Müller cells in vivo it would be essential to demonstrate that cultured cells show a similar expression of glial markers to that known to occur in vivo. This paper reports the results of an immunocytochemical study of Müller cells cultured from adult rabbit retina with particular reference to the antigenic expression of glial markers.

Materials and Methods

Cell Culture

Culture of adult rabbit Müller cells was carried out by a minor modification of the method of Burke and Foster.6 Eyes were obtained from adult albino and pigmented rabbits of both sexes; all procedures reported here conformed to the ARVO Resolution on the Use of Animals in Research. The anterior portions of the eyes were incised circumferentially about 2 mm posterior to the limbus. After removal of the vitreous body the posterior eye cup was washed with Dulbecco phosphate buffered saline (PBS) and the
Antibodies

Polyclonal rabbit anti-human CAC was purchased from Calbiochem (LaJolla, CA) and monoclonal mouse anti-GFAP and monoclonal mouse anti-vimentin antibodies from Boehringer Mannheim. Polyclonal anti-bovine GFAP and polyclonal anti-bovine S-100 protein antibodies raised in the rabbit were obtained from Dakopatts (Bucks, UK). Monoclonal anti-neurofilament peptide antibody was kindly donated by Dr. R. N. Wood. Antibodies were used at the following dilutions throughout the experiment; polyclonal anti-CAC 1:100, monoclonal anti-GFAP 1:20, polyclonal anti-GFAP 1:80, anti-vimentin 1:80, anti-S-100 1:50, anti-neurofilament polypeptide 1:50.

For the immunofluorescence studies fluorescein isothiocyanide conjugated goat anti-mouse IgG (GAM-FITC) and goat anti-rabbit IgG (GAR-FITC) were purchased from Sigma Chemical Co. (St. Louis, MO). Tetramethylrhodamine-isothiocyanate conjugated swine anti-rabbit IgG (SAR-TRITC) was obtained from Dakopatts (Bucks, UK). All of these antibodies were used at a dilution of 1:40.

The cultured cells were treated with 0.125% trypsin in PBS for 5–10 min at room temperature or with proteinase-K (50 μg/ml) for 5–10 minutes on ice. This procedure detached most cells from the plate. Any remaining adherent cells were detached with a fine jet of GMEM with 10% fetal calf serum containing soya bean trypsin inhibitor (600 μg/ml) or, where appropriate, an inhibitor of proteinase-K (phenylmethylsulphinyl fluoride, 40 μg/ml). Where cells remained adherent they could be collected after gentle mechanical separation using a spatula. This avoided the use of higher concentrations of proteolytic enzymes or excessive time in the preparation, both of which were found to result in nonspecific immunocytochemical reactions. Detached cells were centrifuged at 1500 rpm for 10 min and following this the supermatant GMEM was replaced with a fixative composed of 2% paraformaldehyde and 0.25% glutaraldehyde buffered with 0.1 M cacodylate at pH 7.2. After fixation for 30 min cells were washed briefly with PBS and then dehydrated in graded concentrations of ethyl alcohol (1–2 hr in each of 65%, 80% and 90% ethyl alcohol). Subsequently the cells were
transferred to a mixture of 90% ethyl alcohol and Lowicryl K4M resin (Chemische Werke Lowe, Waldkaiburg, FRG) 2:1 for 2 hr, 1:1 for 1 hr and 1:2 for 2 hr. Finally the cells were left in pure resin for 10–16 hr and subsequently embedded in the resin using gelatin capsules. Polymerization was induced by exposure to an ultraviolet lamp (Agar Aids, Stanstead, Essex) for 8–12 hr. All procedures after fixation were performed at −20°C.

Ultrathin sections were made with an ultramicrotome (LKB 8800 Ultrotome III; Bromma, Sweden) and placed on nickel grids. Sections were stained as previously described. The grids were inverted on drops of 1% bovine serum albumin for 30 min to prevent nonspecific reactions, and following this the sections were reacted with the primary antibody (except anti-CAC) at the previously noted dilutions for 16 hr and were then labelled with the protein-A gold complex (Janssen, Olen, Belgium), diluted 1:40. Sections were then fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide and examined with a Phillips EM301 transmission electron microscope (Phillips Analytical, Cambridge, UK).

**Results**

**Culture**

After the second passage, cells with epithelioid characteristics emerged from the explants and grew relatively quickly. Round, polygonal, or large, flattened cells were seen infrequently on the plates. After the third passage highly homogeneous confluent cultures were obtained (Fig. 1). These consisted predominantly of epithelioid cells but spindle-shaped cells were occasionally seen, especially in semiconfluent areas. Once confluent cultures had been obtained cells maintained their morphology up to 60 days in culture (eight passages).

**CAC**

More than 90% of cultured cells labelled with anti-CAC. The staining pattern is demonstrated in Figure 2.

**GFAP**

Immunofluorescence studies showed that 70–90% of cultured cells labelled positively with either monoclonal or polyclonal anti-GFAP antibody. The inten-
Table 1. The results of immunofluorescence labelled with anti-GFAP antibody

<table>
<thead>
<tr>
<th>Fixative</th>
<th>2% Paraformaldehyde</th>
<th>Acetone</th>
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<tbody>
<tr>
<td>Monoclonal anti-GFAP antibody</td>
<td>± (±)</td>
<td>+ (++)</td>
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<tr>
<td>Polyclonal anti-GFAP antibody</td>
<td>++ (+++)</td>
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- = Negative
± = Nonreproducible result
+ = Weakly positive
++ = Positive
+++ = Strongly positive
( ) = Denotes addition of Triton-X

Intensity of the fluorescence however was variable depending on the exact procedure used (Table 1). The most definite and reproducible labelling was obtained with acetone fixation and treatment with Triton-X using the polyclonal anti-GFAP antibody. The staining pattern is illustrated in Figure 3.

On immunoelectron microscopy most cells showed a specific expression of GFAP to both antibodies. Gold particles could be identified in the cytoplasm particularly along the intermediate glial filaments (Fig. 4). Hardly any specific labelling could be seen in the nucleoplasm, in the matrix of the mitochondria, or within the endoplasmic reticulum.

Vimentin

Eighty to ninety percent of cultured cells labelled strongly with anti-vimentin antibody irrespective of the staining procedure used. The pattern of reactivity was essentially similar to that of GFAP (Fig. 5).

In double labelling experiments all of the vimentin-positive cells also labelled with anti-GFAP. Immunostaining with anti-vimentin antibody was however invariably stronger in intensity than that with anti-GFAP antibody (Fig. 6A,B). Immunoelectron
Fig. 5. Immunofluorescence staining of cultured cells (third passage) labelled with a monoclonal anti-vimentin antibody. Fibre-like staining is seen in cytoplasm. Bar: 5 µm.

Microscopy showed that vimentin was also localized along the intermediate glial filaments (Fig. 7) while only background staining was noted in the nucleoplasm or in the matrix of the mitochondria.

**S-100 Protein**

Fifty to seventy percent of cultured cells showed positive anti-S-100 antibody staining of the cytoplasm with immunofluorescence while 30-50% showed a positive staining reaction in the nucleoplasm. Positive staining of the nucleoplasm was always accompanied by positive cytoplasmic reaction. The pattern of cytoplasmic staining, unlike that with anti-GFAP or anti-vimentin, was diffuse. When present, positive staining of the nucleoplasm was patchy or granular (Fig. 8). Immunoelectron microscopy showed that the cytoplasm of most cells was labelled with anti-S100 protein antibody. Gold particles were diffusely identifiable throughout the cytoplasm except within the endoplasmic reticulum (Fig. 9). In many cells both the nucleoplasm and the cytoplasm were labelled but approximately 20-30% of cells were seen in which only the cytoplasm was labelled.

**Controls**

*Negative controls:* Light and electron microscopy failed to show any immunocytochemical reactivity to anti-neurofilament polypeptide antibody nor was there any significant staining when rabbit or mouse control sera were used. At the EM level after reaction with normal rabbit serum only a few gold particles were seen randomly distributed and not differing from the background distribution (Fig. 10).

*Positive controls:* Rabbit retinal tissue showed positive staining with anti-CAC, GFAP, vimentin and S-100 protein (Fig. 11A-D). The medullated area was not labelled with CAC (Fig. 11A), while GFAP expression was restricted to the medullated streak where both astrocytes and oligodenrocytes are present (Fig. 11B). Positive staining with anti-vimentin could be clearly identified with Müller cells (Fig. 11C), anti-S100 protein-labelled cells in the inner retina, including Müller cells, but photoreceptor cells were not labelled (Fig. 11D).

**Discussion**

Various techniques for the culture of isolated Müller cells from adult mammalian retina have been...
Fig. 7. Protein A-gold immunoelectron micrograph of a cultured cell (third passage) labelled with anti-vimentin antibody. Gold particles are specifically localized along intermediate glial filaments between lobes of an indented nucleus (N). Arrows indicate nuclear membrane. Bar: 0.5 \( \mu \text{m} \).

described.\textsuperscript{23-25} Sarthy\textsuperscript{24} obtained Müller cells in culture from degenerating adult rat retina exposed to constant light for 3-7 days. Roberge et al\textsuperscript{25} added the supernatant from mitogen-activated spleen cells to obtain long-term cultures of Müller cells from adult rat retina. Burke and Foster\textsuperscript{8} advocated a simpler method of Müller cell culture using intact adult rabbit retina. We used a minor modification of Burke and Foster's technique and confirmed that the technique allowed the relatively long-term culture of Müller cells from this source. An advantage of using the anangiotic rabbit retina as a source of Müller cells is the possibility of excluding astrocytes by the selection of an avascular area of retina\textsuperscript{13} and oligodendrocytes by the selection of nonmedullated retina. Burke and Foster confirmed that cells cultured by their technique were Müller cells by using tritiated thymidine and autoradiography to localize the proliferating cells in the isolated retina and in the early outgrowth in organ culture. Furthermore, a high proportion of cells in our cultures are labelled with CAC, which has been considered to be specifically expressed in Müller cells but not in astrocytes.\textsuperscript{11,19,20} This enzyme was not detectable in the medullated area of the rabbit retina, supporting the view that it is a specific Müller cell marker in rabbits also. A preliminary description of this result has been published.\textsuperscript{29}

In the present study most of the cultured cells which labelled positively with glial markers did not label with a neuronal marker, implying that the cultured cells were indeed of Müller cell origin. Although most of the cultured cells expressed the glial markers used, not all did so. The cells not labelled might conceivably have been in a different functional state from that of the majority or possibly suffering insufficient antigenic preservation. An alternative explanation might be contamination with some other cell type although the latter, for reasons already discussed, is unlikely.

Previous reports have suggested that Müller cells in situ in intact mammalian retina, including rabbit, lack detectable GFAP expression.\textsuperscript{11,30} Our results in rabbit retinal tissue showed GFAP labelling only in

Fig. 8. Immunofluorescence staining of cultured cells (third passage) with anti-S-100 protein antibody. Cytoplasm is diffusely stained and nucleoplasm is patchily stained (arrow). Bar: 2 \( \mu \text{m} \).
the area of the medullated streak (ie, myelinated nerve fibers associated with blood vessels) but not over Müller cells, thus confirming previous reports.

In contrast to the results of studies of intact mammalian retina, GFAP has been reported as immunohistologically identifiable in cultured adult rat Müller cells. However, were unable to obtain reproducible results in cultured Müller cells from rabbit retina.

Differences in the staining procedures and in the antibodies employed may be important. Dixon and Eng obtained the best GFAP staining in developing
Müller cells when they used fixatives containing a minimal concentration of aldehyde. In our own study the best staining was seen when cells were fixed with acetone rather than paraformaldehyde, suggesting disruption of antigenicity by the aldehyde. Additionally, the best staining could be obtained by labelling with polyclonal rather than monoclonal antibody, a finding which agrees with earlier studies on rat Müller cells. In the case of double labelling experiments the antigenic expression of vimentin was invariably stronger than that of GFAP, irrespective of the staining procedure and in spite of the use of a monoclonal anti-vimentin antibody. Thus Müller cells appear to contain easily detectable amounts of vimentin and the use of an anti-vimentin antibody is an efficient way of identifying Müller cells, irrespective of the staining procedure used. GFAP may be present in less detectable amounts in Müller cells and its demonstration more dependent on the exact immunohistological procedure used.

In the present study immunoelectron microscopy has shown clearly that both GFAP and vimentin are localized along the intermediate glial filaments and it is interesting that in our Müller cell cultures, antigenic expression of GFAP, vimentin and S-100 protein was maintained through numerous passages (up to five in our experiments).

The question as to why Müller cells in culture might express GFAP while Müller cells in the intact retina do not is interesting. It is known that Müller cells in situ accumulate GFAP in response to neurological injury, retinal degeneration, or retinal detachment can also result in antigenic increase. The suggestion that the pattern of proteins synthesized by cells in tissue slices may be characteristic of stressed tissue might at least in part explain the differences in antigenic expression between the in situ and in vitro situation. An additional difference in antigenic expression from that previously reported in the intact retina was the absence of S-100 from the nucleoplasm of some 20-30% of cells. Again the differences found may be explicable by the changed metabolism of cultured cells as against those in situ.

Whatever the reasons, the present study clearly indicates that cultured Müller cells from adult rabbit retina have immunocytochemical characteristics which differ from those of Müller cells in situ.

Key words: Müller cell, culture, carbonic anhydrase C, glial fibrillary acidic protein, vimentin, S-100 protein, immunocytochemistry

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References

Fig. 11. Immunofluorescence staining of adult rabbit retina labelled with anti-CAC (A), GFAP (B), anti-vimentin (C) and anti-S-100 protein (D). CAC is unstained at the medullated area (arrows). The staining of GFAP is restricted to the medullated streak (triangles). Bar: 5 μm.