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Aquaporin-1-facilitated keratocyte migration in cell culture and in vivo corneal wound healing models

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ABSTRACT

Aquaporin-1 (AQP1) water channels are expressed in corneal keratocytes, which become activated and migrate following corneal wounding. The purpose of this study was to investigate the role of AQP1 in keratocyte migration. Keratocyte primary cell cultures from wildtype and AQP1-null mice were compared, as well as keratocyte cultures from pig cornea in which AQP1 expression was modulated by RNAi knockdown and adenovirus-mediated overexpression. AQP1 expression was found in a plasma membrane pattern in corneal stromal and cultured keratocytes. Osmotic water permeability, as measured by calcein fluorescence quenching, was AQP1-dependent in cultured keratocytes, as was keratocyte migration following a scratch wound. Keratocyte migration in vivo was compared in wildtype and AQP1 knockout mice by histology and immunofluorescence of corneal sections at different times after partial-thickness corneal stromal debridement. AQP1 expression in keratocytes was increased by 24 h after corneal debridement. Wound healing and keratocyte appearance near the wound margin were significantly reduced in AQP1 knockout mice, and the number of neutrophils was increased. These results implicate AQP1 water permeability as a new determinant of keratocyte migration in cornea.

1. Introduction

Several aquaporin (AQP) type water channels are expressed in the eye, including AQP0 in lens fiber cells, AQP1 in corneal endothelium, keratocytes, ciliary epithelium and lens epithelium, AQP3 in corneal and conjunctival epithelium, AQP4 in ciliary epithelium and retinal Muller cells, and AQP5 in corneal epithelium (reviewed in Takata et al., 2004; King et al., 2004; Verkman et al., 2008). AQP0 deletion or mutation in mice and humans produces cataracts (Berry et al., 2000; Shiels et al., 2001). Evidence from phenotype comparisons in AQP knockout mice suggests AQP involvement in corneal transparency (Thiagarajah and Verkman, 2002), lens transparency (Ruiz-Ederra and Verkman, 2006), regulation of intraocular pressure (Zhang et al., 2002), light signal transduction and retinal edema (Da and Verkman, 2004; Li et al., 2002), and the repair of corneal epithelial wounds (Levin and Verkman, 2006). These functions suggest the possibility of pharmacological modulation of AQP expression or function in the therapy of glaucoma, cataracts, and corneal and retinal injury. The role of AQP1 in keratocytes is unknown.

The corneal stroma consists of a precisely organized extracellular matrix of collagen fibrils and proteoglycans, including decorin, lumican, keratocan and mimecan, in which is embedded a low density of keratocytes (Meek and Boote, 2004). The functions of keratocytes include generation of the extracellular stromal matrix, and maintenance of corneal transparency and shape (Fini, 1999). Keratocytes normally remain quiescent, but upon injury become stimulated to initiate cell death or to transform into a repair phenotype in which they take on fibroblast-like morphological characteristics including a fusiform shape, multiple nucleoli and absence of cytoplasmic granules, or myofibroblast characteristics, with enlarged size and expression of alpha smooth muscle actin (αSMA) (Fini and Stramer, 2005; West-Mays and Dwivedi, 2006). Migration of keratocytes through the dense corneal stroma is an important process in their response to injury.

Here, we investigated the hypothesis that AQP1 expression in keratocytes facilitates their migration toward a wound. This hypothesis was motivated by our discovery of the involvement of AQPs in cell migration, originally in endothelial cells (Saadoun et al., 2005a), and subsequently in various other cell types (reviewed in Papadopoulos et al., 2008). We proposed that AQPs facilitate cell migration by increasing water permeability at the leading edge of migrating cells, accelerating the formation of lamellipodial

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protrusions in response to actin depolymerization and ion uptake. We found remarkable impairment in the migration of AQP4-deficient glial cells in brain toward a stab wound (Auguste et al., 2007), where glial cells are required to migrate through a dense parenchyma having narrow extracellular spaces, similar to the requirements for migration of keratocytes in corneal stroma. Here, to study AQP1-dependent keratocyte migration, we established keratocyte cell culture models of wound repair and a mouse model of corneal stromal wounding.

2. Material and methods

2.1. Eyes for cell culture and corneal wound studies

Keratocyte cell cultures were generated from murine and porcine corneas. Pig eyes were obtained from Pork Power Farms (Turlock, CA) within few hours after death. Transgenic mice deficient in AQP1 in a CD1 genetic background were generated by targeted gene disruption as described (Ma et al., 1998). All experimental methods and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the University of California, San Francisco Institutional Animal Care and Use Committee (IACUC).

2.2. Keratocyte cell culture

Primary cultures of keratocytes were generated from fresh pig and mouse eyes as described previously for monkey keratocytes, with modifications (Kawakita et al., 2006). Globes were washed in 70% methanol for 2 min followed by 3 washes in sterile HCO_3^--free medium (product 18045-088, Invitrogen, Carlsbad, CA). Under a laminar flow hood, corneas were dissected, taking care to exclude the limbus, and digested in 3 ml dispase II (2.4 U/ml, Roche, Mannheim, Germany) at room temperature while shaking at 140 rpm for 2–3 h. Partially digested corneas from pig were cut in 6–8 pieces and the remaining inner layers (Descemet's membrane, endothelium, Bowman's membrane and epithelium) were mechanically removed with a scalpel (#21, Fisher Scientific, Los Angeles, CA). Partially digested corneas from mice were retained on a 100 μm pore size strainer (Fisher) and inner corneal layers were removed by peeling using two forceps. The remaining corneal stroma (from pig and mice) was incubated for 8–16 h at 37 °C while shaking with DMEM containing 1 mg/ml collagenase A, 20 mM HEPES (both from Sigma), 50 mg/ml gentamicin, and 1.25 μg/ml amphotericin B (both from Invitrogen) in a 15 ml tube. Afterwards, cells were resuspended in keratinocyte serum-free medium (KSFM, Invitrogen) and seeded at a density of 10^5 cells/cm^2 on collagen type I plates (BD Biosciences, Bedford, MA) at 37 °C under 5% CO_2. Keratocyte morphology was maintained in KSFM medium, but became fibroblast-like upon replacement with DMEM containing 10% FBS (Invitrogen) for 5–7 days. For some experiments pig keratocytes were treated with pig AQP1 siRNA or with AQP4 or Kir4.1 siRNA (negative controls) (Dharmacon, Lafayette, CO) at 80–95% confluence using RNAiMAX transfection reagent (Invitrogen). Transfection efficiency was optimized and confirmed using siGLO transfection fluorescent indicators (Dharmacon), and reduction in AQP1 protein expression was confirmed by immunofluorescence and immunoblot analysis using AQP1 antibody (Chemicon, Temecula, CA). Experiments were done at 48–72 h after transfections. In some experiments pig keratocytes were infected with recombinant adenovirus encoding AQP1 (AQP1-Ad) at 1000 pfu/cell, custom generated by ViraQuest (North Liberty, IA). Experiments were done at 72 h after viral infection.

2.2.1. Osmotic water permeability

Keratocytes were seeded on collagen-coated coverslips and their cytoplasm was labelled with calcein by incubation with 10 μM calcein-AM (Molecular Probes, Eugene, OR) for 30 min at room temperature. Osmotic water permeability was measured from the kinetics of calcein fluorescence in response to changing perfusate osmolalities from 300 (PBS) to 150 mOsm (PBS 1:1 diluted with water), as described (Solenov et al., 2004). Calcein fluorescence was measured using a Nikon TE-2000 inverted fluorescence microscope equipped with 40× objective lens and photomultiplier, amplifier, and analog-to-digital converter. The time course of fluorescence in response to osmotic gradients was fitted to a single exponential function with time constant, τ: $F(t) = A + B\,e^{-t/\tau}$, where A and B are related to system sensitivity and background signal.

2.2.2. In vitro wound healing assay

In vitro wound healing was analyzed in confluent cell monolayers incubated in DMEM containing 10% FBS grown on collagen type I plates (BD Biosciences) as described (Saadoun et al., 2005a). Briefly, cells were scraped in an approximately 300-μm wide strip using a standard 200-μl pipette tip. The wounded monolayers were washed twice to remove non-adherent cells. Phase contrast micrographs at 4× were taken just after cells were scraped and after 24 h. Wound healing was quantified as the area covered by the wound edges over 24 h.

2.2.3. In vivo wound healing

Mice were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol (avertin, 125 mg/kg; Sigma–Aldrich, St. Louis, MO), and proparacaine (0.5%) was applied to the corneas of both eyes. A previously reported model of epithelial debridement was used with modifications (Stramer et al., 2003; Mohan et al., 1998). Under an operating microscope, the central epithelium was debrided by applying an Alger Brush II (Alger equipment Co., Lago Vista, TX) for 20 s within a 0.5-mm diameter area, which disrupts Bowman's membrane and results in removal of the epithelium and anterior part of the stroma. Buprenorphine (0.1 mg/kg) was administered for pain relief after surgery. Mice were allowed to recover in a heated cage to maintain normothermia. Mice were euthanized by anesthesia and cervical dislocation at 5 min, 24, 48 and 72 h after injury, and eyes were enucleated and fixed in PBS containing 4% paraformaldehyde for at least 4 h at 4 °C. After fixation tissues were processed through graded concentrations of ethanol, followed by Citrisolv (Fisher), and embedded in paraffin. Five serial sagittal sections (5 μm thickness, 10 μm between sections) at the central cornea were cut on a microtome, parallel to the optic nerve. Only eye sections with a proper sagittal orientation were analyzed, using as selection criteria iris aperture and presence of the optic nerve head. At least five mice of each genotype were analyzed at each study time point. H&E-stained corneas were imaged using a brightfield microscope (Leica, Heidelberg, Germany) equipped with a digital camera (Spot, Sterling Heights, MI). Three fields of ~0.025 cm^2 from the central cornea at 400× total magnification were imaged, located at the site of wound (w), ~50 μm adjacent to the wound (a) and ~1 mm distal to the wound (d). The numbers of keratocytes and polymorphonuclear leukocytes in the 3 regions were determined before and at different times after injury.

2.3. Histology and immunofluorescence

Eye sections were deparaffinized in Citrisolv and rehydrated in graded ethanols. After epitope retrieval with citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6, 30 min, 95–100 °C), sections were blocked with 1% bovine and 5% goat sera and incubated with