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Hyaluronan cable formation by ocular trabecular meshwork cells

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ABSTRACT

Hyaluronan (HA) in the ocular trabecular meshwork (TM) is a critical modulator of aqueous humor outflow. Individual HA strands in the pericellular matrix can coalesce to form cable-like structures, which have different functional properties. Here, we investigated HA structural configuration by TM cells in response to various stimuli known to stimulate extracellular matrix (ECM) remodeling. In addition, the effects of HA cable induction on aqueous outflow resistance was determined. Primary TM cell cultures grown on tissue culture-treated plastic were treated for 12–48 h with TNFα, IL-1α, or TGFβ2. TM cells grown on silicone membranes were subject to mechanical stretch, which induces synthesis and activation of ECM proteolytic enzymes. HA structural configuration was investigated by HA binding protein (HAbp) staining and confocal microscopy. HAbp-labeled cables were induced by TNFα, TGFβ2 and mechanical stretch, but not by IL-1α. HA synthase (HAS) gene expression was quantitated by quantitative RT-PCR and HA concentration was measured by ELISA assay. By quantitative RT-PCR, HAS-1, -2, and -3 genes were differentially up-regulated and showed temporal differences in response to each treatment. HA concentration was increased in the media by TNFα, TGFβ2 and IL-1α, but mechanical stretch decreased pericellular HA concentrations. Immunofluorescence and Western immunoblotting were used to investigate the distribution and protein levels of the HA-binding proteins, tumor necrosis factor-stimulated gene-6 (TSG-6) and inter-α-inhibitor (IαI). Western immunoblotting showed that TSG-6 and IαI were increased by TNFα, TGFβ2 and IL-1α, but mechanical stretch reduced their levels. The underlying substrate appears to affect the identity of IαI·TSG-6·HA complexes since different complexes were detected when TM cells were grown on a silicone substrate compared to a rigid plastic surface. Porcine anterior segments were perfused with 10 μg/ml polyinosinic:polycytidylic acid (polyI:C), a potent inducer of HA cables, and outflow rates were monitored for 72 h. PolyI:C had no significant effect on outflow resistance in porcine anterior segments perfused at physiological pressure. Collectively, *HAS* gene expression, HA concentration and configuration are differentially modified in response to several treatments that induce ECM remodeling in TM cells. In ocular TM cells, our data suggests that the most important determinant of HA cable formation appears to be the ratio of HA chains produced by the different HAS genes. However, the act of rearranging pericellular HA into cable-like structures does not appear to influence aqueous outflow resistance.

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Hyaluronan (HA) is a large, negatively charged glycosaminoglycan (GAG) chain that is composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine (Toole, 2004). The final HA chain can range from 2000 to 25,000 disaccharide units in length. HA is synthesized by three related hyaluronan synthase (HAS) enzymes called HAS1, HAS2 and HAS3 (Itano et al., 1999). These HASs are located at the cell membrane. The activity of each HAS gene directly correlates to the size and amount of HA synthesized. HAS2 produces abundant high molecular weight (MW) HA, HAS1 produces small amounts of high MW HA, while HAS3 synthesizes low MW HA, but in large amounts (Itano et al., 1999). Newly synthesized HA is extruded directly from the cell into the extracellular matrix (ECM). Once it is secreted, HA can remain tethered to the cell surface via receptors, which generates a voluminous cell-type specific pericellular matrix that influences various properties of the cell (Toole, 2004). Pericellular HA can inhibit ligand access to receptors, limit phagocytosis and cause cell cycle arrest (Stern et al., 2006).

Two configurations of HA have been described: globular HA, which is the classical form detected in the pericellular matrix, and HA cable-like structures. HA cable formation was first described in

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intestinal mucosal smooth muscle cells treated with the viral mimetic, polyinosinic-polycytidylic acid (polyI:C) (de la Motte et al., 2003). HA cables have subsequently been detected in several other cell types including proximal tubular epithelial cells, epidermal keratinocytes, fibroblasts, and airway smooth muscle cells (Evanko et al., 2009; Jokela et al., 2008; Lauer et al., 2009; Selbi et al., 2006b). HA cables typically arise from the peri-Golgi and individual HA strands become cross-linked and complexed into HA cables (Day and de la Motte, 2005). HA cables appear to function as scaffolds, which could prevent loss of ECM components during tissue remodeling or act as a template for matrix regeneration (Evanko et al., 2009). The biological properties of HA cables differs from that of pericellular HA since cables are adhesive for leukocytes (de la Motte et al., 2003). Thus, HA cables have a unique architecture and different functional activities than pericellular globular HA.

Little is known about the biological processes involved in HA cable formation. Previously, it was suggested that HA cable formation was a self-contained latent response that was independent of protein synthesis since cycloheximide, a protein synthesis inhibitor, induced cable formation within 2–4 h of treatment (Hascall et al., 2004). Since then, several factors have been proposed to influence cable formation including modifying the activity of each HAS gene to alter the ratio of individual HA strands coalesced into the HA cable, increasing the number of cellular protrusions that could stabilize the cables as they assemble, altering pericellular matrix HA concentration, regulating the levels of ECM proteins that cross-link individual HA chains into cables, or a combination of all these (Day and de la Motte, 2005; Evanko et al., 2009; Kultti et al., 2006; Selbi et al., 2006b).

HA chains can be stabilized by numerous HA-binding proteins called hyaladherins (Toole, 1990). A well-studied HA binding complex is that formed by inter-α-inhibitor (IαI) (Salier et al., 1996; Zhuo et al., 2004). IαI is assembled in the Golgi apparatus and is composed of two heavy chains (HC1 and HC2) bound to the chondroitin sulfate chain of the proteoglycan, bikunin (Zhao et al., 1995; Zhuo et al., 2004). IαI is a donor of HCs, which are enzymatically transferred to HA chains by an intermediary called tumor necrosis factor-stimulated gene-6 (TSG-6) (Milner et al., 2006). Transfer of HCs to HA chains is thought to stabilize the HA matrix since individual HA chains can be cross-linked into HA assemblies (Milner et al., 2006; Toole, 2004). The proteoglycan, versican, is another hyaladherin that is associated with HA cables (Evanko et al., 2009).

The trabecular meshwork (TM) in the anterior segment of the eye is the tissue responsible for establishing and maintaining intraocular pressure (IOP) within a normal range. Elevated IOP (>22 mmHg), which is a primary risk factor for glaucoma, is caused by a blockage in the conventional aqueous humor outflow pathway in the TM (Stamer and Acott, 2012). A normal homeostatic response to elevated IOP initiates degradation of the existing ECM by matrix metalloproteinases (MMPs) (Acott et al., 2014; Keller et al., 2009a). Concomitantly, deposition of new ECM facilitates the egress of aqueous humor from the anterior chamber, which in turn reduces elevated IOP. The role of HA in the regulation of IOP was first demonstrated in the 1950s (Barany and Scotchbrook, 1954; Francois et al., 1956). Since then, other studies have shown that enzymatic degradation of HA by hyaluronidases increases outflow in some, but not all, animal species (Grant, 1963; Hubbard et al., 1997; Keller et al., 2008; Knepper et al., 1984; Sawaguchi et al., 1993). Furthermore, HA concentration was lower in glaucoma TM than age-matched controls (Knepper et al., 1996a). Our most recent data demonstrated that RNAi silencing of *HAS1* and *HAS2* genes decreased HA concentration and reduced outflow through the human TM (Keller et al., 2012b). Thus, several lines of evidence point toward HA concentration as an important factor in aqueous outflow.

Various stimuli are known to increase or reduce aqueous outflow through the TM by inducing ECM remodeling. For instance, mechanical stretch, which mimics the stretch and distortion that is placed on TM cells *in situ* during pressure fluctuations, induces secretion and activation of MMPs (Bradley et al., 2001). Additionally, the inflammatory cytokines, tumor necrosis factor-α (TNFα) and interleukin-1α (IL-1α), are released following laser trabeculoplasty, a common surgical treatment for glaucoma patients (Bradley et al., 2000). Application of these cytokines to anterior segment perfusion culture increased outflow rates (Bradley et al., 2006). Conversely, transforming growth factor-β2 (TGFβ2), levels of which are increased in the aqueous humor of glaucoma patients, (Fleenor et al., 2006; Gottanka et al., 2004; Inatani et al., 2001; Tripathi et al., 1994) decreased outflow in perfusion culture. However, it is not known how these external stimuli affect HA concentration or configuration in TM cells. Our main objectives of this study were to investigate whether HA cable-like structures are formed by ocular TM cells and to determine which factors appear to be important for HA cable formation. Results from this study will provide further insight into the role of HA in aqueous outflow resistance and in glaucoma.

1. Material and methods

1.1. TM cell culture and treatments

Primary TM cell cultures were established from TM tissue dissected from 20 porcine eyes (Carlton Packing, Carlton, OR) as described previously (Polansky et al., 1979; Stamer et al., 1995). All pieces of dissected TM tissue were placed in a T25 flask and cultured in Dulbecco's Modified Eagle's Medium (DMEM), 1:1 mixture of high and low glucose, containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-fungizone to allow the cells to populate the surface of the flask. When confluent, the mixed population of porcine TM cells was passaged to a maximum passage number of 5.

For cytokine treatments, cells were grown to confluence for at least 3 days in 6-well plastic tissue culture plates with serum-containing medium, washed with phosphate-buffered saline (PBS) and then placed into serum-free DMEM. The following cytokines were added: 10 ng/ml recombinant porcine TNFα, 10 ng/ml recombinant porcine IL-1α (R&D Systems, Minneapolis, MN), or 5 ng/ml activated TGFβ2 (Invitrogen, Carlsbad, CA). For mechanical stretch experiments, TM cells were cultured in 6-well FlexCell plates (BioFLex Int, Hillsborough, NC). These plates contain a flexible silicone elastomer membrane coated with collagen type I. Cells were grown to confluence, washed with PBS, placed into serum-free DMEM and a push pin was placed beneath the membrane of the well (Bradley et al., 2001). A weight was applied to the lid of the plate, pushing the plate downward on the push pin and stretching the collagen-coated silicone membrane and attached cells. This produces a constant pressure with approximately 10% stretch/distortion (Bradley et al., 2001).

1.2. Immunofluorescence and confocal microscopy

TM cells were plated on FlexCell membranes in serum-containing medium for at least 3 days, placed in serum-free medium and then treated with the cytokines and mechanical stretch for a further 3 days. Immunofluorescence and confocal microscopy were performed as detailed previously (Keller et al., 2009b, 2008). Briefly, membranes were fixed in 4% paraformaldehyde and blocked in CAS universal blocking buffer (Invitrogen). In some experiments, ice-cold methanol or a mixture of acetic acid, ethanol

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