Isolation and Characterization of Porcine Müller Cells
Myofibroblastic Dedifferentiation in Culture

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Purpose. To characterize phenotypic and antigenic changes in isolated Müller cells during proliferation in extended culture.

Methods. Müller cells were isolated from porcine retina by sequential papain and DNase digestion, trituration, and density gradient centrifugation. The identity of the isolated cells was confirmed by immunodetection of carbonic anhydrase II (CA-II), cellular retinaldehyde-binding protein (CRALBP), glial fibrillary acidic protein (GFAP), vimentin, and α smooth muscle actin (αSMA). Continuously proliferating cells established in culture were examined for changes in the expression of these antigens.

Results. Primary cultures of purified Müller cells, incubated under routine culture conditions, were proliferative and lost immunodetectable CRALBP within 2 weeks. The expression of CA-II also diminished with time, but at an apparently lower rate than that of CRALBP. Loss of GFAP expression was even more gradual and was complete by passage 5. De novo expression of αSMA was detectable in some cells within 12 days in culture and by all cells by passage 5. During this period, vimentin expression remained qualitatively unchanged.

Conclusions. Isolated porcine Müller cells in culture undergo a phenotypic dedifferentiation to a fibroblast-like cell, which includes loss of detectable CRALBP, CA-II, and GFAP, and they acquire expression of the myoid marker αSMA. Invest Ophthalmol Vis Sci. 1996; 37:740-752.

The leading cause of failure to correct retinal detachment surgically is the development of a postoperative complication known as proliferative vitreoretinopathy (PVR). This is an anatomic disorder in which forming sheets or strands of cellular connective tissue can alter visual acuity and, in more severe cases, deform and detach the retina.1 The pathogenesis of this disorder follows the order of events commonly associated with wound repair.2 Frequently, there is an initiating event, such as trauma, or the formation of a retinal defect, such as a tear. Cells dispersed (or recruited) into the vitreous cavity proliferate, migrate, and ultimately contract preexisting vitreal or newly synthesized matrix into dense connective tissue strands and epiretinal membranes. Tractional forces arising from these tissues are transmitted to the retina and result in the described anatomic defects.3

Much attention has been focused on the cellular content of proliferative scar. A number of immunohistochemical studies4–13 have identified cells present in proliferative tissues, including retinal pigment epithelial cells, glia (including astrocytes and Müller cells), macrophages, and a ubiquitous fibroblast-like cell type of uncertain origin, commonly referred to as a myofibroblast. This cell is so named because it shares features with myocytes found in contracting dermal or tendinous granulation tissue. This includes expression of α-smooth muscle actin, an actin isoform thought to be necessary for the generation of tractional forces associated with wound closure.14–16

Of the cells involved in the pathogenesis of PVR, the role of Müller cells in this disorder is probably the least understood. Müller cells are highly specialized glia known to play important roles in the maintenance of normal retinal physiology.17 Considerable evidence suggests that Müller cells also can function as a reac-
tive cell type in retinal wound repair. By virtue of their transretinal orientation, Müller cells are positioned to respond to a number of initiating events in retinal pathology, such as retinal detachment or even the presence of mitogens in the vitreous.\textsuperscript{18-22} Studies\textsuperscript{20,21,23} examining Müller cell responses in animal models of experimental retinal detachment have documented reactive changes in the Müller cells, including proliferation, hypertrophy, migration, and altered expression of enzyme and intermediate filament proteins.

The immediate goal of this investigation was to characterize Müller cell phenotypic changes during transition from a "quiescent," nonproliferative phenotype to an "activated," rapidly proliferating cell. To study these changes, we have isolated and purified Müller cells from freshly dissociated porcine retina and introduced the cells into culture. These cells were examined systematically for changes in morphology and expression of established enzyme and cytoskeletal markers before and after isolation and during both early and late proliferative stages of culture. Our results indicate that Müller cells undergo a "myoid"-type differentiation, which includes loss of enzyme and cytoskeletal markers and acquired expression of the myoid-type actin cytoskeleton.

**METHODS**

**Isolation and Culture of Porcine Müller Cells**

Porcine eyes were harvested from pentobarbital-anesthetized animals, each weighing 30 to 40 pounds, and transported to the laboratory in ice-cold saline containing antibiotics. Methods used for securing animal tissue were humane and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Each eye was hemisected with a circumferential cut posterior to the ora serrata, and the retinas were removed essentially intact after dissection at the optic nerve. Retinas were transferred to ice-cold L-15 (Gibco, Grand Island, NY), washed several times with additional medium to remove loosely adherent material, and stored on ice until use.

Retinal cells were released using a papain dissociation protocol modified from the procedure reported by Gillette and Dacheux.\textsuperscript{24} Cooled retinas were transferred to 10 ml of L-15 containing 17 U/ml papain (Sigma Chemical, St. Louis, MO) preactivated for 30 minutes with 2 mM cysteine and 1 mM ethylenediaminetetraacetic acid at 34°C. Digestion was for 60 minutes at 34°C, after which the digestion medium was removed and the retina washed twice with fresh L-15. The retinas were then incubated in L-15 containing 150 U/ml of deoxyribonuclease I (DNase, #D-5025; Sigma) for an additional 30 minutes at 34°C. The retinas were dissociated by trituration (repeated passage) of the tissue through a borosilicate tissue culture pipette (10 ml). After 10 cycles through the pipette, the tissue fragments were permitted to settle for 10 minutes, and the top 8 ml of supernatant was removed and stored on ice. This procedure was repeated five to six times until the retina was almost completely dissociated, leaving primarily vascular elements and fibrous material.

Phase-contrast microscopic examination of the individual supernatants revealed that the cellular composition varied with each trituration. Typically, the first three supernatants contained large numbers of photoreceptors but relatively few morphologically recognizable Müller cells, and they were discarded. Generally, fractions 4 to 6 were enriched for Müller cells. Supernatants with the highest numbers of Müller cells were combined for further processing. Cells were pelleted, resuspended in L-15 containing 10% fetal bovine serum and layered on a 10-ml continuous density gradient composed of 0% to 50% Percoll (Pharmacia LKB Biotechnology, Uppsala, Sweden) in normal saline (0.9% wt/vol NaCl) for separation at 500g for 5
minutes. Optimal purification was achieved by sequential centrifugation through two gradients.

After recovering from the second density gradient, cells were resuspended in growth medium composed of Dulbecco’s minimal essential medium (Gibco) containing 20 mM HEPES, 10% fetal bovine serum, and 1% Pen/Strep/Fungisone (Gibco) and were introduced into culture. Cells were maintained at 37°C in a humidified atmosphere composed of 5% CO₂ and 95% air. Routine cell culture was in 75-cm² tissue culture flasks coated with 0.1 mg/ml monomeric type I collagen (Vitrogen; Celltrix, Santa Clara, CA) dissolved in 0.012 M HCI.

Preparation of Retinal and Cell Extracts

Extracts were prepared from freshly isolated porcine retina, dissected as described above but frozen in -70°C isopentane immediately after isolation. The frozen retina was added directly to 5 ml of an extraction cocktail containing 0.15 M NaCl, 0.01 M Tris, pH 8, with 10 mM dithiothreitol, 1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid and 50 μM of a saturated solution of phenylmethylsulfonyl fluoride dissolved in ethanol. The retina was homogenized with 10 strokes of a Dounce tissue homogenizer. Insoluble material was removed by microcentrifugation for 2 minutes, and the supernatant was aliquoted and stored at -70°C until use.

Extracts of 5th-passage Müller cells were performed using the same extraction cocktail as for the intact retina: 75-cm² tissue culture flasks containing 5th-passage Müller cells at approximately 80% confluence were washed several times with serum-free medium and scraped, using a rubber policeman, into 2 ml of the extraction cocktail. The suspension was homogenized, centrifuged, aliquoted, and stored as described above.

Electrophoresis and Western Blot Analysis

Proteins were separated on a 5% to 15% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions. Transfer of electrophoretically separated proteins to nitrocellulose was for 2 hours at 100 V, after which the membranes were blocked using 2% bovine serum albumin (fraction V; Sigma) in Tris-saline (0.01 M Tris/0.15 M NaCl, pH 8). Membranes were probed in a slotted, blotting chamber with primary antibodies, and horseradish peroxidase-conjugated (HRP) secondary antibodies were diluted in Tris-saline containing 0.05% Tween-20 for 2 hours each at room temperature with 3 X 5-minute washes between and after antibody treatment. Color development was with diaminobenzidine, per the manufacturer’s (Sigma) recommendations.

Immunofluorescence and Phase-Contrast Microscopy

Retinal chops were prepared from eyecups dissected as above and fixed with 2% paraformaldehyde in 0.1 M Na₂HPO₄, pH 7, for 2 hours at room temperature. After fixation the retina was removed, transferred to filter paper, and sectioned into 100-μm chops using a McIlwain Tissue Chopper (Brinkman Instruments, Westbury, NY). Cells attached to coverslips were fixed with 2% paraformaldehyde for 1 hour at room temperature. Chops and coverslips were permeabilized by a 10-minute treatment with phosphate-buffered saline (PBS; 0.01 M Na₂HPO₄, 0.15 M NaCl, pH 7.4) con-
Myofibroblastic Dedifferentiation of Müller Cells

FIGURE 3. Phase-contrast photomicrographs of dissociated retinal cells during purification of Müller cells. Porcine retina were digested with papain-DNase, and the cells were released by trituration. Pooled fractions enriched in Müller cells (A) were purified further by centrifugation through two sequential 0% to 50% Percoll density gradients (B and C, respectively) as described in Methods. Magnification, X125.

taining 0.1% Triton X-100. Nonspecific sites were blocked with 2% serum from the same species as the secondary antibody, diluted in PBS, for 60 minutes at room temperature. Primary and secondary antibody treatments were for 60 minutes at room temperature using 0.2% secondary serum, diluted in PBS, with 3 × 5-minute washes in between. Photomicrographs were taken with a Nikon (Melville, NY) Optiphot equipped with epifluorescence illumination and phase-contrast optics using a 35-mm camera. For experiments in which the percentages of labeled cells were determined, the numbers of fluorescent cells within each field were determined first, followed by a count of total cells by phase-contrast using an objective of the same power (X10).

Immunonochemicals

Primary antibodies used for Western blots and immunofluorescence localization studies included sheep anti-human carbonic anhydrase II (Serotec, Oxford, UK) rabbit anti-bovine cellular retinaldehyde-binding protein (a generous gift from Dr. John Saari, University of Washington School of Medicine, Seattle, WA), rabbit anti-bovine glial fibrillary acidic protein (GFAP) (Dako A/S, Glostrup, Denmark), rabbit anti-human GFAP (Chemicon International, Temecula, CA), monoclonal mouse anti-GFAP, clone G-A-5 (Sigma), monoclonal mouse anti-porcine vimentin, clone V9 (Dako A/S), and monoclonal mouse anti-α smooth muscle actin, clone IA4 (Sigma, St. Louis, MO). Secondary antibodies used included rhodamine-conjugated rabbit anti-sheep (Chemicon), fluorescein- and rhodamine-conjugated donkey anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA), rhodamine-conjugated goat anti-mouse (Sigma), HRP-conjugated rabbit anti-sheep (Cappell, West Chester, PA), HRP-conjugated goat anti-rabbit (Southern Biotechnology Associates, Birmingham, AL), and HRP-conjugated goat anti-mouse antibodies (Fisher Biotech, Atlanta, GA). Normal sera from rabbit, donkey, and goat were purchased from The Binding Site, Birmingham, England.

RESULTS

Characterization of Immunoprobes Against CA-II, CRALBP, Glial Fibrillary Acidic Protein, and Vimentin

As a preliminary step in the isolation of porcine Müller cells, we examined the reactivity of the specific antibodies to porcine retinal antigens in a detergent extract of freshly isolated retina. Western blots of retinal extracts (15 μl) were probed using antibodies raised against carbonic anhydrase-II (CA-II; MWt 29,115 kDa), cellular retinaldehyde-binding protein (CRALBP; MWt 36,474 kDa), GFAP (MWt 49,880 kDa), vimentin (MWt 53,713 kDa), and α smooth muscle actin (αSMA; MWt 42,108 kDa). Adjacent lanes were probed using two dilutions of primary antibody, the second at a 10-fold lower concentration than the first, followed by the appropriate HRP-conjugated secondary antibody. In each case except αSMA, a major immunoreactive band was observed at the relative position appropriate for the antigen in question (Fig. 1). Although there was no visible response to the anti-
FIGURE 4. Indirect immunofluorescence microscopic localization of CA-II, CRALBP, GFAP, and vimentin in purified porcine Müller cells. Porcine Müller cells purified by density gradient centrifugation as in Figure 3, attached to coverslips during a 2-hour incubation at 37°C, were fixed, permeabilized, and probed with primary antibodies against CA-II (A), CRALBP (B), GFAP (C), and vimentin (D). Detection was with rhodamine-conjugated secondary antibodies. Magnification, ×106. CA-II = carbonic anhydrase II; CRALBP = cellular retinaldehyde-binding protein; GFAP = glial fibrillary acidic protein; αSMA = α smooth muscle actin.

αSMA antibody, a similar gel in which 100 μl of extract was loaded revealed a faint reactive band (not shown).

To confirm that these immunoprobes localized antigens to the retinal Müller cells, 100-μm sections of formaldehyde-fixed porcine retina were probed with the same panel of polyclonal and monoclonal antibodies as in Figure 1 and were detected with rhodamine-conjugated secondary antibodies. As with the Western blots, four of the primary antibodies were strongly positive. Antibodies against CA-II, CRALBP, GFAP, and vimentin were localized to large transretinal cells, and their morphologies were consistent with that of Müller cells (Fig. 2). As suggested by the Western blots, the monoclonal antibody against αSMA did not localize to Müller cells, but fluorescence was detected occasionally in the wall of larger blood vessels (not shown).

We also examined the staining patterns produced by two other commercially available anti-GFAP antibodies. The different suppliers' polyclonal rabbit (Chemicon) and mouse monoclonal antibodies (clone G-A-5; Sigma) produced similar results by Western blot analysis. However, only the polyclonal rabbit antibody produced a staining pattern in porcine retina similar to that presented in Figure 2C. The monoclonal antibody did not produce detectable labeling of the Müller cells but was weakly reactive with astrocytes in the nerve fiber layer (not shown).

Isolation and Characterization of Porcine Müller Cells

Müller cells that were mechanically dissociated from the papain–DNase-digested retina, as detailed in the Methods section, were recognized easily by phase-contrast microscopy. Specific morphologic features included a long cell body, centrally located nucleus, and prominent end foot. However, within a few minutes of dissociation, the cells began to round (Fig. 3A).

Purification of the dissociated cells was accomplished by sequential centrifugation through Percoll density gradients. Formed during centrifugation, the Müller cell layer was typically the second and most prominent layer from the top of the gradient. In the example presented, the starting material (Fig. 3A), representing the pooled cells released by trituration, is enhanced by the first density gradient (Fig. 3B), but many smaller, apparently non-Müller cells are still present. A second, identical, density gradient greatly improved the purity of the preparation (Fig. 3C).

The identity of the isolated, purified Müller cells was confirmed by indirect immunofluorescence using the same panel of antibodies as in Figures 1 and 2.
Myofibroblastic Dedifferentiation of Müller Cells

FIGURE 5. Phase-contrast photomicrographs of Müller cells during proliferation in culture. Müller cells purified from dissociated retina, as in Figure 3, were incubated under culture conditions. Phase-contrast photomicrographs were taken after 24 hours (A), 5 days (B,C), 12 days (D,E), 15 days (F), and 22 days (G). Magnifications, (A to F) X100; (G) X20. CA-IT = carbonic anhydrase II; CRALBP = cellular retinaldehyde-binding protein; GFAP = glial fibrillary acidic protein; αSMA = α smooth muscle actin.

Purified cells were resuspended in serum-free medium and incubated at 37°C on glass coverslips at a density of 10,000 cells per coverslip. After a 2-hour incubation to permit cell adhesion, the coverslips were fixed and probed as described in the Methods section. The majority of the dissociated, purified cells were reactive for all four of the antigens tested (Fig. 4A–D). The percentages of cells labeled by each antibody were calculated from counts performed by naive observers counting more than 500 cells per coverslip for each antigen. The percentages of cells judged to be labeled were 681 of 714 (95.4%) for CA-IT, 582 of 583 (98.9%) for CRALBP, 779 of 820 (95%) for GFAP, and 687 of 690 (99.6%) for vimentin. Also, 0 of 540 (0%) cells were judged to be labeled on coverslips probed for αSMA, as were 0 of 514 (0%) in negative controls in which the primary antibodies were omitted (not shown).

Growth of Porcine Müller Cells in Culture

Primary Cultures. To examine the morphologic changes and antigenic expression in primary cultures, freshly isolated Müller cells were seeded onto glass coverslips at low density and incubated under routine culture conditions for varying lengths of time. Depending on the preparation, 10% to 30% of the initially adherent cells developed intracellular vacuoles and detached from the culture surface within several days (not shown). The remaining cells began to proliferate within 2 to 3 days of incubation. By 5 days of incubation, single cells (Fig. 5A) had proliferated into small round clusters of cells, or foci (Figs. 5B, 5C). Foci progressively increased in cell number until they became round or oval masses of cells with the general appearance of large multinucleate cells (Fig. 5D). These rounded structures eventually became polygonal and had greater delineation of individual cell boundaries (Figs. 5E, 5F). At this stage, the cells located at the periphery of the foci possessed a morphology distinct from those cells in the center because they exhibited a higher degree of spreading on the tissue culture surface. At 22 days in culture, with continued cell proliferation, the foci began to coalesce as the cultures approached confluence (Fig. 5G).

We examined the cultured Müller cells for the presence of the specific markers at 12 and 22 days in culture.
Coverslips of cells incubated for 12 days (Fig. 5E) were fixed and probed as above for the panel of enzyme and cytoskeletal markers used in Figures 1, 2, and 4. The reactivity of the cells to anti-CA-II continued to be fairly strong and included intense cytoplasmic and nuclear staining in all cells (Fig. 6A). In contrast, the intensity of stain for CRALBP was, by this time, diffuse and considerably weaker, requiring photographic exposures four to five times longer than anti-CA-II-probed cells to generate the faint images presented in Figure 6B. In the case of the cytoskeletal proteins, antibodies against GFAP (Fig. 6C) and vimentin (Fig. 6D) generated intense fibrillar staining patterns in all cells within each focus. Interestingly, by 12 days, there was weak but detectable reactivity in a few cells within each focus to the monoclonal antibody against αSMA. When present the staining pattern was predominantly short, linear strands or rod-like structures (Fig. 6E).

Additional coverslips of cells prepared as above, but incubated for 22 days (as in Fig. 5G), were fixed and stained using the same panel of antibodies. The tightly packed cells near the center of each cluster were unchanged in reactivity to CA-II from the 12-day samples, whereas the larger cells at the periphery had considerably less intense cytoplasmic staining but retained the prominent nuclear localization (Fig. 7A). The staining intensity for CRALBP remained weak and otherwise unchanged from the 12-day samples (Fig. 7B). All cells in the cluster, regardless of position or morphology, remained reactive to antibodies against both GFAP (Fig. 7C) and vimentin (Fig. 7D). However, in contrast to the uniformity of the vimentin-dependent fluorescence, the staining intensity for GFAP now varied among the cells. Finally, cells probed with αSMA were substantially more reactive by 22 days. At least 50% of the cells possessed some reactivity with the antibody, generally in the form of linear stress fiber-like arrays (Fig. 7E).

These data suggested that, at this stage in culture, some cells should contain both GFAP and αSMA. This was confirmed experimentally with double-stained coverslips. Photomicrographs taken of well-spread cells near the periphery confirmed that indeed some cells possessed recognizable filamentous GFAP (Fig. 7F) and αSMA-positive stress fibers (Fig. 7G; arrow indicates same cell).
Myofibroblastic Dedifferentiation of Müller Cells

**FIGURE 7.** Indirect immunofluorescence microscopic localization of CA-II, CRALBP, GFAP, vimentin, and αSMA in porcine Müller cells after 22 days in culture. Dissociated, purified Müller cells were seeded onto coverslips and incubated for 22 days, after which the cells were fixed, permeabilized, and probed with primary antibodies against CA-II (A), CRALBP (B), GFAP (C, F), vimentin (D), and αSMA (E, G). Detection was with rhodamine-conjugated secondary antibodies. Micrographs F and G are of the same field from a coverslip doubled-probed with antibodies against GFAP and αSMA. In this case, detection of GFAP antibody was with a fluorescein-conjugated secondary antibody. The arrowhead indicates the same cell in each photo. Magnification, ×125. CA-II carbonic anhydrase II; CRALBP = cellular retinaldehyde-binding protein; GFAP = glial fibrillary acidic protein; αSMA = α smooth muscle actin.

**Passage 2.** Our observations of the primary cultures (Fig. 7) suggested that spatial positioning within a proliferating cluster may relate to the variability in expression of CA-II and GFAP. To investigate this further, parallel primary cultures of cells in tissue culture flasks, which were nearly confluent by 22 days in culture, were released with trypsin–ethylenediaminetetraacetic acid to create a single cell suspension, as occurs in routine subcultivation. These cells, now referred to as passage 2 cells, were seeded onto coverslips at 50% of confluent density. The coverslips were incubated for 5 days, after which they were fixed and probed for the Müller cell maker proteins as above. The most readily discernible change in these cultures was in the morphology of the cells; all the cells assumed a more fibroblast-like morphology, previously observed only in the cells at the periphery of the foci. Cytoplasmic fluorescence for CA-II was weak or nonexistent, but the nuclear fluorescence persisted (Fig. 8A). The staining intensity for CRALBP was unchanged from the primary cultures (Fig. 8B), whereas the GFAP-dependent filamentous fluorescence, still...
FIGURE 8. Indirect immunofluorescence microscopic localization of CA-II, CRALBP, GFAP, vimentin, and aSMA in passage 2 Müller cells. Primary cultures of Müller cells (22 days) were released with trypsin-ethylenediamine-tetraacetic acid as per routine subculture, seeded onto coverslips, and incubated for 5 days, after which the cells were fixed, permeabilized, and probed with primary antibodies against CA-II (A), CRALBP (B), GFAP (C), vimentin (D), and aSMA (E). Detection was with rhodamine-conjugated secondary antibodies. Magnification, ×96. CA-II = carbonic anhydrase II; CRALBP = cellular retinaldehyde-binding protein; GFAP = glial fibrillary acidic protein; aSMA = α smooth muscle actin.

αSMA (E). Detection was with rhodamine-conjugated secondary antibodies. Magnification, ×96. CA-II = carbonic anhydrase II; CRALBP = cellular retinaldehyde-binding protein; GFAP = glial fibrillary acidic protein; aSMA = α smooth muscle actin.

present in the majority of the cells, was noticeably decreased within the population (Fig. 8C). Approximately 50% of the cells remained intensely positive for GFAP, whereas the rest of the cells possessed only weak fibrillar staining. Anti-vimentin fluorescence, in contrast, remained unchanged and continued to be uniformly positive throughout the cultures (Fig. 8D). Finally, the frequency of αSMA-positive cells approached 100%. However, like that of GFAP, the fluorescence intensity also varied between individual cells. Approximately 40% of the cells possessed intensely positive stress fibers, whereas the majority were faint to moderately positive (Fig. 8E).

Passage 5. Extended subcultivation of these cells did not produce any obvious changes in cell morphology, suggesting that the morphology observed at passage 2 was essentially stable. A final examination of the same cells at culture passage 5 for the Müller cell antigens confirmed the endpoint of the trend we observed in the previous examinations. Passage 4 cells were released and seeded onto coverslips at 50% of confluent density. After 5 days in culture, the coverslips were fixed and probed as above. CA-II fluorescence (Fig. 9A) and CRALBP-dependent (Fig. 9B) fluorescence were weak or nonexistent in the cell cytoplasm, whereas the nuclear staining associated with anti-CA-II persisted. At this stage, no cells possessed recognizable GFAP-positive filamentous structures, though the cells remained weakly reactive (Fig. 9C). One hundred percent of the cells were intensely positive for both vimentin (Fig. 9D) and αSMA (Fig. 9E).

To confirm the changes in protein expression suggested by the results of the indirect immunofluorescence localization studies, we prepared detergent extracts of passage 5 cultures and performed Western blots using the same antibodies and protocols used to probe the extract of porcine retina presented in Figure 1. For this blot, the amount of extract loaded onto the gel (0.11 ml) was adjusted to produce vimentin staining of the same visual intensity as in Figure 1 because the expression of this marker appeared to be least altered during the extended period in culture (Fig. 10; lanes 7, 8). Under these conditions, there was no detectable immunoreactivity with the a-
Myofibroblastic Dedifferentiation of Müller Cells

FIGURE 9. Indirect immunofluorescence microscopic localization of CA-II, CRALBP, GFAP, vimentin, and αSMA in passage 5 Müller cells. Trypsin-released passage 4 Müller cells were seeded onto coverslips and incubated for an additional 5 days, after which the cells were fixed, permeabilized, and probed with primary antibodies against CA-II (A), CRALBP (B), GFAP (C), vimentin (D), and αSMA (E). Detection of primary antibodies was with rhodamine-conjugated secondary antibodies. Magnification, ×48. CA-II carbonic anhydrase II; CRALBP = cellular retinaldehyde-binding protein; GFAP = glial fibrillary acidic protein; αSMA = α smooth muscle actin.

tibodies against CA-II, CRALBP, or GFAP (Fig. 10; lanes 1 to 6). This analysis also confirmed the expression of αSMA, indicated by the appearance of a prominent band at approximately 42 kDa (Fig. 10; lanes 9, 10). A Western blot in which a 5-fold higher amount of cell extract was loaded did not detect CA-II, CRALBP, or GFAP (not shown). These results indicate that the antigens were not present in the cells in detectable amounts and that positive nuclear staining for CA-II does not correlate with the presence of detergent-extractable enzyme.

DISCUSSION

The ultimate goal of these studies was to determine the potential contribution of Müller cells to fibroproliferative retinal disease. It was anticipated that "activated," or actively proliferating, Müller cells, such as cells responding to retinal detachment, would have characteristics different from those of their quiescent counterparts recently removed from normal retina. For this reason, we first examined the phenotypic changes occurring in Müller cells after introduction into culture. To exclude population changes resulting from overgrowth of the culture by non-Müller cells in the initial isolates, we used methods that enabled characterization of the cell population immediately after isolation. Expression of the repertoire of Müller cell antigens would still resemble that of the cells in situ. This also allowed more confident characterization of the antigenic changes occurring through the initial and latter stages of culture. Our decision to use porcine, rather than human or primate, cells for this study was based on a number of factors, including the availability of high-quality tissue of consistent age, the anatomic and genetic similarities between human and porcine tissues, and the potential of this species to serve as a cost-effective model for experimentation in situ.

In the current study, Müller cells were dissociated from enzyme-digested porcine retina and were purified using sequential density gradient centrifugation, a method used previously by other investigators, to isolate morphologically recognizable Müller cells.
The purified cells, characterized 2 hours after isolation, were 95.4%, 99.8%, 95%, and 99.6% reactive to the four antibodies used to identify the cells. If we assume that each nonreactive percentage represented unique cells, then, in the worst case, the non-Müller cells in the isolate would be 10.2% of the isolate. Because it is more likely that the nonreactive cells represented the same cells, a more reasonable approximation of the percent contamination in the final preparation is 5% or less.

We observed that the isolated cells were nonproliferative for 2 to 3 days and then entered a phase of relatively rapid proliferation in distinctive clusters or foci. At 12 days in culture, the proliferating Müller cells lost detectable CRALBP but continued to react with antibodies against CA-II, GFAP, and vimentin. At this stage, we also detected minor expression of αSMA. With continued culture for 22 days, the cells near the free margins of the growing foci assumed a fibroblast-like morphology. This change correlated with loss of detectable CA-II, diminished expression of GFAP, and increased expression of αSMA. When the cells were dissociated into a single-cell population, the conversion of the cells to fibroblast-like morphology was complete, and the reduction in GFAP expression was more pronounced. By passage 5, CA-II, CRALBP, and GFAP were undetectable by indirect immunofluorescence localization or Western blot analysis of cell lysates. At this same stage, αSMA was present in all cells. Notably, expression of vimentin remained qualitatively unchanged throughout the study.

We anticipated that GFAP expression would follow the pattern most widely reported for most species, that is, little or no expression until cells were introduced into culture. However, we detected GFAP in porcine retinal Müller cells and astrocytes in situ, as well as in the cultured cells. This result was achieved with a polyclonal antibody and was confirmed with a second polyclonal antiserum supplied by a different vendor. Conversely, porcine retina probed with a monoclonal antibody produced no reaction within Müller cells and a weak, but detectable, reaction within astrocytes. All three antibodies produced similar reactions in Western blots of retinal extract. We found that the monoclonal antibody did not recognize GFAP in the formaldehyde-fixed tissue with the same avidity as the polyclonal antibodies, a finding consistent with at least one previous report, and that porcine Müller cells do express detectable amounts of GFAP. This also suggests that astrocytes in porcine retina probably contain higher quantities of GFAP than Müller cells. We are aware of no published studies of porcine retina with which we can confirm these observations.

The experimental methods used in this study did not yield quantitative data about antigen expression. We can neither confirm nor deny previous reports that introduction of Müller cells into culture causes a significant upregulation of GFAP and vimentin expression. However, our results clearly demonstrate that GFAP expression, in contrast to that of vimentin, is ultimately lost during extended culture. The same is true for both CA-II and CRALBP, which is not surprising in light of previous reports. Lewis and coworkers reported that, in response to retinal detachment, cat Müller cells lose expression of CA-II, CRALBP, and glutamate synthetase. Subsequent studies revealed that decreased expression can be detected within 7 days of retinal detachment, a time frame consistent with our current observations.

We were particularly intrigued by the de novo expression of αSMA. Earlier reports described a similar onset of expression of other cell types, including lipocytes, lens epithelial cells, and retinal pigment epithelial cells. This actin isoform, normally expressed in smooth muscle, serves as a marker for myoid differentiation in fibrocontractive tissue and has been demonstrated...
Myofibroblastic Dedifferentiation of Müller Cells

strated to be necessary for tractional force generation by fibroblasts.15,16 We recently reported37 that in porcine retinal pigment epithelial cells maintained as continuously proliferating cultures, the onset of expression is coincident with the acquisition of enhanced fibrotic potentials, including adhesion to collagen and the ability to generate tractional forces. Together, these results suggest that Müller cells in culture should acquire the ability to generate tractional forces, an activity we have observed in a study to be published at a later date.

In conclusion, we have demonstrated that Müller cells have the capacity to undergo phenotypic transformation or dedifferentiation that involves the loss of some established cell markers and the acquisition of αSMA, yielding myoid or myofibroblast-like cells. Because the ubiquitous αSMA-expressing fibroblast identified in epiretinal membranes is of uncertain origin, we are tempted to speculate that these could arise from phenotypically altered Müller cells in addition to the other cells known to express αSMA. However, this hypothesis is valid only if porcine Müller cells mimic human cells. It should be pointed out that the tissue culture conditions under which Müller cells undergo these changes are substantially different from the intraocular environment. The nutrient-rich growth medium and growth factor-laden whole blood serum are optimized to produce maximum, sustained cell growth. It is more likely that during intraocular proliferation, the relevant bioactive peptides and growth factors are available to varying degrees and that levels are transient in nature, a relationship we confirmed in a recent study40 of the levels of contraction-stimulating activity in human pathologic vitreous using fibroblasts as target cells. Thus, it is possible that the conditions necessary to induce these phenotypic changes in Müller cells might not occur. The relevance of these current observations to human ocular pathology will only become clear with continued study.

Key Words
αSMA, glial fibrillary acidic protein (GFAP), Müller cells, myofibroblast, proliferative vitreoretinopathy

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