The Effects of Hyaluronic Acid on Canine Posterior Lens Capsule Opacification *In Vitro*

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Introduction

The normal crystalline lens is isolated by a basement membrane, the lens capsule. A single layer of cuboidal lens epithelial cells (LEC) lines the anterior lens capsule and continuously secretes the lens capsule. The majority of the lens is composed of fibers that are arranged in a central nucleus and a peripheral cortex. Cataract is defined as any opacity of the lens and is the most common cause of visual impairment in both humans and dogs (Andersson et al. 1994; Buyukmihci et al. 1987; Cheng et al. 2001; Schaumberg et al. 1998). Cataracts develop secondary to endocrine disorders (Basher et al. 1995), inherited or congenital causes (Davidson et al. 2007; Priester et al. 1972), toxins (Davidson et al. 2007), electrolyte imbalances (Davidson et al. 2007), electric shock (Davidson et al. 2007), trauma (Davidson et al. 2007), drugs (Heywood 1971), radiation (Ching et al. 1990; Lett et al. 1989; Roberts et al. 1987), nutritional deficits (Ranz et al 2002), retinal atrophy Barnett 1970), intraocular inflammation (Albert et al. 1976; Bistner 1973; Buyukmihci et al. 1987), and from age-related degeneration (Davidson et al. 2007). When the lens becomes cataractous, the LECs may migrate and proliferate aberrantly on the posterior lens capsule (Apple et al. 1992). Other changes that occur during cataractogenesis include the formation of bladder or Wedl cells which are swollen LECs, as well as myofibroblastic differentiation of the LECs to form capsular plaques. Advanced cortical cataracts have a decreased amount of LEC resulting from cell degeneration. Fluid from lytic lens proteins may accumulate in small clefts formed between degenerative fibers and is associated with vacuolization of lens fibers and pyknotic nuclei.

Cataract surgery is currently the only accepted treatment for cataracts and related vision loss and is the current standard of care. Extracapsular cataract extraction by phacoemulsification with subsequent intraocular lens (IOL) implantation is the most frequently performed ophthalmic
surgical procedure, with a success rate of greater than 95% (Beebe 2003; Davison et al. 1999). During cataract surgery, part of the anterior lens capsule is removed by a capsulectomy. The lens fibers are removed via phacoemulsification, and the lens capsule may be polished to decrease the number of residual LECs prior to IOL implantation. Viscoelastic is used to inflate the anterior chamber as well as to inflate the lens capsule prior to IOL implantation. Quality of life following cataract surgery is markedly improved, as reported by humans who have undergone cataract surgery. There is also the perception, by owners, of an improved quality of life in canine companions that have had cataract surgery.

Complications of cataract surgery include retinal detachment, glaucoma, incisional dehiscence, intraocular infection, IOL decentration, and the most common postoperative complication, posterior capsule opacification (PCO) (Noble 2002). Posterior capsule opacification refers to opacities of the posterior lens capsule following cataract surgery that can lead to diminished vision either directly by the LECs or indirectly by posterior capsule wrinkles and striae or by IOL decentration that can induce visual aberrations (Apple et al. 1972). Following cataract surgery, LEC may migrate across the inner surface of the lens capsule, across the IOL optic, or they may migrate around the capsulotomy edge onto the outer surface of the capsule. When these cells migrate to the posterior portion of the lens capsule, they may create PCO. The LECs may also show increased metabolic activity, become vacuolated, and take on the appearance of clear pearl-shaped cells or vacuoles, termed Elschnig’s pearls.

Phacoemulsification, IOL placement, and residual or new growth of lens cortical material can all lead to blood-aqueous barrier breakdown and intraocular inflammation (Nishi and Nishi 2002). It is well established that intraocular inflammation and surgical disruption of the anterior lens capsule initiate cell signaling where the primary response of any remaining anterior and
equatorial LEC is to undergo epithelial-mesenchymal transition (EMT) (Awasthi et al. 2008; Chandler et al. 2007). This results in the posterior migration and proliferation of the LEC that is associated with PCO formation with subsequent vision impairment. EMT refers to a change in phenotype from a cuboidal epithelial cell to fibroblastic morphology accompanied by aberrant basement membrane synthesis (Awasthi et al. 2008; Chandler et al. 2007). However, this process is not completely understood for any species. The pathway to PCO formation is very complex and, therefore, offers numerous sites for possible inhibition chemically, pharmacologically, or mechanically.

The impact of PCO on vision includes decreased visual acuity, diplopia, glare, and decreased contrast sensitivity (Buehl et al. 2005; Buehl et al. 2005; Cheng et al. 2001; Claesson et al. 1994; Jose et al. 2005; Sunderraj et al. 1992). PCO can also cause a decrease in visual acuity by opacification of the visual axis. Opaque PCO, also called capsular fibrosis or scarring, that occurs in the visual axis may impair vision as much as the original cataractous lens (Buelg et al. 2005). PCO is seen in up to 50% of adult humans up to five years postoperatively (Apple et al. 1992; Mootha et al. 2004; Schaumberg et al. 1998), in up to 43% of children one year postoperatively (Metge et al 1989), and in up to 100% of children (Apple et al. 1992) and dogs within one year after cataract surgery (Bras et al. 2006).

Hyaluronan, also known as hyaluronic acid or hyaluronate, is a uniformly repetitive, linear glycosaminoglycan composed of disaccharides of glucuronic acid and N-acetylglucosamine (Toole 2001). Although hyaluronic acid (HA) is distributed ubiquitously in vertebrate tissues, both in the embryo and in the adult, its organization with respect to cells is variable. In adult tissues such as the vitreous humor, synovial fluid and dermis, it clearly plays an extracellular, structural role based on its unique hydrodynamic properties. However, during
dynamic cellular events such as inflammation, wound repair, tissue development and cancer, HA also interacts with cells and influences their behavior in a variety of ways (Lee and Spicer 2008; Toole 2004; Toole 2001; Turley et al. 2002; Vigetti et al. 2008).

There are at least three fundamentally different ways in which HA can influence normal and abnormal cell behavior. First, due to its biophysical properties, unbound HA has a profound effect on the biomechanical properties of extracellular and pericellular matrices in which cells reside (Toole 2001). Second, HA forms a multivalent template for specific interactions with other pericellular macromolecules, thus contributing to the assembly, structural integrity and physiological properties of matrices (Toole 2001). In these two ways, HA makes the pericellular matrix more conducive to cell shape changes required for cell division, movement and morphogenesis, and thus contributes to cell behavior in what has been called a ‘permissive’ fashion. A third function of HA is to interact with cell surface receptors that transduce intracellular signals and influence cellular form and behavior directly (Bourguinon et al. 2001; Day and Prestwich 2002; Herrlich et al. 2000; Noble 2002; Toole 2004). Thus HA also has an ‘instructive’ influence on cell behavior.

The biological functions of HA are essentially mediated by cell surface HA receptors, including CD44 and the receptor for HA mediated motility (RHAMM). The CD44 ectodomain is responsible for the binding of HA, and the cytoplasmic domain regulates specific signaling and interacts with cytoskeletal proteins (Bourguinon et al. 2001). Using immunohistochemistry, Saika et al (Saika et al. 1998) determined that CD44 is ubiquitously expressed in human LEC; CD44 expression is unknown in the canine lens. RHAMM is an 85-kDa protein expressed in most tissues, distributed in multiple compartments including cell surface, cytoskeleton, mitochondria and the cell nucleus (Turley et al. 2002). RHAMM binds HA and also acts as a
microtubule-associated protein interacting with the actin cytoskeleton (Assmann et al. 1999; Turley et al. 2002). To date, expression of RHAMM has not been examined in the lens. The relative contribution of the two types of HA receptors and the intracellular signaling pathways involved in HA-mediated effects in LEC remain unknown. It is well established in other tissue types that HA will bind with cell receptors CD44 and RHAMM and increase cellular migration and proliferation (Bourguinon et al. 2001; Kayastha et al. 1999; Panagopoulos et al. 2008). In patients with cancer, HA concentrations are usually higher in malignant tumors than in corresponding benign or normal tissues, and in some tumor types the level of HA is predictive of malignancy (Kayastha et al. 1999). In patients with breast and ovarian carcinomas as well as B-cell lymphoma, high levels of HA, CD44 and RHAMM expression in the stroma are consistent with low survival rates (Kayastha et al. 1999; Nagel et al. 2010).

Viscoelastics are used to inflate the anterior chamber and lens capsule intraoperatively during cataract surgery and HA is a common component of these viscoelastics. There are various viscoelastics used in intraocular surgery including 1.2% HA, 2.0% HA, chondroitin sulfate or hydroxypropylmethylcellulose (HPMC). Viscoelastics are generally removed at the conclusion of cataract surgery; however, removal of these viscoelastics is never complete. HA has been shown to induce EMT in other cell types such as meningiomas and thus, the introduction of exogenous HA in the form of viscoelastics during cataract surgery may promote LEC migration and proliferation and subsequently, PCO formation. This study was performed to evaluate if canine LECs are capable of responding to hyaluronic signaling of CD44 and RHAMM as well as to evaluate if lens epithelial cell migration and proliferation will be increased using viscoelastics with higher hyaluronic acid content.
Materials and Methods

Samples:

Normal eyes were obtained by enucleation from dogs in good general health that were humanely euthanized at a local animal shelter for population control purposes. All dogs used in this study were estimated to be between one and eight years of age, based on dentition and thickness of the anterior lens capsule. Globes were collected within one hour of death and placed in dilute betadine solution until dissection. Normal lenses and lens capsules were obtained after dissection and placed in 10% neutral-buffered formalin.

Cataractous lens capsules were obtained from dogs undergoing elective cataract surgery at The Ohio State University College of Veterinary Medicine. These capsules were harvested from immature, mature, and hypermature cataracts. The most common etiologies for the cataracts were diabetes mellitus and inherited. These capsules were then used for immunohistochemistry, western blots, and qualitative real-time polymerase chain reaction.

Positive Controls for CD44 and RHAMM

A total of five paraffin-embedded neoplasms from dogs were utilized in this study for CD44. One canine squamous cell carcinoma, one canine complex mammary adenoma, one canine mammary carcinoma, one canine mixed mammary tumor, and one canine cutaneous hemangiosarcoma. To evaluate RHAMM, seven paraffin-embedded neoplasms from dogs were utilized in this study for RHAMM. Three normal testicles, a sertoli cell tumor, and three squamous cell carcinomas were evaluated. Samples were obtained from the Department of Veterinary Biosciences, at The Ohio State University and were diagnosed by various board-certified veterinary pathologists.
**Immunohistochemistry of CD44 and RHAMM**

The standard avidin-biotin-peroxidase complex (ABC) technique was used with diaminobenzidine (DAKO, Carpinteria, CA) as the chromagen and Mayers hematoxylin (Signet Laboratories, Dedham, MA) as the counterstain. Paraffin-embedded samples were sectioned (5um) and placed on Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA). Five cataractous lens capsules and six normal lens capsules were evaluated for the presence of CD44. Sixteen cataractous lens capsules and eight normal lens capsules were evaluated for the presence of RHAMM. Slides were decorated in xylenes, rehydrated through a graded series of alcohols, and rinsed in phosphate-buffered saline (PBS). Slides were incubated with peroxidase block (DAKO) for ten minutes at room temperature, washed in PBS, and then incubated with a protein block (DAKO) for twenty minutes at room temperature. Sections were incubated for one hour at 37° C in a humidified chamber with one of the following primary antibodies diluted in antibody diluent (DAKO): CD44 (Santa Cruz Biotechnology, INC., Santa Cruz, CA) diluted to 1:400 or RHAMM (Santa Cruz Biotechnology, INC., Santa Cruz, CA) diluted 1:50. Sections were then rinsed in PBS and incubated with anti-rat secondary antibody (Biogenex, San Ramon, CA) solution for thirty minutes at room temperature. Slides were washed a final time in IX PBS before incubation with the chromagen and counterstain. Slides were washed again with tap water until clear and then dehydrated. Slides were gradually passed through 70%, 85%, 100% ethanol until a final two-minute incubation in xylenes. Slides were then coversliped with Permount mounting solution.

Following staining, the slides were evaluated by the authors (DJH, HLC). The slides were assessed for staining intensity and location.
qRT-PCR of Normal and Cataractous Lens for CD44 and RHAMM

RNA was extracted from normal and cataractous anterior lens capsule samples according to the suggested protocol using Absolutely RNA MicroPrep Kit (Stratagene, La Jolla, CA). The ImPromII Reverse Transcriptase kit (Promega, Madison, WI) was used to synthesize the first strand of cDNA. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using the Mx3000p Multiplex Quantitation System (Stratagene) as follows: 95°C for fifteen minutes, then forty cycles at 94°C for thirty seconds, 60°C for thirty seconds, and 70°C for thirty seconds, using the QuantiTect SYBR Green PCR kit (Stratagene). Primers to amplify sequences of CD44 and RHAMM were designed based on previously published sequence data. Primers to amplify the sequence for glyceraldehydes-3-phosphate dehydrogenase (GAPDH; housekeeping control) were based on previously published sequence data. Primers employed were:

CD44 Forward: 5’-CCA AGA CAG TTC CAG GGT GT-3’
CD44 Reverse: 5’-TCG ACT ATT GAC AGC GAT GC-3’
RHAMM Forward: 5’-TCT GCA CTT TTC TCA GCC CTG GT-3’
RHAMM Reverse: 5’-TGC TGC TCA CAG TCA AGC CAC T-3’
GAPDH Forward: 5’-GCC GTG GAA TTT GCC GT-3’
GAPDH reverse: 5’-GCC ATA AAT GAC CCC TTC AT-3’

All samples were run in duplicate three separate times. The threshold cycle value was calculated for each sample by the instrument software. The relative amount of CD44, RHAMM, and HPRT mRNA was calculated using the LinRegPCR software (v. 11.x; JM Ruijter, S van der
Velden, A Ilgun, Amsterdam, the Netherlands). The results were expressed as the ration of the target gene (CD44 or RHAMM) to the HPRT housekeeping gene.

**Primary canine lens epithelial cell cultures**

Anterior lens capsules with adherent LEC were incubated in trypsin (0.25% trypsin and 1X EDTA, Gibco, Carlsbad, CA) for five minutes at 37° C. After incubation, the solution and lens capsule were centrifuged for two minutes at 0.3 x g. Fluid was decanted and supplemented DMEM (10% fetal bovine serum and 1% antibiotic/antimycotic [Gibco]) was then added. The solution, including the lens capsule, was transferred to a laminin-coated culture flask (Beckton-Dickinson, Franklin Lakes, NJ) and incubated in a humidified incubator at 37° C and 5% CO₂. LEC were grown until 90% confluence prior to re-plating.

**Scratch model to induce epithelial-mesenchymal transition**

Lens epithelial cells in each well were allowed to grow to 90% confluence in unsupplemented DMEM in a twelve-well laminin coated culture dish. A vertical one mm scratch was then made on the cellular surface. Cells were then treated with unsupplemented DMEM, 0.012 mg/mL of HA, 0.02 mg/mL of HA, and 1 mg/mL of HA (n=6) and allowed to recover for sixteen hours. The HA (Sigma) was prepared in unsupplemented DMEM. Digital images were taken immediately after the initial scratch was made and sixteen hours after healing. ImageJ (National Institutes of Health) was then used to quantify the cellular migration and proliferation.

**Mock Cataract Surgery**
A stab incision was made into the anterior chamber avoiding the lens. The cornea was completely removed with Steven’s tenotomy scissors and the iris was removed using Vannas scissors. An anterior capsulorhexis was performed using Vannas scissors exposing the lens nucleus and cortex. The lens nucleus and cortex were removed using a lens loop. All capsules were irrigated with PBS and aspirated prior to incubation with viscoelastic agent or the control to remove residual lens fibers and LECs. However, not all LECs were removed prior to capsule dissection. For the control group (n=6 capsules), the lens capsule was irrigated a second time using PBS. The remaining eyes were then divided into three treatment groups (n=6 capsules per group) and treated with either 2% hyaluronic acid viscoelastic (Acri.Tec, Hennigsdorf, Germany), 1.2% hyaluronic viscoelastic (Acri.Tec), or HPMC viscoelastic (contains no hyaluronic acid) (Acri.Tec,). The various viscoelastic agents were left in the lens capsule for five minutes, then the viscoelastic was aspirated and then irrigated with PBS to ensure removal of treatment. Capsules were then removed from their zonular and vitreal attachments with Vannas scissors and placed in culture dishes with five milliliters of unsupplemented DMEM. The plates were then placed in a 37°C / 5% carbon dioxide incubator. Phase contrast microscopy pictures were taken of the capsules immediately after adding cell culture medium, and every twenty-four hours after the treatment to monitor LEC migration onto the posterior capsule.

Results

Immunohistochemistry of CD44 and RHAMM

Antibody specificity was confirmed using canine corneal epithelial cell lysate and canine MCDK cell lysate in western blot analysis. Of six normal lens capsules, three normal lens capsules exhibited positive expression (Figure 1) and three normal lens capsules were negative
for expression of CD44. All five cataractous lens capsules that were evaluated were positive for CD44 expression (Figure 2). Eight normal lens capsules were evaluated for RHAMM expression and of these, seven demonstrated immunoreactivity (Figure 3) and one was negative. Sixteen cataractous lens capsules were evaluated for RHAMM expression and of these samples, fifteen were positive for RHAMM expression (Figure 4) and one sample was negative. All lens epithelial cells that were positive for CD44 and RHAMM staining exhibited cytoplasmic staining, which was similar to what was seen in our positive controls.

**qRT-PCR of Normal and Cataractous Lens for CD44 and RHAMM**

Both cataractous and normal lens capsules were evaluated for mRNA expression of CD44 or RHAMM relative to GAPDH. Normal lens capsules showed an mRNA expression of 6.1 for CD44 and 5.9 for RHAMM as compared to GAPDH. In contrast, cataractous lens capsules showed an mRNA expression of 10.0 for CD44 and 7.0 for RHAMM as compared to GAPDH.

**Scratch model to induce epithelial-mesenchymal transition**

Lens epithelial cell cultures were evaluated immediately after creation of a scratch (referred to as time zero) and sixteen hours after creating the scratch. A one-way Analysis of Variance with a Bonferroni Multiple Comparison Test was performed to evaluate differences in the area of the scratch devoid of cells between the four treatment groups (DMEM only, 0.012 mg/mL HA, 0.02 mg/mL HA, 1.0 mg/mL HA). All time zero cultures compared with time sixteen within the same treatment group showed a statistically significant difference between the area of the scratch devoid of cells. There was a significant difference (p<0.001) between the area
of the scratch at time sixteen hours of the group receiving DMEM compared with the area of the
scratch at time sixteen hours of cells treated with 1.0mg/mL of HA (Figure 5).

Mock Cataract Surgery

Capsules were evaluated by phase contrast microscopy to evaluate the extent of migration
and proliferation of LECs from the anterior capsule to the posterior lens capsule. Our time period
to allow contact of the LECs with HA was five minutes. Many times, surgeons are able to
remove the viscoelastic with less than five minutes of LEC contact time; however, we felt this
was a reasonable amount of time that would correlate surgically in the clinical setting. Cells
from the control group (PBS only) were consistently found only on the anterior lens capsule
(Figure 6). Cells from the HPMC group, the viscoelastic material that contains no HA, migrated
to the same extent as the control group, as LEC consistently remained on the anterior lens
capsule after three days (Figure 7). Cells from the 1.2% HA treated group showed minimal
migration onto the posterior lens capsule, however did not cross the midline, or visual axis, of
the posterior lens capsule. Cells from the 2.0% HA treated group showed extensive migration
onto the posterior lens capsule and frequently migrated beyond the posterior capsule midpoint
into the region of the visual axis and achieved 90% confluence on the posterior capsule.

Discussion

Posterior capsule opacification affects 50% of adult human (Apple et al. 1992; Mootha et
al. 2004; Schaumberg et al. 1998) and up to 43% of children (Metge et al. 1989) and one 100%
of dogs (Bras et al. 2006) after cataract surgery within one year. Due to this high incidence of
postoperative PCO formation, surgical and medical techniques are being investigated to address
this vision-impairing complication. Hyaluronic acid is a common component of surgical viscoelastics, however to our knowledge, this compound has not been evaluated as to its effects on the LECs and whether it has the ability to influence posterior proliferation and migration. In this study we evaluated HA cell surface receptors CD44 and RHAMM and confirmed their presence in canine LECs. We also evaluated the ability of canine LECs to respond to HA, utilizing a scratch test model as well as a mock cataract surgery on canine cadaver eyes.

The biological functions of HA are essentially mediated by cell surface HA receptors, CD44 and RHAMM. CD44 comprises a large family of transmembrane glycoproteins that exhibit extensive molecular heterogeneity. The CD44 ectodomain is responsible for the binding of HA, and the cytoplasmic domain regulates specific signaling and interacts with cytoskeletal proteins. CD44 expression was previously unknown in the canine lens. RHAMM is an 85-kDa protein expressed in most tissues, distributed in multiple compartments including cell surface, cytoskeleton, mitochondria and cell nucleus (Turley et al. 2002). RHAMM binds to HA and also acts as a microtubule-associated protein interacting with the actin cytoskeleton (Assmann et al. 1999; Schaumberg et al. 1998). Previous to this study, RHAMM expression has not been evaluated in the lens of any species. In this study, immunohistochemistry was performed and identified the presence of CD44 and RHAMM in both normal and cataractous LECs. Saika et al. previously determined that CD44 is present in human LECs (Saika). Therefore, canine LECs have the ability to respond to HA signaling. It has been well established in other tissue types that HA increases cellular migration and proliferation through CD44 and RHAMM signaling pathways (Bourguinon et al. 2008; Panagopoulos et al. 2008; Toole 2004). In patients with various forms of cancer, HA concentration is usually higher in malignant tumors than in corresponding benign or normal tissues and in some tumor types the level of HA is predictive of
malignancy (Kayastha et al. 1999; Nagel et al. 2010). Since migration and proliferation can be induced, and since CD44 and RHAMM are expressed in LEC, these cells may respond by increasing migration and proliferation.

qRT-PCR for CD44 and RHAMM expression in normal and cataractous LEC were evaluated in this study. This technique demonstrated that CD44 and RHAMM mRNA expression is present in both normal and cataractous LEC as compared to GAPDH. Cataractous lens capsules showed a four-fold increase in CD44 mRNA expression and a negligible increase in RHAMM mRNA expression as compared to normal lens capsules.

Following sixteen hours, the scratch test model showed that there was a significant difference in the area devoid of cells between the control group versus the LEC treated with 1.0 mg/mL of HA. This study showed that there was not a significant difference between any of the groups except for the time sixteen hours for the control group versus time sixteen hours for the 1.0 mg/mL HA. However, there was a trend of increasing rate of scratch wound closure in each group as HA content increased. Therefore, in vitro, LECs respond to HA by increasing the rate of migration and proliferation, and the response appears to be concentration dependent.

The mock cataract surgery model showed that, subjectively, in lens capsules treated with viscoelastics with higher HA content greater migration of LECs onto the posterior lens capsule occurred. The results of this study suggest that higher concentrations of HA in viscoelastic materials used in clinical cataract surgery could potentially increase PCO in vivo.

This study demonstrates that CD44 and RHAMM are present in canine LECs and therefore, LECs have the ability to respond to HA signaling. Further in vivo studies are warranted to evaluate if viscoelastics both with and without HA increase the extent of PCO.

Reference List


