In Vitro Characterization of a Spontaneously Immortalized Human Müller Cell Line (MIO-M1)

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Purpose. To characterize a spontaneously immortalized human Müller cell line and to determine whether it retains the characteristics of primary isolated cells without undergoing differentiation in vitro.

Methods. An immortalized cell line obtained from human retina was investigated for the expression of known markers of Müller cells, including cellular retinaldehyde binding protein (CRALBP), glutamine synthetase, epidermal growth factor receptor (EGF-R), α-smooth muscle actin (α-SMA), and glial fibrillary acidic protein (GFAP). Also examined were the morphologic features of these cells, by scanning and transmission electron microscopy, and their functional characteristics, by electrogenic responses to glutamate. In addition, comparative studies were made of these cells with primary cultures of freshly isolated human Müller cells.

Results. The cells expressed CRALBP, EGF-R, glutamine synthetase, and α-SMA, as judged by confocal microscopy and Western blot analysis of cell lysates. Western blot analysis did not detect GFAP in cell lysates, but confocal microscopy showed that occasional cells expressed GFAP after detachment from the monolayer. The morphologic features of the cells examined, as judged by scanning and transmission electron microscopy, resemble those of cells derived from primary cell cultures. They possess villous projections on their apical surfaces and contain loose bundles of microtubules aligned parallel to one another and the long axis of the cell process. Characteristically, they contain abundant deposits of glycogen particles that do not differ from those seen in primary isolated cells. Preliminary recordings with intracellular electrodes revealed that these cells have properties similar to those described for mammalian Müller cells and depolarize in response to L-glutamate without significant change in membrane resistance, consistent with the well-established electrogenic uptake of this amino acid.

Conclusions. A spontaneously immortalized Müller cell line was characterized that retains the characteristics of primary isolated cells in culture. To the authors' knowledge, it constitutes the first human Müller cell line reported in the literature. It has been named MIO-M1 (Moorfields/Institute of Ophthalmology-Müller 1) after the authors' institution. Availability of this human cell line will facilitate studies designed to obtain a better understanding of the role of Müller cells in normal and pathologic conditions. (*Invest Ophthalmol Vis Sci.* 2002;43: 864–869)

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Müller cells are astrocyte-like radial glial cells that extend vertically throughout the retina, although their nuclei are usually in the middle of the inner nuclear layer.[1] The distal border of Müller cells is marked by the outer limiting membrane, which consists of junctional processes of Müller cells and photoreceptors, whereas the proximal border of the cells is marked by the inner limiting membrane, consisting of the Müller cell membrane and a basement membrane.[1] They are considered to be the principal glial cells of the retina, because of their ability to perform functions that astrocytes, oligodendrocytes, and ependymal cells effect in other regions of the central nervous system.[2] They stabilize the complex retinal architecture, provide an orientation scaffold, give structural and metabolic support to retinal neurons and blood vessels, and prevent aberrant photoreceptor migration into the subretinal space. In vitro, Müller cells promote extensive neurite outgrowth from rods,[3] express several neurotransmitter receptors, including γ-aminobutyric acid type B ($GABA_B$) receptor,[4] and various types of glutamate transporters,[5] which facilitate glutamate uptake to keep its extracellular concentration below neurotoxic levels.[6] Glutamate uptake is voltage dependent, and cell depolarization slows down or even reverses uptake of this amino acid.[7] Müller cells also express glutamine synthetase, an enzyme that is involved in detoxification of ammonia and glutamate and operates in concert with the L-glutamate–L-aspartate transporter (GLAST), to terminate the neurotransmitter action of glutamate, and that is responsible for the supply of cells with glutamine.[8]

Müller cells are thought to play an important role in pathologic processes of retinal wound healing and neovascularization, and massive local proliferation of Müller cells is a key feature of retinal proliferative disorders. They are found in the occluded lumen of retinal capillaries during retinal vein occlusion[9] and exhibit profound changes in expression of constitutive and inducible reactive molecules during disease processes, as judged by histologic studies.

Alteration in Müller cell behavior and phenotype are often seen in animal models of retinal proliferation, and profound changes are observed in retinal tissue from patients with various retinal disorders, including proliferative vitreoretinopathy,[10,11] proliferative diabetic retinopathy,[12,13] macular holes and macular pucker,[14,15] age-related macular degeneration,[16,17] and inherited macular dystrophies.[18] Elucidation of the mechanisms that lead to the development of retinal disease would be aided by a better understanding of the cellular processes that precede any pathologic changes.

Investigations of Müller cell functions in vitro have been laborious, because of the difficulty in obtaining pure cell populations and the tendency of these cells to differentiate rapidly in culture.[19] Although various Müller cell lines have been reported in the literature, these have been derived from rat retina,[19,20] and to our knowledge, no human Müller cell lines are currently available. Herein we report the characterization of a cell preparation derived from human retina, which after several passages in vitro retained the characteristic Müller cell morphology. The phenotypic features and electrogenic response to L-glutamate of this cell line paralleled those of freshly isolated cells. The availability of this human cell line should

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advance studies into the cell biology and function of Müller cells in normal and pathologic retina.

Materials and Methods

Müller Cell Isolation and Culture

Müller cells were isolated from eyes obtained from Moorfields Hospital Eye Bank after obtaining consent for research use and local ethics committee approval. The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Müller cells that became spontaneously immortalized derived from an eye of a 68-year-old female corneal donor 36 hours after death. We have named these cells MIO-M1 after our institution, Moorfields/Institute of Ophthalmology-Müller 1. Cells were isolated by a slight modification of an established method.[19] Briefly, retina was vigorously pipetted, followed by incubation with trypsin-EDTA (5% trypsin, 2% EDTA; GibcoBRL, Paisley, Scotland, UK) for 20 minutes at 37°C, and filtration through a stainless-steel sieve. Cells were washed and cultured to confluence in DMEM containing L-glutamax I (GibcoBRL) and 10% fetal calf serum (FCS; GibcoBRL). Müller cells were identified by their characteristic morphology under phase-contrast microscopy and by their expression of glutamine synthetase, glial fibrillary acidic protein (GFAP), α-smooth muscle actin (α-SMA), vimentin, cellular retinaldehyde binding protein (CRALBP), and epidermal growth factor receptor (EGF-R),[21–25] as judged by immunocytochemical staining or Western blot analysis of cell lysates. Subclones of the cell line were obtained by limiting dilution, and three of them (clones 1, 5, and 14) were analyzed for the expression of Müller cell markers. Chromosome examination to confirm the human origin of the cells was performed using standard G-banding techniques by Cytogenetic DNA Services Ltd., London, UK.

Confocal Microscopy Analysis of Müller Cell Marker Expression

Müller cells were cultured for 48 hours in fibronectin-coated (5 μg/mL) glass chamber slides (NalgeNunc, Inc., Roskilde, Denmark), fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 10 minutes, and incubated for 3 hours with primary antibodies diluted in 0.5% blocking reagent (Roche Molecular Biochemicals, Lews, UK) in Tris-buffered saline (TBS; pH 7.5). These included a monoclonal anti-CRALBP antibody (B2, a kind gift of John C. Saari, University of Washington, Seattle, WA); goat polyclonal anti-glutamine synthetase (clone C-20, Santa Cruz Biotech, Santa Cruz, CA); monoclonal anti-EGF-R (clone 29.1, Sigma, Poole, UK); monoclonal anti- α-SMA; clone 1A4, Sigma), and monoclonal anti-GFAP (clone 6F2; Dako, Glostrup, Denmark). Mouse IgG isotypes matching those of the test antibodies (Sigma) were used as the negative control. After incubation with primary antibody, specimens were washed in TBS, followed by incubation for 30 minutes with rabbit anti-mouse antibodies conjugated with FITC or rhodamine (Santa Cruz Biotech). Slides were then washed and counterstained with 4′,6′-diamino-2-phenylindole (DAPI) for 1 minute and mounted on glass slides (Vectashield mounting medium; Vector Laboratories, Burlingame, CA). Fluorescent images were recorded using a confocal microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) operating in multitrack mode for FITC, DAPI, and rhodamine-Cy3 fluorochromes.

Western Blot Analysis

Confluent cell monolayers were lysed with radioimmune precipitation assay buffer (RIPA buffer: 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 158 mM NaCl, and 50 mM Tris [pH 7.2]), followed by centrifugation of the lysates at 13,000 rpm for 5 minutes and storage of the supernatants at −85°C until use. Aliquots of Müller cell lysates (1.5 mg/mL) were resolved on 7% Tris-acetate polyacrylamide gels (NuPAGE; Invitrogen, Groningen, The Netherlands) for 60 minutes at 150 V in Tris-acetate running buffer (50 mM Tris, 50 mM tricine, and 0.1% SDS [pH 8.3]; Invitrogen). Proteins were then transferred to nitrocellulose membranes and blocked with 2% blocking reagent (in TBS, pH 7.4). Immunodetection was performed using the same antibodies as for confocal analysis. Immunocomplexes were detected by enhanced chemiluminescence (ECL; Amersham, Amersham, UK) after incubation with goat antiserum against rabbit or mouse IgG coupled to horseradish peroxidase (Santa Cruz Biotech). Images were analyzed and processed using an image reader (LAS-1000 Pro, ver. 2.1; Fuji, Bedford, UK).

Transmission Scanning and Electron Microscopy Analyses

Comparison was made between a primary culture of Müller cells at passage 3 (3173 cells) and MIO-M1 cells at passage 43 (after approximately 129 divisions). Cells were grown in 24-well tissue culture plates (NalgeNunc) and fixed overnight in a mixture containing 3% glutaraldehyde and 1% paraformaldehyde, buffered to pH 7.4 with 0.07 M sodium cacodylate-HCl. Cells were washed three times with cacodylate buffer (pH 7.4), osmicated for 2 hours with a 1% aqueous solution of osmium tetroxide, rinsed in deionized water, and dehydrated through ascending grades of alcohol (50%–100%, 10 minutes per step). For transmission electron microscopy, after four changes of 100% ethanol, wells were filled with Araldite resin and cured at 60°C. Semithin and ultrathin sections were cut using a microtome (Ultracut S; Leica, Cambridge, UK) fitted with the appropriate grade of diamond knife. After sequential contrasting with 1% uranyl acetate and lead citrate, thin sections were viewed and photographed using a transmission electron microscope (model 1010; JEOL, London, UK), operating at 80 kV. For scanning electron microscopy, cells were fixed and dehydrated to 100% ethanol for transmission microscopy. After dehydration cells were critical point dried, sputter coated with gold, and examined in a scanning electron microscope (6100SEM; JEOL), operating at 15 kV.

Electrophysiology Studies

Immortalized Müller cells were subcultured in 35-mm tissue culture dishes (NalgeNunc) for 24 to 48 hours before electrophysiological studies. Recordings were made from seven Müller cells in four different preparations in DMEM at room temperature, using sharp intracellular electrodes filled with 1 M K-acetate (resistance: 135–190 MΩ). Cells were impaled under visual control, and recordings were made in current-clamp mode. Membrane resistance was monitored by observing the voltage response to hyperpolarizing current pulses (−0.1 or −0.2 nA). L-Glutamate was applied to the bathing medium from a 500-mM solution in DMEM, with a micropipette (tip diameter, ~10 μm) positioned approximately 500 μm from the recording site. Ejections were made using 300-ms pressure pulses repeated (1–2-sec intervals) 2 to 10 times.

Results

Morphologic and Karyotypic Features of MIO-M1 Cells

Phase-contrast micrographs showed that MIO-M1 cells in nonconfluent monolayers spread throughout the culture plate surface, displaying a bipolar morphology, a rough membrane appearance, and the formation of cytoplasmic projections (Fig. 1A). When confluent, they acquired an elongated shape and adopted a fibroblast-like morphology, although they retained their rough membrane appearance (Fig. 1B).

Karyotypic analysis of MIO-M1 cells confirmed that these cells were of human derivation, displaying two X chromosomes, in accordance with the female origin of the cells. They display structural abnormalities observed in cells in long-term culture, with a near triploid modal chromosome number (71 ± 5) and a satellite chromosome. Analysis of the metaphases showed that all the chromosomes could be assigned to the groups of the human karyotype (Fig. 1C).

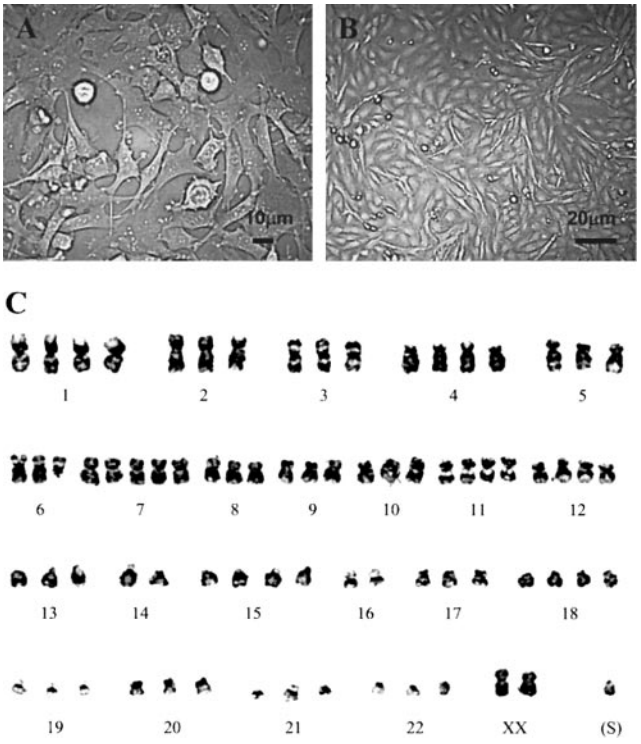

FIGURE 1. Phase-contrast appearance and karyotype of the Müller cell line MIO-M1. (**A**) Subconfluent monolayer of Müller cell line MIO-M1 exhibiting long cytoplasmic projections and bipolar morphology. Intracellular granular appearance was often observed in these cultures. (**B**) Confluent monolayer of Müller cells in culture. Spindle shape and fibroblast-like morphology were characteristic of these cells at confluence. (**C**) Karyotypic features of MIO-M1 cells showing a polyploid chromosome number, the X human chromosomes, and a satellite chromosome (S). Original magnification, (**A**) ×200; (**B**) ×100.

Expression of Müller Cell Markers

Confocal microscopy analysis of MIO-M1 cells showed that they express known markers of glial Müller cells. They stained positively for EGF-R and glutamine synthetase, and simultaneous expression of both molecules was observed in the majority of cells (Fig. 2A). They also stained for CRALBP, with a characteristic coarse, granular cytoplasmic and perinuclear staining (Fig. 2B), and for α-SMA (Figs. 2C, 2D), with a pattern characteristic of cytoskeleton staining. Although the majority of cells did not express GFAP, staining for this molecule was observed on a few cells that appeared to have detached from the monolayer (Fig. 2D).

These results were supported by Western blot analysis of cell lysates, which identified the expression of EGF-R, vimentin, CRALBP, and glutamine synthetase by MIO-M1 cells at passage 45 and by three clones derived from the original cell preparation at passage 18 (clones 1, 14, and 5; Fig. 3). We were unable to detect GFAP in cell lysates of culture monolayers (not shown).

Scanning and Transmission Electron Microscopy Analysis

Scanning electron microscopy showed that there was a high degree of correspondence between the Müller cell line MIO-MI at passage 43 and a primary Müller cell culture (named 3173) at passage 3 (Fig. 4). At confluence, both types appeared as a loosely packed aggregation of elongated cells with numerous intercellular gaps (Figs. 4A, 4B). Both cell preparations exhibited bipolarity when confluent (Figs. 4A, 4B). At subconfluence, they displayed a pronounced bipolar morphology in

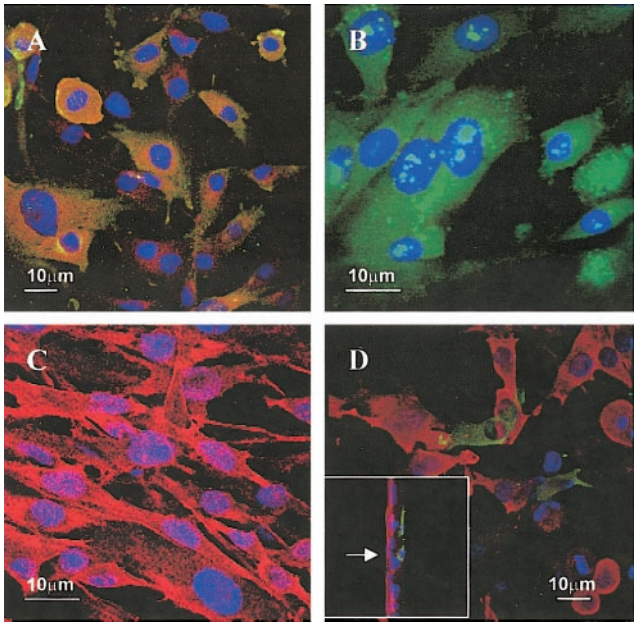

FIGURE 2. Confocal microscopy analysis of Müller cell markers. MIO-M1 cells showing the expression of characteristic markers of Müller cells. (**A**) EGF-R (FITC-stained) and glutamine synthetase (rhodamine-stained). (**B**) CRALBP (FITC). (**C**) Smooth muscle actin (Cy5). (**D**) α-SMA (Cy5) and GFAP (FITC). *Inset*: side view of the same cell monolayer showing the expression of GFAP (FITC) by detached cells. *Arrow*: bottom of the culture dish. Original magnification, (**A**, **D**) ×400; (**B**, **C**) ×630.

which the distinctly rounded-up perikaryon occupied a central position between oppositely directed processes of roughly similar size and elaboration (Figs. 4C, 4D). Contact between neighboring cells in these cultures was achieved by small-diameter filopodial connections that arose from the perikaryon and its processes (Figs. 4E, 4F). In both cell preparations, a large proportion of cells exhibited microvillous projections of varying length but uniform diameter on their apical surface (Figs. 4G, 4H).

Transmission electron microscopy confirmed the flattened nature of the two cell preparations at confluence. We did not observe specialized gap or adherent junctions between cells. Typically, both Müller cell preparations reached a maximum thickness of 4 μm at the nucleus and tapered to less than 1 μm at the periphery. There was a high similarity between the nuclei of the two cell preparations in size, number, and appearance of nucleoli and in distribution of heterochromatin and euchromatin (Figs. 5A, 5B). As in most cultured cells, the

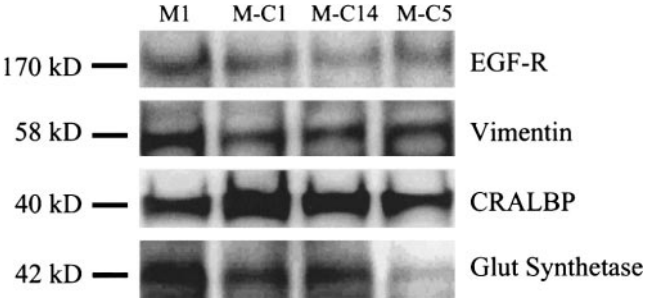

FIGURE 3. Western blot analysis of Müller cell markers. Bands showing the presence of characteristic Müller cell markers in whole-cell lysates of MIO-M1 monolayers (passage 45; *lane 1*) and three clones derived from MIO-M1 cells at passage 18 (labeled M-C1, M-C14, and M-C5; *lanes 2–4*).

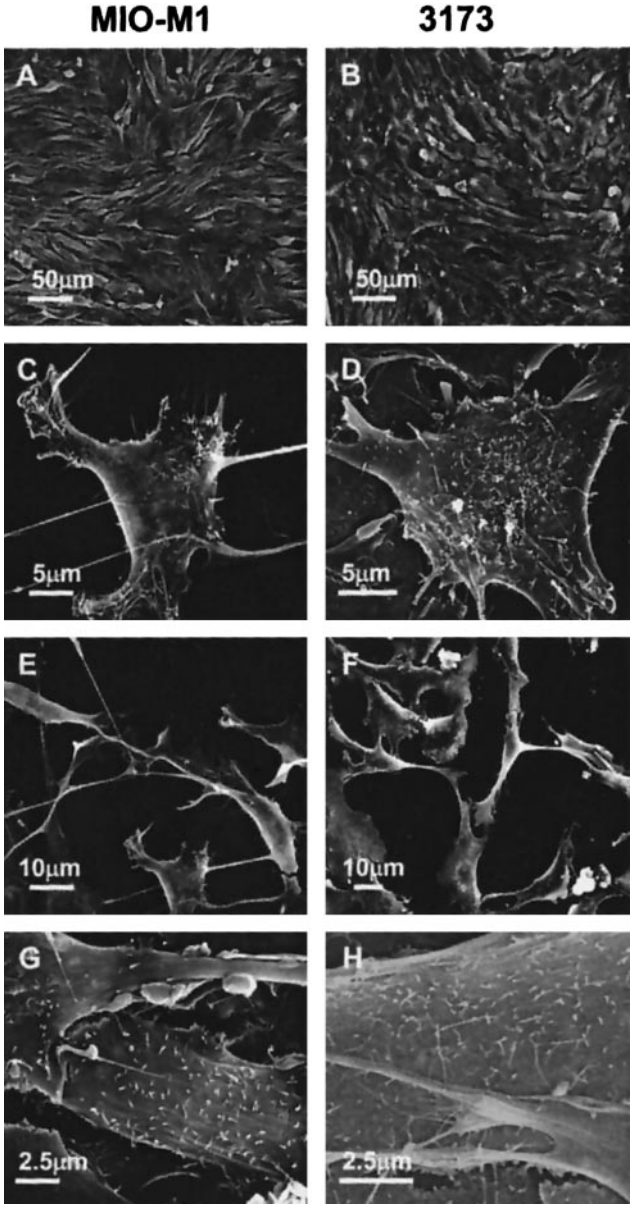

FIGURE 4. Comparison of the MIO-M1 cell line with a primary Müller cell culture (3173) by scanning electron microscopy. Photomicrographs showing morphologic similarities between the Müller cell line MIO-M1 at passage 43 and a preparation of freshly isolated 3173 cells at passage 3. (**A**, **B**) Confluent monolayers displaying elongated shape and loosely packed appearance. (**C**, **D**) Scattered cells exhibiting cytoplasmic projections. (**E**, **F**) Cells exhibiting a bipolar morphology and tubular processes extending to adjacent cells. (**G**, **H**) Details of villous projections on apical surface.

nucleus was close to a Golgi system and several mitochondria. Centrioles and the occasional microtubule (not shown) were also observed in this region (Figs. 5C, 5D). The distribution of perinuclear organelles was strikingly similar in both cell preparations (Figs. 5C–F). This was particularly evident in the Golgi apparatus, which was surrounded by small vesicles and mitochondria (Figs. 5C, 5D). Neither cell preparation exhibited evidence of a well-developed microtubule-organizing center at confluence. However, when cells were subconfluent and in the process of colonizing the dish, we observed large processes containing loose bundles of microtubules aligned parallel to one another and the long axis of the cell process (Figs. 5E, 5F). Small concentrations of intermediate filaments were also observed in both cell preparations (Figs. 5E, 5F). Of the three cytoskeletal elements present in these cells, actin filaments were the most abundant and frequently observed. In each type, actin bundles were concentrated in submembranous locations at cell margins and cell matrix–substrate adhesions.

Electrophysiological Response to Glutamate

Electrophysiological recordings of individual Müller cells cultured in monolayers showed that MIO-M1 cells had resting potentials of -54 ± 14.6 mV and apparent input resistances of 24 ± 5.2 MΩ (Fig. 6). Application of L-glutamate caused a reversible depolarization of membrane potential by 6.1 ± 5.0 mV in all cells. Membrane resistance was not significantly modified during glutamate application ($97\% \pm 8.7\%$ of control).

DISCUSSION

This study describes the characterization of a spontaneously immortalized human Müller cell line, named MIO-M1 after our institution, Moorfields/Institute of Ophthalmology-Müller 1, and which to our knowledge constitutes the first human cell line of this nature reported in the literature. All the parameters investigated, including morphologic appearance under phase-contrast and transmission electron microscopy, expression of various cell markers, and response to glutamate were consistent with those reported in the literature for glial Müller cells. Phase-contrast microscopy showed that the morphologic features of MIO-M1 cells resembled those of rat,[18] cat,[26] and rabbit[27] retinal cells. Examination of Müller cell markers by confocal microscopy and Western blot analysis of cell lysates revealed that these cells expressed well-documented markers of Müller cells.[21–25] These include EGF-R, glutamate synthetase, CRALBP, α-SMA, and vimentin (Figs. 2, 3). Although few cells stained for GFAP, we could not detect this molecule by Western blot analysis of cell lysates, which we attributed to a low expression of this intermediate filament protein. This is in accordance with various reports that in mammalian Müller cells, GFAP is found at low levels or is completely absent,[25,28] but that expression of this molecule increases dramatically in culture[27] and with injury.[26,29] The absence of high levels of GFAP suggests that these cells do not exhibit characteristics of activation in vitro, unlike that observed with cells isolated from rat[18] and cat[26] retinas.

Müller cells and retinal astrocytes share several characteristics in vitro, including morphology, expression of GFAP, α-crystallin, carbonic anhydrase, and glutamine synthetase.[30,31] However, they differ in that astrocytes do not express CRALBP.[23,31] Based on the methodology used to isolate Müller cells from retina, it is possible that photoreceptors and other neural cells, as well as astrocytes, may have contaminated the original culture. Because neural cells do not survive for long periods in culture, the possibility remains that the cells that became immortalized could have been astrocytes. However, astrocytes express high levels of GFAP[29,30] and do not express CRALBP,[23] for which reason our present observations further support the classification of the cell line MIO-M1 as Müller cells, rather than astrocytes.

To date, there have been no reports on the appearance of cultured Müller cells viewed by scanning electron microscopy. It is of interest that MIO-M1 cells in culture exhibited villous projections on their apical surfaces and tubular processes from which finer processes emerged (Fig. 4). Müller cells have been shown to exhibit tubular processes that wrap neurons in the ganglion cell layer,[1,2,19] and it is possible that these processes observed in vitro on the MIO-M1 cell line reflected these characteristics.

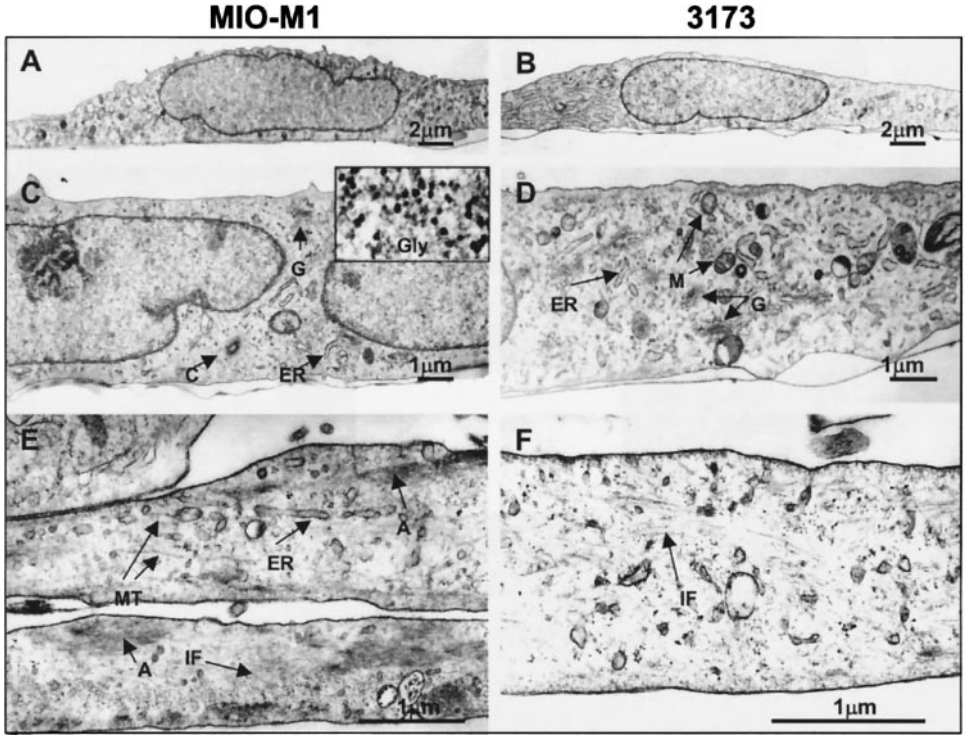

FIGURE 5. Comparison of the MIO-M1 cell line with a primary Müller cell culture by transmission electron microscopy. Photomicrographs showing morphologic similarities between the Müller cell line MIO-M1 at passage and a preparation of freshly isolated 3173 cells at passage 3. (**A**, **B**) Size and appearance of nuclei and distribution of heterochromatin and euchromatin are similar in both cell preparations. (**C**, **D**, *arrows*) Perikarya in both cell preparations contain elaborate Golgi systems (G), centrioles (C), mitochondria (M), and cisterns of endoplasmic reticulum (ER). Glycogen particles were also observed in the perikarya of MIO-M1 cells (*inset*). (**E**, **F**, *arrows*) Microtubules (MT), actin (A), and intermediate filaments (IF) were all observed in both cell preparations.

Unlike the reports of others that rabbit Müller cells in culture show gap and tight junctions,[32,33] after careful examination of the transmission electron microscopy preparations, we did not detect the presence of these specialized junctions in these cells. Although this may be due to differences in our methodology or in the species from which Müller cells derive, the present observations are in accordance with other reports that cat Müller cells do not exhibit gap junctions between them[34] and further support the suggestion that Müller cells do not form gap junctions in the mammalian retina.[35] To our knowledge, there are no reports in the literature that demonstrate this type of specialized junction in human cells in culture. Our findings that the MIO-M1 cells were rich in intermediate filaments and glycogen deposits and that they contained bundles of microtubules aligned parallel to one another and the long axis of the cell process corresponds to features reported for glial Müller cells.[19,34,35] Because these cells expressed low levels of GFAP, it is possible that vimentin may constitute the main component of the abundant intermediate filaments observed by electron microscopy (Fig. 5).

Our preliminary intracellular recordings of MIO-M1 cells revealed that these cells had basic electrophysiological properties similar to those previously described for mammalian Müller cells in vitro,[36,37] although the resting membrane potentials of cells in our study were slightly less hyperpolarized than those of others.[37,38] This difference could be due to differences in the extracellular medium composition, in that our medium contained slightly higher levels of K^+. The finding that the cells depolarized in response to L-glutamate is consistent with an electrogenic uptake for this amino acid, as has

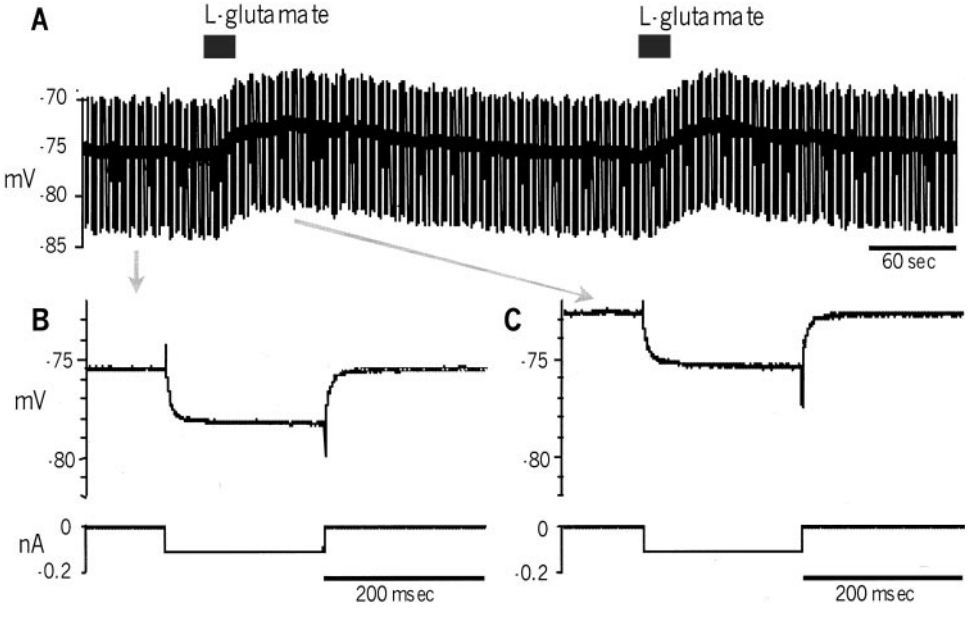

FIGURE 6. Electrophysiological response of MIO-M1 cells to L-glutamate. (**A**) Continuous voltage record obtained through intracellular microelectrodes showing that L-glutamate, applied at the times indicated by *marker bars* (six ejections), evoked a depolarizing response. Transient upward and downward deflections in the trace are due to regular injections of current through the recording electrode. (**B**) Voltage response due to injection of −0.1 nA through the recording electrode, before the application of L-glutamate. (**C**) Voltage response to current injection after application of L-glutamate. Note the depolarization of the membrane potential but the absence of effect of L-glutamate on the magnitude of the voltage response to current injection.

been described previously for Müller cells.[20,38,39] It is noteworthy that we did not see significant changes in input resistance during L-glutamate application. It is, however, likely that recordings made in the soma with a sharp microelectrode would be dominated by the high levels of somal potassium currents seen by others,[37,39] and thus L-glutamate–induced currents would be masked.

We conclude that the cell line MIO-M1 retained the phenotypic and functional characteristics of Müller cells in vitro. The availability of this human cell line will greatly facilitate biological and biochemical studies designed for better understanding of the role of these cells in normal and pathologic situations. It will also provide a tool for the investigation of pharmacologic agents that have the potential to treat and prevent retinal proliferative disease.

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