Changes in Membrane Conductance Play a Pathogenic Role in Osmotic Glial Cell Swelling in Detached Retinas

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Detachment of the neural retina from the pigment epithelium may be associated with tissue edema; however, the mechanisms of fluid accumulation are not understood. Because retinal detachment is usually not accompanied by vascular leakage, we investigated whether the osmotic swelling characteristics of retinal glial (Müller) cells are changed after experimental detachment of the porcine retina. Osmotic stress, induced by application of a hypotonic bath solution to retinal slices, caused swelling of Müller cell bodies in 7-day-detached retinas, but no swelling was inducible in slices of control retinas. Müller cell somata in slices of retinal areas that surround local detachment *in situ* also showed osmotic swelling, albeit at a smaller amplitude. The amplitude of osmotic Müller cell swelling correlated with the decrease in the K⁺ conductance, suggesting a causal relationship between both gliotic alterations. Further factors implicated in Müller cell swelling were inflammatory mediators and oxidative stress. We propose that a dysregulation of the ion and water transport through Müller cells may impair the fluid absorption from the retinal tissue, resulting in chronic fluid accumulation after detachment. This knowledge may lead to a better understanding of the mechanisms involved in retinal degeneration after detachment. (Am J Pathol 2006, 169:1990–1998; DOI: 10.2353/ajpath.2006.060628)

Detachment of the neural retina from the pigment epithelium is a major cause of vision loss, with approximately 15,000 new cases of nontraumatic retinal detachment every year in the United States.¹ In recent years, retinal detachment has become part of a surgical procedure for treating age-related macular degeneration by macular translocation. Likewise, a number of proposed retinal therapies, such as transplantation of pigment epithelium or photoreceptors, electronic retinal implants, or injection of trophic factors or vectors into the subretinal space, may include short- and long-term detachments. Though reattachment surgery has a success rate of over 90% in producing morphological recovery, often there are visual deficits that persist for long time periods after retinal reattachment, suggesting that functional impairments of retinal cells are not recovered. Experimental detachment causes complex cellular responses in the neural retina.² The damage to the outer segments and the apoptotic death of photoreceptor cells,^{3,4} as well as the morphological and biochemical alterations of inner retinal neurons,^{5–8} are associated with activation of macro- and microglial cells.^{9–13} Among the various gliotic responses being characteristic for the detached retina, Müller glial cells display a decrease in their K⁺ conductance.^{14–16} The decrease in K⁺ conductance is associated with a decrease in the gene and protein expression of the major K⁺ channel subtype of the cells, inwardly rectifying Kir4.1 channel.¹⁶ It has been suggested that impaired retinal K⁺ clearance, normally performed by Müller cells by means of their K⁺ channels,¹⁷ may contribute to neuronal hyperexcitation and glutamate toxicity and, therefore, to neuronal degeneration in the detached retina.^{14,16}

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Various studies suggest that retinal detachment may be associated with fluid accumulation in the retinal tissue. Intra- and extracellular edema in and around Müller cells is an early alteration in the detached retina of the pig.¹⁸ Experimental detachment of the primate retina causes edematous swelling and cystoid degeneration of the inner retinal layers.^{19,20} Cystoid fluid-filled spaces have been described to develop in the detached human retina.²¹ Optical coherence tomography performed before reattachment surgery often demonstrated edema in the macular tissue,^{22,23} even in cases when the macula remained attached.²⁴ Fluid accumulation within the retinal tissue may contribute to neuronal degeneration and to the decrease in visual acuity after detachment.

The fluid accumulation in the retina suggests that the water homeostasis in the detached retina is altered. Generally, two cell types are implicated in the water homeostasis of the neural retina. The subretinal space, which encloses the photoreceptor segments, is dehydrated by the pigment epithelium,²⁵ whereas the inner retina is dehydrated by transcellular water transport through Müller cells.^{26,27} The water transport through pigment epithelial and Müller cells is tightly coupled to a transcellular transport of ions, especially of K^+ and Cl^- . In distinct membrane domains of Müller cells, eg, around blood vessels, Kir4.1 and aquaporin-4 water channels are co-localized, suggesting that the transglial water transport is predominantly coupled to K^+ currents that flow through the cells into the blood.²⁶ It has been suggested that the down-regulation of Kir4.1 channels in Müller cells under pathological conditions will disrupt this water transport—in addition to the transglial K^+ clearance currents—through the cells.^{27,28}

The formation of chronic edema is caused by an imbalance between the fluid inflow into the tissue and the fluid absorption from the tissue. Normally, retinal detachment is not associated with vascular leakage, suggesting that the fluid accumulation in the detached retina is caused by other mechanisms. We hypothesize that an impairment of the fluid absorption function of Müller cells may contribute to the fluid accumulation in the detached retina. To investigate whether Müller cells alter the water transport across their plasma membranes after detachment, we determined the volume changes of the cells in response to hypotonic stress (a situation resembling hypoxia-induced intracellular edema in the brain). We found that osmotic stress causes swelling of Müller cells in detached retinas but not in control retinas. Furthermore, we found that the decrease in K^+ conductance characteristically for Müller cells of detached retinas^{14–16} is significantly correlated with the alteration in the osmotic swelling characteristics of the cells.

Materials and Methods

Materials

Mitotracker Orange (chloromethyltetramethylrosamine) was purchased from Molecular Probes (Eugene, OR). Adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), uridine triphosphate (UTP), triamcinolone

acetone (9 α -fluoro-16 α -hydroxyprednisolone), prostaglandin E_2 (PGE₂), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), *N*-nitrobenzylthioinosine (NBTI), *N*⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS2179), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and all other substances used were purchased from Sigma-Aldrich (Taufkirchen, Germany). The following antibodies were used: mouse anti-glutamine synthetase (1:250; Chemicon), mouse anti-vimentin (1:400, V9 clone; Immuno-tech, Marseille, France), rabbit anti-cyclooxygenase-2 (1:100; Cayman Chemical, Ann Arbor, MI), rabbit anti-Kir4.1 (1:200; Alomone Labs, Jerusalem, Israel), Cy3-conjugated goat anti-rabbit IgG (1:400; Dianova, Hamburg, Germany), and Cy2-coupled goat anti-mouse IgG (1:200; Dianova).

Surgical Procedure

All experiments were performed in accordance with applicable German laws and with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Twelve young adult domestic white pigs (17–22 kg; both sexes) were used. Twenty-four hours before and after surgery, the food intake of the animals was restricted, with free access to water. Intramuscular azaperone (15 mg/kg; Cilag-Janssen, Neuss, Germany), atropine (0.2 mg/kg; Braun, Melsungen, Germany), and ketamine (3 mg/kg; Ratiopharm, Ulm, Germany) were administered for premedication. By stepwise application of ketamine (5 mg/kg) and propofol (5 mg/kg; Ratiopharm), a totally intravenous anesthesia was performed. Deltajonin (Delta Select, Pfullingen, Germany) was continuously infused via a vein of the ear. Pulse rate and pO₂ were monitored during anesthesia, and O₂ (3 l/min) was applied.

Rhegmatogenous detachment was created in one eye per animal; the other eye served as nonoperated control. The pupils of the eyes were dilated by topical tropicamide (1%; Ursapharm, Saarbrücken, Germany) and phenylephrine hydrochloride (5%; Ankerpharm, Rudolstadt, Germany), and a lateral canthotomy was created. Hemostasis was achieved with wet-field cautery. After pars plana sclerotomy, a circumscribed vitrectomy was performed in the area of the future detachment, and balanced salt solution (Delta Select) was infused into the eye to replace the vitreous. Thin glass micropipettes attached to 250- μ l glass syringes (Hamilton, Reno, NV) were used to create a retinal detachment by subretinal injection of saline, followed by 0.25% sodium hyaluronate in saline (Healon; Pharmacia & Upjohn, Dübendorf, Switzerland). The retina ventral of the optic nerve head was detached, whereas the dorsal retina remained attached. After surgery, gentamicin (5 mg) and dexamethasone (0.5 mg) were injected subconjunctivally. The lateral canthotomy was closed with 5-0 silk sutures, and atropine (1%) eye drops were instilled into the conjunctival sac. After a survival time of 7 days, the animals were anesthetized as described, the eyes were excised, and the animals were sacrificed by intravenous T61 (embutramid mebezonium

iodide; 0.65 ml/kg body weight; Hoechst, Unterschleißheim, Germany). To investigate whether operation procedure per se alters osmotic swelling of glial cells, vitrectomy (without retinal detachment) was performed in one further animal, and the retinas and cells were investigated at 7 days after surgery.

Electrophysiological Recordings

Whole-cell patch-clamp recordings were performed using Müller cells acutely isolated in papain and DNase I-containing solutions, as described previously.¹⁴ The cell suspensions were stored in serum-free modified Eagle's medium at 4°C (up to 6 hours) before use. Voltage-clamp records were performed at room temperature using the Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and the ISO-2 computer program (MFK, Niedernhausen, Germany). Patch pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) and had resistances between 4 and 6 mega Ω when filled with the intracellular solution that contained 10 mmol/L NaCl, 130 mmol/L KCl, 1 mmol/L CaCl₂, 2 mmol/L MgCl₂, 10 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.1. The signals were low-pass filtered at 1, 2, or 6 kHz (eight-pole Bessel filter) and digitized at 5, 10, or 30 kHz, respectively, using a 12-bit analog/dialog converter. The recording chamber was continuously perfused with extracellular solution consisting of 135 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 1 mmol/L Na₂HPO₄, 10 mmol/L HEPES-Tris, and 11 mmol/L glucose, pH 7.4.

To evoke K⁺ currents, depolarizing and hyperpolarizing voltage steps of 250-ms duration, with increments of 10 mV, were applied from a holding potential of -80 mV. The membrane capacitance of the cells was measured by the integral of the uncompensated capacitive artifact (filtered at 6 kHz) evoked by a hyperpolarizing voltage step from -80 to -90 mV when Ba²⁺ (1 mmol/L) was present in the bath solution. Membrane potentials were measured in the current-clamp mode.

Immunohistochemistry

Isolated retinas were fixed in 4% paraformaldehyde for 2 hours. After several washing steps in buffered saline, the tissue was embedded in saline containing 3% agarose (w/v), and 70- μ m-thick slices were cut by using a vibratome. The slices were incubated in 5% normal goat serum plus 0.3% Triton X-100 in saline for 2 hours at room temperature and, subsequently, in primary antibodies overnight at 4°C. After washing in 1% bovine serum albumin in saline, the secondary antibodies were applied for 2 hours at room temperature. The lack of unspecific staining was demonstrated by negative controls omitting the primary antibodies (not shown). Images were recorded with the confocal laser scanning microscope LSM 510 Meta (Carl Zeiss GmbH, Jena, Germany) at single planes; excitation and emission settings were held constant for all images acquired.

Müller Cell Swelling

The experiments were performed at room temperature. To determine volume changes of Müller glial cells *in situ* evoked by hypotonic stress, the cross-sectional area of Müller cell somata in the inner nuclear layer of retinal slices was measured. Acutely isolated retinal slices (thickness, 1 mm) were placed in a perfusion chamber and loaded with the vital dye Mitotracker Orange (10 μ mol/L). It has been shown that Mitotracker Orange is taken up selectively by Müller glial cells in the retina, whereas neurons remain unstained.²⁹ The dye was dissolved in extracellular solution that contained 136 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L HEPES-Tris, and 11 mmol/L glucose, pH 7.4. A gravity-fed system with multiple reservoirs was used to perfuse the recording chamber continuously with extracellular solutions; the hypotonic solution was added by fast changing of the perfusate. The hypotonic solution contained 60% of control osmolarity and was made by adding distilled water to the extracellular solution. Ba²⁺ (1 mmol/L) was preincubated for 10 minutes in extracellular solution before it was applied within hypotonic solution, and blocking substances were preincubated for 15 minutes before hypotonic challenge. The slices were examined by using the LSM. Mitotracker Orange was excited at 543 nm, and emission was recorded with a 560-nm long-pass filter. During the experiments, the Mitotracker Orange-stained cell somata in the inner nuclear layer were recorded at the plane of their largest extension. To assure that the maximum soma areas were precisely recorded, the focal plane was continuously adjusted during the course of the experiments.

Data Analysis

To determine the extent of soma swelling, the cross-sectional area of Mitotracker Orange-stained cell bodies in the inner nuclear layer of retinal slices was measured using the image analysis software of the LSM. Bar diagrams display the mean cross-sectional areas of Müller cell somata that were measured after a 4-minute perfusion of the hypotonic solution, in percentage of the soma area measured before osmotic challenge (100%). The amplitude of the steady-state inward K⁺ conductance was measured at the end of the 250-ms voltage step from -80 to -140 mV. Statistical analysis was made using SigmaPlot (SPSS Inc., Chicago, IL) and the Prism program (Graphpad Software, San Diego, CA); significance was determined by Mann-Whitney *U*-test for two groups and by analysis of variance followed by comparisons for multiple groups. Data are expressed as means $\pm$ SEM.

Results

K⁺ Currents and Membrane Capacitance of Isolated Müller Cells

As shown recently,¹⁶ Müller cells isolated 7 days after surgery from detached porcine retinas displayed a

Figure 1. Experimental detachment in porcine eyes causes alterations in the K^+ conductance and membrane capacitance of Müller glial cells. The data were determined in cells derived from nonoperated and vitrectomized control eyes, detached retinas, and peri-detached (attached) retinal areas from operated eyes. **A:** Representative traces of whole-cell K^+ currents in cells isolated from a nonoperated control retina, a detached retina, and a peri-detached retinal area of an operated eye. Voltage steps were applied from a holding potential of -80 mV to de- and hyperpolarizing potentials between -180 and $+40$ mV (250 ms, 20-mV increment). Small bars at left indicate zero-current levels. **B:** Mean amplitude of the inward K^+ currents. The steady-state currents were measured at the end of the 250-ms voltage step from -80 to -140 mV. **C:** Resting membrane potential. **D:** Cell membrane capacitance. Each bar represents values obtained in 55 to 58 cells from 11 pigs (except the data from the vitrectomized control eye, which are from five cells). * $P < 0.05$; *** $P < 0.001$ significant differences versus unoperated control.

strong decrease in the amplitude of their inward K^+ currents (Figure 1A), in the mean by 85% compared with control (Figure 1B). Moreover, Müller cells isolated from peri-detached retinal areas (ie, attached areas that surrounded the local detachment *in situ*) displayed a decrease of their K^+ currents, in the mean by 63%. Cells

from vitrectomized control eyes (without retinal detachment) displayed current amplitudes similar to the native controls (Figure 1B). The decrease of the K^+ conductance was associated with a decrease in the membrane potential of Müller cells (Figure 1C). The cell membrane capacitance, which is a marker of the cell membrane area, was significantly enlarged in Müller cells isolated from detached retinas compared with cells from nonoperated control eyes (Figure 1D). Cells isolated from peri-detached retinal areas displayed a weaker, but still significant, increase in their membrane capacitance, whereas cells from vitrectomized eyes (without retinal detachment) showed a normal membrane capacitance (Figure 1D). The data suggest that gliotic alterations occur also in attached retinal areas that surround local detachments in operated eyes, albeit at a lower degree.

Osmotic Müller Cell Swelling

The swelling of Müller cell somata was investigated in acutely isolated retinal slices (Figure 2A) by perfusing the slices with a hypotonic solution that contained 60% of control osmolarity. Exposure to hypotonic solution did not alter the size of Müller cell bodies in retinal slices from nonoperated and vitrectomized control eyes (Figure 2, B–D). However, perfusion of slices from detached retinas caused a time-dependent increase in the size of Müller cell bodies (Figure 2B). After a 4-minute perfusion with hypotonic solution, the cross-sectional area of Müller cell bodies in detached retinas increased by $12.3 \pm 0.7\%$ ($P < 0.001$) (Figure 2C). Interestingly, Müller cell somata in slices from peri-detached retinal areas showed a similar swelling on hypotonic stress (Figure 2B), albeit with a significantly ($P < 0.001$) lower amplitude than cells from detached retinas; as a mean, cells in the peri-detached

Figure 2. Experimental detachment in porcine eyes changes the osmotic swelling characteristics of Müller glial cells. Retinal slices were perfused with a hypotonic solution, and the cross-sectional area of the Müller cell somata in the inner nuclear layer was recorded and is expressed in percentage of the value obtained before hypotonic challenge (100%). **A:** Example of vital dye-stained Müller cells in an acutely isolated slice of a nondetached retina. The arrows mark Müller cell somata; the arrowheads indicate Müller cell endfeet. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; PRS, photoreceptor segments. Scale bar = $10 \mu\text{m}$. **B:** Exposure to hypotonic solution induced time-dependent swelling of Müller cell bodies in slices from a detached retina and from a peri-detached retina, which was located near the local detachment in the operated eye *in situ*. Hypotonic challenge had no effect on the size of Müller cell bodies in a slice from a nonoperated control eye. However, Müller cell bodies in the control retina displayed osmotic swelling when Ba^{2+} ions (1 mmol/L) were present in the bathing solution. The data are means of three cells under each condition. The images display original records of a dye-filled Müller cell body in a slice of a detached retina, recorded before and during hypotonic exposure. Scale bar = $5 \mu\text{m}$. **C:** Mean cross-sectional area of Müller cell bodies after 4 minutes of hypotonic exposure in the absence and presence of Ba^{2+} (1 mmol/L). The cells were recorded in slices of nonoperated control eyes, detached retinas, and peri-detached retinal areas from operated eyes. Each bar represents values obtained in 12 animals; per animal, four to 13 cells were recorded. **D:** Mean Müller cell soma area of cells in slices from a vitrectomized control eye in the absence and presence of Ba^{2+} (1 mmol/L). The bars represent values from seven and eight cells, respectively. * $P < 0.001$ significant differences versus data obtained before hypotonic challenge (100%). • $P < 0.001$ significant differences.

Figure 3. Correlation between the amplitude of the inward K^+ conductance and the amplitude of osmotic swelling of Müller cell bodies. The data were determined in isolated Müller cells and slices, respectively, from detached and peri-detached retinal areas, and from retinas of nonoperated control eyes. Each point represents the mean value of data obtained in one animal.

retina swelled by $6.5 \pm 1.0\%$ ($P < 0.001$) (Figure 2C). Müller cell bodies in slices from control, detached, and peri-detached retinas swelled on hypotonic stress when K^+ channel-blocking Ba^{2+} ions were present in the bath solution (Figure 2, B–D). The data indicate that Müller cells in detached and, to a lower extent, peri-detached retinal areas are more sensitive to osmotic stress than cells in control retinas and lack the ability of rapid volume regulation under hypotonic conditions.

Relationship between Reduction of K^+ Conductance and Cell Swelling

We show here that Müller cells in detached retinas decrease their K^+ conductance (Figure 1, A and B) and are more sensitive to osmotic stress conditions. The observation that K^+ channel-blocking Ba^{2+} ions induce osmotic swelling in cells from control retinas (Figure 2, B–D) suggests a causal relationship between the alterations of both physiological parameters during detachment. To support this assumption, we determined whether there is a correlation between the decrease of the K^+ conductance and the extent of cellular swelling in Müller cells of 11 operated animals. Figure 3 displays a scatter plot of both parameters obtained in cells from operated and control eyes for each animal. As shown, there was a negative correlation between both parameters; the smaller the mean K^+ currents of Müller cells, the larger was the amplitude of cellular swelling under hypotonic conditions ($r = -0.805$; $P < 0.001$). The data suggest that changes in the expression or functional state of K^+ channels play a pathogenic role in osmotic swelling of Müller cells in detached retinas. However, because cells from different retinal areas (eg, from control retinas and peri-detached retinal areas) may show different swelling amplitudes despite similar K^+ currents (Figure 3), other causative factors—in addition to the decrease of func-

Figure 4. Involvement of inflammatory mediators and oxidative stress in evoking Müller cell swelling. **A:** Triamcinolone acetonide (triam; 100 $\mu\text{mol/L}$) inhibited the hypotonic swelling of Müller cell bodies in detached retinas and in control retinas in the presence of Ba^{2+} (1 mmol/L), arachidonic acid (AA; 10 $\mu\text{mol/L}$), PGE_2 (30 nmol/L), or H_2O_2 (50 $\mu\text{mol/L}$). **B:** The inhibitor of the phospholipase A_2 4-bromophenacyl bromide (bromo; 300 $\mu\text{mol/L}$) and the cyclooxygenase inhibitor indomethacin (indo; 10 $\mu\text{mol/L}$), respectively, blocked the osmotic swelling of Müller cell bodies in detached retinas and in control retinas in the presence of Ba^{2+} (1 mmol/L). **C:** The reducing agent dithiothreitol (DTT; 3 mmol/L) inhibited the swelling of cells in detached and control retinas. Each bar represents values obtained in five to eight cells. $^{**}P < 0.01$; $^{***}P < 0.001$ significant differences versus control (100%). $^{\bullet}P < 0.05$; $^{\circ}P < 0.01$; $^{\circ\circ}P < 0.001$ significant blocking effects.

tional K^+ channels—may contribute to the alteration of glial swelling characteristics after retinal detachment. Such factors may be inflammatory mediators and oxidative stress.^{30,31}

Involvement of Inflammatory Mediators and Oxidative Stress in Müller Cell Swelling

The anti-inflammatory glucocorticoid triamcinolone acetonide is used clinically for the rapid resolution of retinal edema.^{32,33} To investigate whether triamcinolone inhibits the hypotonic swelling of Müller cell bodies in detached retinas, we applied this steroid and found that it fully prevented the cell swelling under hypotonic conditions (Figure 4A). Likewise, triamcinolone inhibited the osmotic swelling of Müller cell bodies in control retinas, which was evoked by hypotonic challenge in the presence of Ba^{2+} (Figure 4A). The data suggest that inflammatory mediators may be a causative factor of osmotic Müller cell swelling.

The action of arachidonic acid and of its metabolites, especially PGE_2 , has been causally implicated in the development of retinal edema^{34,35} and in swelling of brain astrocytes and Müller cells.^{30,31,36} To determine whether the osmotic swelling of Müller cells in detached retinas is caused by formation of arachidonic acid due to activation of phospholipase A_2 (PLA_2), the selective PLA_2 inhibitor 4-bromophenacyl bromide was tested. As shown in Figure 4B, inhibition of PLA_2 prevented osmotic swelling in slices of detached retinas and of control retinas in the presence of Ba^{2+} . Likewise, inhibition of the enzyme cyclooxygenase by indomethacin blocked the osmotic Müller cell swelling (Figure 4B). Application of arachidonic acid or PGE_2 to slices of control retinas induced Müller cell swelling under hypotonic conditions in the absence of Ba^{2+} ; this swelling was inhibited in the presence of triamcinolone (Figure 4A).

It is known that gliotic Müller cell responses in detached retinas are, at least in part, caused by hypoxic

conditions.¹² Hypoxia in the detached retina may cause oxidative stress. To reveal whether acute oxidative stress plays a role in osmotic swelling of Müller cells, we tested whether a reducing agent inhibits the swelling in detached retinas and whether application of H₂O₂ to control retinas may induce Müller cell swelling. As shown in Figure 4C, the osmotic swelling of Müller cell bodies in detached retinas, and in control retinas in the presence of Ba²⁺, was fully inhibited in the presence of dithiothreitol, a cell-permeable reducing agent. In control retinas under hypotonic conditions, H₂O₂ induced cellular swelling, which was not observed in the absence of H₂O₂ (Figure 4A). The swelling-inducing effect of H₂O₂ was fully abolished by triamcinolone (Figure 4A). The data suggest that both arachidonic acid metabolites and oxidative stress are involved in evoking Müller cell swelling under hypotonic conditions.

Purinergic Inhibition of Müller Cell Swelling

It has been shown that a purinergic signaling mechanism exists in the rodent retina that inhibits osmotic swelling of Müller cells.³¹ This autocrine signaling mechanism involves the release of endogenous ATP and adenosine and the consecutive activation of P2Y₁ and A1 adenosine receptors. Furthermore, it has been shown that triamcinolone acetate inhibits osmotic swelling of rat Müller cells via stimulation of endogenous adenosine signaling.³⁷ To determine whether such an inhibitory purinergic mechanism is also present in the porcine retina, we tested various agonists and antagonists of purinergic receptors. The purinergic receptor agonists ATP, ADP, UTP, and adenosine blocked the osmotic swelling of Müller cell bodies in detached retinas when they were applied simultaneously with the hypotonic challenge (Figure 5A). The swelling-inhibitory effects of ATP, UTP, and adenosine in detached retinas were blocked by the selective antagonist of adenosine A1 receptors, DPCPX (Figure 5B). Furthermore, the inhibitory effect of triamcinolone was blocked in the presence of the A1 receptor antagonist (Figure 5B), suggesting that this steroid mediates its effect on glial cell swelling via stimulation of endogenous adenosine signaling. This assumption is supported by the observation that the effect of triamcinolone was also blocked by the inhibitor of nucleoside transporters, NBTI (Figure 5C), suggesting that triamcinolone evokes transporter-mediated release of adenosine from retinal cells, which subsequently activates adenosine A1 receptors. The swelling-inhibitory effect of UTP was blocked by the selective inhibitor of P2Y₁ receptors, MRS2179, and by NBTI (Figure 5C), suggesting that UTP evokes the release of both ATP and adenosine from retinal cells. The inhibitory effect of adenosine on the osmotic swelling of rat Müller cells may be mediated by A1 receptor-evoked opening of ion channels in Müller cell membranes³¹; the extrusion of ions is associated with a water flow out of the cells and, therefore, inhibition of cellular swelling. A similar mechanism of A1 receptor-mediated inhibition of Müller cell swelling in the detached porcine retina is suggested by the observation that a Cl⁻

Figure 5. Purinergic inhibition of osmotic Müller cell swelling in the detached porcine retina. **A:** The purinergic receptor agonists ATP, ADP, UTP, and adenosine, respectively (each at 10 μ mol/L), blocked the osmotic swelling of Müller cell bodies in detached retinas. **B:** The inhibitory effects of ATP (10 μ mol/L), UTP (10 μ mol/L), adenosine (10 μ mol/L), and triamcinolone (triam; 100 μ mol/L) on the Müller cell swelling in detached retinas were each blocked by the selective antagonist of adenosine A1 receptors, DPCPX (100 nmol/L). **C:** The effects of UTP (10 μ mol/L) and triamcinolone (triam; 100 μ mol/L) were blocked by the selective inhibitor of nucleoside transporters, NBTI (10 μ mol/L). In addition, the selective inhibitor of P2Y₁ receptors, MRS2179 (30 μ mol/L) blocked the effect of UTP. **D:** The swelling-inhibitory effect of adenosine (10 μ mol/L) was blocked by the Cl⁻ channel blocker NPPB (100 μ mol/L). Each bar represents values obtained in four to eight cells. ** P < 0.01, *** P < 0.001 significant differences versus control (100%). ** P < 0.01; *** P < 0.001 significant blocking effects. ° P < 0.05; °° P < 0.01; °°° P < 0.001 significant inhibition of the agonist effects.

channel blocker, NPPB, largely prevented the effect of adenosine (Figure 5D), suggesting that adenosine evoked opening of Cl⁻ channels in Müller cells.

Immunocytochemical Visualization of Proteins That Contribute to Müller Cell Swelling

We describe that a decrease in the K⁺ conductance, the occurrence of inflammatory mediators, and oxidative stress are factors that contribute to the osmotic swelling of Müller cells in the detached retina. As recently shown,¹⁶ the immunoreactivity of the Kir4.1 protein, which is the major K⁺ channel subtype expressed by Müller cells,³⁸ is altered in detached retinas compared with controls. Whereas in control retinas the immunoreactivity for Kir4.1 is prominently localized at the inner limiting membrane and around the blood vessels, this prominent localization was absent in detached retinas (Figure 6A). The data suggest that the decrease in the K⁺ conductance of Müller cells (Figure 1, A and B) is caused, at least in part, by a mislocation of Kir4.1 protein.

We showed that inhibition of the enzyme cyclooxygenase prevented the osmotic swelling of Müller cell bodies in detached retinas (Figure 4B). To determine whether cyclooxygenase-2 (COX-2) is located to Müller cell bod-

ies in the porcine retina, we immunostained slices of control and detached retinas. Control retinas displayed a low-level expression of COX-2 immunoreactivity predominantly in the ganglion cell and outer plexiform layers (Figure 6B). This staining pattern is in agreement with previously published data that describes a neuronal localization of COX-2 immunoreactivity in the rodent retina, ie, in the outer plexiform layer and in retinal ganglion cells.³⁹ Detached retinas displayed an apparent increase in the immunoreactivity for COX-2, which was partially co-localized with Müller cell fibers. Interestingly, the inner nuclear layer, which contains the somata of Müller cells was largely devoid of COX-2 immunoreactivity in control retinas, whereas a significant expression was found after detachment (Figure 6, B–D). The immunoreactivity for COX-2 was partially co-localized with vimentin-expressing Müller cell fibers and was expressed in Müller cell somata (Figure 6D). The data suggest that in control retinas, COX-2 is expressed in neuronal cells, whereas after detachment an up-regulation of COX-2 occurs in both neuronal cells and Müller cells.

Discussion

Detachment of the human retina,^{21–24} or of the retina of different mammalian species,^{18–20} is frequently associated with a fluid accumulation within the retinal tissue. Commonly, retinal detachment is not accompanied by vascular leakage, suggesting that the fluid accumulation is caused by other mechanisms. We did not find extravasated albumin immunoreactivity around retinal vessels in slices of 7-day-detached porcine retinas (not shown), excluding the presence of vascular leakage at this time. We assume that the fluid absorption function of Müller cells is impaired after detachment; a decrease in the fluid absorption may contribute to the formation of chronic edema. Because the water transport through Müller cells is coupled to the K^+ clearance currents which flow through the cells,^{26,27} a down-regulation of functional K^+ channels after detachment should not only disturb the retinal K^+ homeostasis but also the water absorption from the retinal tissue. Here, we describe that the decrease in Müller cell K^+ conductance after detachment is correlated with an increase in osmotic swelling of the cells under osmotic stress conditions. Such an osmotic swelling was not observed in cells from control retinas. The observation that pharmacological blockade of K^+ chan-

Figure 6. Experimental detachment of the porcine retina changes the immunoreactivities of various proteins involved in Müller cell swelling. The slices were derived from nonoperated control retinas and from detached retinal areas at 7 days after surgery. **A:** Immunoreactivity for Kir4.1. The arrows and the arrowhead mark the prominent expression of the Kir4.1 immunoreactivity around vessels and at the inner limiting membrane, respectively, in the control retina. **B:** Immunoreactivity for COX-2. Cell nuclei were stained with Hoechst 33258 (blue). The arrows point to Müller cell fibers that pass through the IPL. **C:** Immunoreactivities for COX-2 and glutamine synthetase (GS) in the INL. **D:** Immunoreactivities for COX-2 and vimentin. The arrowheads point to somata of two vimentin-expressing Müller cells with COX-2 expressing somata. Note the thick vimentin-positive Müller cell fibers in the detached retina, likely reflecting the hypertrophy of the cells. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bars = 20 μ m.

nels by Ba^{2+} ions induces osmotic swelling in cells of control retinas additionally supports the assumption of a causal relationship between the alterations in membrane conductance and the osmotic swelling characteristics. Furthermore, we found that, in addition to altered membrane currents, inflammatory mediators and oxidative stress are implicated in mediation of acute cellular swelling. Both factors are assumed to be pathogenic factors in retinopathies which are associated with retinal edema, such as diabetic retinopathy. We suggest that the fluid accumulation in detached retinas is caused by the gliotic dysregulation of the ion and water transport through Müller cells, which may impair water extrusion from the retina into the blood and, thus, may contribute to a fluid accumulation in detached retinas.

We found that functional K^+ channels, in particular Kir4.1, are required for the volume homeostasis of Müller cells under hypotonic stress. Kir channels of Müller cells display a high open probability between 0.8 and 0.9 at the resting membrane potential⁴⁰; this high open probability allows prompt transmembrane fluxes of K^+ ions (and, therefore, water) in dependence on the momentary osmotic conditions. However, after inactivation of the Kir channels, eg, under pathological conditions or in the presence of Ba^{2+} , the ion permeability of the Müller cell membranes is strongly decreased under resting conditions, and the cells are not able to rapidly release K^+ ions in response to hypotonic stress. The resulting osmotic gradient across the plasma membrane draws water into the cells. We assume that only the opening of additional osmolyte release channels, eg, of Cl^- channels after activation of adenosine A1 receptors, allows the cells to avoid swelling under pathological conditions.

Edema is one major cause of neuronal degeneration and functional impairment in the retina, and resolution of edema aids the restoration of vision. Corticosteroids such as triamcinolone acetonide are used clinically for the rapid resolution of retinal edema.^{32,33} In addition to the inhibitory effect of triamcinolone on vascular leakage,⁴¹ this steroid may inhibit intracellular edema, ie, swelling of glial cells.³⁷ By opening of membrane channels, triamcinolone is suggested to re-establish the fluid clearance function of Müller cells and, thus, may facilitate the resolution of both extra- and intracellular fluid accumulation. We show here that one major action-mechanism of triamcinolone in the detached porcine retina is the inhibition of cellular swelling by activation of an endogenous adenosine signaling pathway. Moreover, we show that various purinergic receptor agonists such as ATP and UTP may inhibit osmotic Müller cell swelling as well. UTP may act on UTP-sensitive purinergic receptors, ie, P2Y_2 and P2Y_4 .⁴² Pharmacological activation of P2Y_2 receptors has been shown to stimulate the subretinal fluid reabsorption and reattachment of experimentally detached retinas⁴³ via activation of ion and water transport through the pigment epithelium.^{44,45} We suggest that application of P2Y_2 receptor agonists during reattachment surgery or during surgery involving temporary detachment (eg, macular translocation) should limit retinal damage not only via stimulation of reattachment but also by stimulation of the Müller cell-mediated fluid absorption from the

retinal tissue. Perhaps the increase in the Ca^{2+} responsiveness on purinergic receptor stimulation previously described in Müller cells of the detached retina^{15,16} may be part of an endogenous protection mechanism that restricts Müller cell swelling.

There is evidence that retinal detachment is associated with a disturbance in retinal circulation. The retinal blood flow rate is decreased in patients with retinal detachment compared with controls.⁴⁶ Retinal circulation times of the detached areas are longer than those of the peri-detached areas, and both are longer than those of normal subjects.⁴⁷ The cause of the decreased blood flow in detached human retinas is unknown. We suggest that swelling of perivascular Müller cell processes may represent one factor that decreases the retinal blood flow via compression of vessels. Moreover, the decrease in the membrane K^+ conductance (Figure 1, A and B) that is predominantly caused by down-regulation of Kir4.1 channels around the vessels (Figure 6A) may restrict the perivascular release of vasodilating K^+ ions.⁴⁸ However, a causal relationship between dysfunction of Müller cells and impaired hemodynamics during detachment remains to be established.

In summary, we show that the decrease in Müller cell K^+ conductance after detachment correlates with an alteration in osmotic swelling characteristics of the cells. This alteration may reflect a change in the transmembrane water transport. A dysregulation of the ion and water transport through Müller cells may impair the fluid absorption from the retinal tissue resulting in chronic fluid accumulation. A1 receptors may constitute a promising target for the development of novel drugs for the resolution of retinal edema, via stimulation of the fluid clearance function of Müller cells.

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