Induction of Posterior Capsule Opacification by Hyaluronic Acid in an Ex Vivo Model

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Purpose. Because hyaluronic acid (HA) is found in many surgical viscoelastic agents, this study aimed to determine (1) if HA receptors are present in the canine lens, (2) if the rate of lens epithelial cell (LEC) migration is altered following treatment with HA, and (3) if introduction of exogenous HA into the lens capsule promotes lenticular migration, thus contributing to posterior capsule opacification (PCO).

Methods. Normal and cataractous canine LECs were evaluated for expression of the HA receptor CD44 and the receptor for HA mediated motility (RHAMM) using immunohistochemistry, immunoblotting, and real-time PCR. Canine LEC were treated with various concentrations of HA, and induction of migration was monitored over time. Commercially available surgical viscoelastics were utilized ex vivo, and rates of PCO formation were analyzed.

Results. Basal protein and mRNA expression of both CD44 and RHAMM was noted. Cataractous canine LEC demonstrated significantly ($P < 0.01$) higher expression of CD44 but not RHAMM. Treatment with higher concentrations of HA resulted in a significant ($P < 0.01$) increase in CD44 mRNA and increased LEC migration in vitro. Use of CD44-neutralizing antibodies confirmed the role of CD44 in HA-induced lenticular migration. Viscoelastic material containing higher concentrations of HA led to increased rates of ex vivo PCO.

Conclusions. Exogenous HA can induce lenticular migration and CD44 expression. Use of surgical viscoelastics that contained HA resulted in increased rates of ex vivo PCO suggesting that judicious selection and use of viscoelastic material during cataract surgery is warranted. (*Invest Ophthalmol Vis Sci.* 2012;53:1835–1845) DOI:10.1167/iovs.11-8735

Cataract, defined as any opacity of the lens, is the most common cause of visual impairment in both humans and dogs.[1–3] Surgery is currently the only accepted method for eliminating cataracts and related vision loss. Phacoemulsification extracapsular cataract extraction (ECCE) with intraocular lens (IOL) implantation is the most frequently performed ophthalmic surgical procedure, with a success rate of greater than 95%.[4,5] During ECCE, surgical viscoelastics play several roles including protecting delicate tissue and cells from trauma, creating and preserving intraocular space, and lubricating and separating tissues. The unique properties of many viscoelastic materials can be attributed to the presence of hyaluronic acid (HA). Viscoelastic agents are typically removed from the eye prior to the close of surgery by using automated irrigation and aspiration. In doing so, the surgeon aims to minimize or avoid any significant changes in postoperative intraocular pressure. Unfortunately, due to the adhesive nature of surgical viscoelastics, complete removal of viscoelastic agents from intraocular tissues, including the lens capsule, is extremely difficult. As a result, it is inevitable that some viscoelastic material will remain in the anterior chamber following cataract surgery.

HA is a large, linear glycosaminoglycan found in the pericellular matrix of numerous cell types.[6] Although 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 vitreous, synovial fluid, and dermis, it clearly plays an extracellular, structural role based on its unique hydrodynamic properties. However, HA can also interact with surface receptors and influence cellular behavior in a variety of ways during inflammation, wound repair, tissue development, and cancer progression.[7–10] The ability of HA to modify cellular responses has been demonstrated in various tissue types, most notably through induction of epithelial-mesenchymal transition and migration.[11–13]

The biological functions of HA are mediated largely by cell surface receptors, CD44, and the receptor for HA-mediated motility (RHAMM).[10] CD44 consists of a large family of transmembrane glycoproteins that exhibit extensive molecular heterogeneity. Binding of HA to CD44 extracellular domains affects cell adhesion to the extracellular matrix, proliferation, and migration.[11,14,15] The intracellular CD44 domain can regulate specific cell signaling, examples of which include interaction with tyrosine kinases and activation of Rho-like GTPases.[10] Previous research has determined that CD44 is expressed in both normal and cataractous human lens epithelial cells (LEC).[16,17] While the presence of CD44 protein was recently documented in the murine lens, expression is currently unknown in the canine lens.[18] RHAMM is an alternatively spliced protein expressed in most tissues and distributed in multiple compartments including the cell surface, cytoskeleton, mitochondria, and cell nucleus.[10] RHAMM is capable of binding HA and is important in tissue injury and repair[19]; interaction with the actin cytoskeleton as a microtubule-associated protein and regulation of Ras GTPases are proposed mechanisms for RHAMM-mediated signaling.[10,20] To date, expression of RHAMM has not been examined in the crystalline lens of any species. Additionally, the relative contribution of the two types of HA receptors and the

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intracellular signaling pathways involved in HA-mediated effects in LEC remains unknown.

Following cataract surgery, the most common long-term complication is posterior capsule opacification (PCO).[21–25] It is well established that postoperatively, the primary response of the remaining LEC is to undergo epithelial-mesenchymal transition, migrate, and proliferate.[24,26,27] Understanding the factors leading to PCO development and formulating new prevention strategies are of critical importance due to the high worldwide prevalence and subsequent vision impairment associated with PCO.[24,25] In other tissue types, it is well documented that HA can increase cellular migration.[11–13] Thus, the introduction of exogenous HA in the form of viscoelastics during cataract surgery may promote LEC migration and, ultimately, PCO formation. This study was designed to test the hypothesis that HA and surgical viscoelastic can modulate lenticular migration using canine in vitro models of PCO formation.

Materials and Methods

Cells and Tissues

Normal eyes were obtained by enucleation from dogs of various breeds in good general health that were humanely euthanized at a local animal shelter for population control purposes; The Ohio State University Institutional Animal Care and Use Committee deemed this research exempt from review. All dogs used in this study were estimated to be between 1 and 8 years of age, based on dentition and thickness of the anterior lens capsule.[28] Globes were collected within one hour of death, placed in dilute povidone-iodine (Betadine) solution, and then rinsed and immersed in PBS until dissection.

Normal anterior lens capsules and whole lenses were obtained by dissection and placed in 10% neutral-buffered formalin or were snap frozen. Cataractous lens capsules were obtained from dogs undergoing elective cataract surgery at The Ohio State University College of Veterinary Medicine. Lens capsules were harvested from mature and hypermature cataracts, which almost universally have LEC that have migrated onto the posterior capsule.[29] Samples chosen for this study had obvious subcapsular plaques or diffuse LEC proliferation onto the posterior lens, evident at the time of surgery. Patients with only nuclear cataract were not included in this study. Cataractous capsules with adherent LEC were placed in 10% neutral-buffered formalin or snap frozen immediately after continuous curvilinear capsulorhexis. Normal and cataractous lens capsules were then evaluated by immunohistochemistry, immunoblotting, and quantitative real-time PCR (qRT-PCR).

Positive Canine Controls for CD44 and RHAMM

Multiple neoplastic and normal canine tissues were used in this study to confirm specificity for CD44 and RHAMM. These included squamous cell carcinoma, complex mammary adenoma, mammary carcinoma, cutaneous hemangiosarcoma, sertoli cell tumor, and normal testicle. Samples were obtained from The Ohio State University College of Veterinary Medicine and were previously diagnosed by board-certified veterinary pathologists.

Immunohistochemistry of Normal Lenses, Cataractous Lenses, and PCO Samples

Standard avidin-biotin-peroxidase complex technique was used, as previously described,[26,30] with diaminobenzidine (DAKO, Carpinteria, CA) as the chromagen and Mayers hematoxylin (Signet Laboratories, Dedham, MA) as the counterstain. Ten normal lenses, 10 cataractous lenses, and 4 PCO samples, all of canine origin, were evaluated with each antibody. Primary antibodies diluted to the following ratios in antibody diluent (DAKO) were CD44 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:400 dilution, RHAMM (Santa Cruz Biotechnology) at 1:50 dilution, or α-smooth muscle actin (αSMA; Spring Bioscience, Freemont, CA) at 1:100 dilution. Sections were subsequently rinsed in PBS and incubated with anti-rat or anti-goat secondary antibodies (Biogenex, San Ramon, CA). Following immunohistochemical staining, slides were evaluated for staining intensity and location.

Immunoblotting

Whole-cell protein was extracted from frozen positive controls, and all test samples using 1X CHAPs lysis buffer (Chemicon, Temecula, CA) as previously described.[26,30] Protein concentration was quantified using the Bradford protein assay (Bio-Rad, Hercules, CA).

Samples ($n = 6$) were denatured (95°C, 3 minutes) in modified SDS sample loading buffer and protein (15 μg) was separated by SDS-PAGE (10% acrylamide, v/v) at 150 V for approximately 1.5 hours. Following electrophoresis, proteins were transferred to a nitrocellulose membrane at 300 mA for 1.5 hours. Nonspecific binding was blocked by incubating the membrane for 6 hours at room temperature in 5% nonfat dry milk diluted in 1× Tris-buffered saline containing 0.1% Tween-20 (TBST). After being blocked, membranes were incubated with either CD44 (1:1000 dilution) or RHAMM (1:50 dilution) in blocking buffer. The membrane was extensively washed with 1× TBST, and secondary antibody was added to blocking buffer for 1 hour at room temperature. Protein was detected using Pierce (Rockford, IL) immunoblotting system. Membranes were stripped (Pierce), and the technique was repeated using anti-β-actin antibody (1:5000 dilution; Sigma-Aldrich, St. Louis, MO). Kodak 1D image analysis software (Kodak Molecular Imaging, New Haven, CT) was used to obtain densitometry readings for all Western blots.

qRT-PCR of Normal and Cataractous LECs for CD44 and RHAMM

RNA was extracted from all 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. qRT-PCR was performed using the Mx3000p Multiplex quantitation system (Stratagene) as follows: 95°C for 15 minutes, then forty cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 70°C for 30 seconds, using the QuantiTect SYBR Green PCR kit (Stratagene). Primers to amplify sequences of canine 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. The forward primer for CD44 was 5′-TATTGCTTCAATGCTTCAGCTCCA-3′ and reverse was 5′-AGGTTGTGTTTGCTCCACCTTCTTGAC-3′; forward primer for RHAMM was 5′-TCTGCACTTTTCTCAGCCCTGGT-3′ and reverse was 5′-TGC TGCTCACAGTCAAGCCACT-3′; forward primer for GAPDH was 5′-GCCGTGGAATTTGCCGT-3′ and reverse was 5′-GCCATAAATGACCCCTTCAT-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 amounts of CD44, RHAMM, and GAPDH mRNA were calculated using LinReg PCR software (version 11.x; JM Ruijter, S van der Velden, A Ilgun, Amsterdam, the Netherlands). Results were expressed as the ratio of the target gene (CD44 or RHAMM) to the GAPDH housekeeping gene.

Primary Canine LEC Cultures

Anterior lens capsules with adherent LECs were incubated in trypsin (0.25% trypsin and 1× EDTA (Gibco, Carlsbad, CA) for 5 minutes at 37°C. After incubation, the solution and lens capsule were centrifuged for 2 minutes at 300*g*. Fluid was decanted and supplemented Dulbecco's modified Eagle medium (DMEM; 10% fetal bovine serum and 1% antibiotic/antimycotic [Gibco]) was then added. The solution,

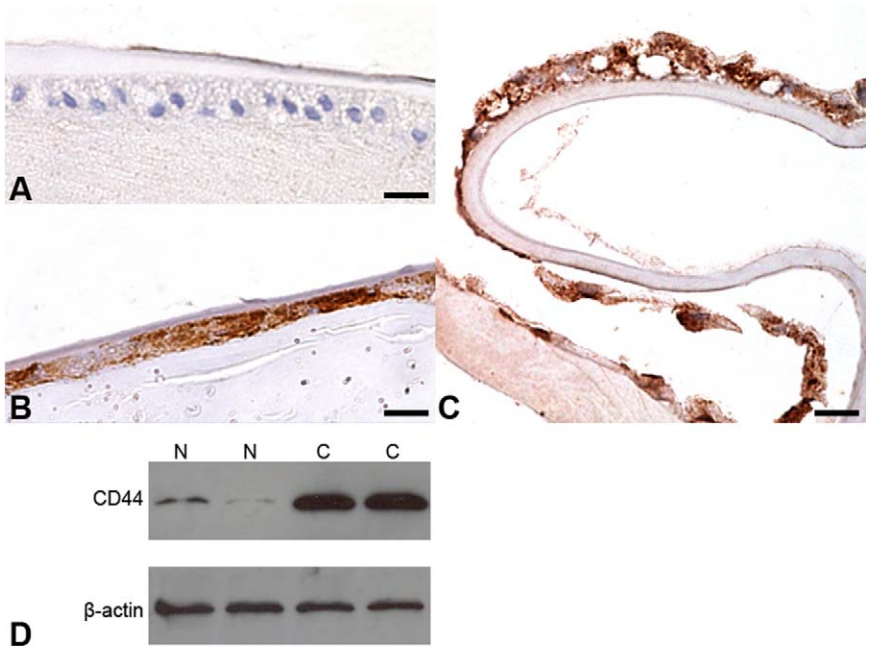

Figure 1. Expression of CD44 protein in canine LEC. (**A**) In normal LEC there is little to no CD44 immunoreactivity, while all LEC (**B**) in cataractous and (**C**) PCO samples demonstrated robust cytoplasmic staining for CD44. (**D**) Western blot analysis of normal (N) canine LEC found little CD44 protein present, while clinical samples of canine cataracts (C) showed increased expression of CD44 protein. Scale bars are equal to 30 μm.

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% CO_2. LEC were grown until 90% confluence prior to replating.

Scratch Model to Induce Cellular Migration

Lens epithelial cells in each well were allowed to grow to 95% confluence in unsupplemented DMEM in 12-well laminin-coated culture dishes. A vertical 1-mm scratch was then made on the cellular surface. To evaluate changes in CD44 and RHAMM expression, cells were treated with unsupplemented DMEM, 0.5 mg/mL of HA, and 1.0 mg/mL of HA ($n = 6$) and allowed to recover for 8 hours at 37°C. The HA (Sigma-Aldrich) was of high molecular weight and prepared in unsupplemented DMEM. Treatment medium was then removed, cells were washed twice with PBS, and the plates were immediately frozen and mRNA was extracted as described above.

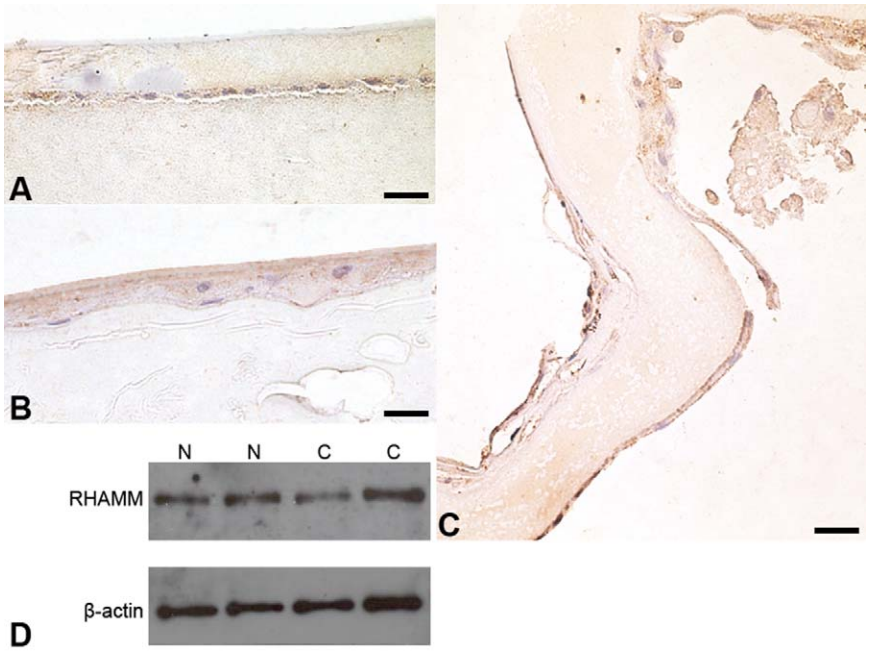

Figure 2. Expression of RHAMM protein in canine LEC. The majority of (**A**) normal LEC had faint RHAMM expression; most LEC in (**B**) cataractous and (**C**) PCO samples consistently demonstrated RHAMM immunoreactivity although intensity varied from faint to moderate. (**D**) Western blot analysis of normal (N) canine LEC and clinical samples of canine cataracts (C) found similar expression of RHAMM protein. Scale bars are equal to 30 μm.

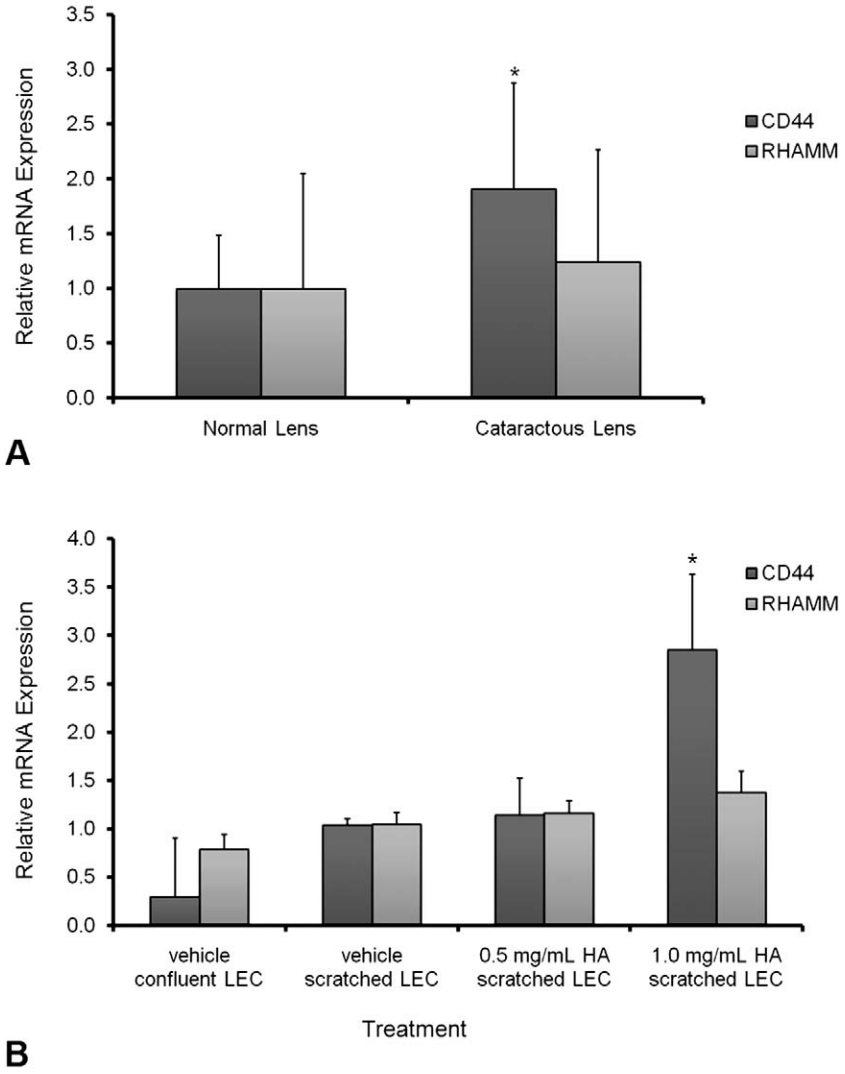

Figure 3. CD44 and RHAMM mRNA expression in canine LEC. Using qRT-PCR, (**A**) basal expression both of CD44 and RHAMM mRNA was detected in normal LEC. In canine cataracts where LEC are undergoing migration, a significant ($P < 0.01$) increase in CD44 expression was noted compared to normal LEC. While a slight increase in RHAMM mRNA expression was found in cataractous samples compared to normal LEC, it was not found to be significant. (**B**) Following treatment with various doses of HA, culture wells containing migrating canine LEC had increased CD44 mRNA compared to confluent control LEC; this increase was significant ($P < 0.01$) when wells with migrating LEC were treated with 1.0 mg/mL HA compared to confluent controls. Eight hours following the initiation of the scratch, LEC treated with 1.0 mg/mL HA had significantly ($P < 0.01$) higher CD44 mRNA compared to vehicle treated LEC. Error bars indicate SEM.

To evaluate cellular migration, we followed the above-described protocol to create a 1-mm scratch. Lens epithelial cells were then treated with unsupplemented DMEM, 0.5 mg/mL HA, and 1.0 mg/mL HA ($n = 6$) and allowed to incubate for 16 hours at 37°C. These concentrations were chosen because maintaining fluidity of the culture media was necessary and higher HA concentrations increased the culture medium viscosity excessively. Digital images were taken immediately after the initial scratch was made and after incubating for 16 hours. ImageJ software (National Institutes of Health, Bethesda, MD) was then used to quantify the initial area of the scratch and the final area of the scratch 16 hours later, herein referred to as initial-SA and final-SA, respectively.

CD44 Neutralization

Neutralizing CD44 functions was carried out by preincubating canine LECs with blocking anti-CD44 antibody (10 μg/mL; IM7, BD Biosciences, San Jose, CA) or normal rat immunoglobulin G (IgG; 10 μg/mL; BD Biosciences) for 30 minutes at 37°C. The cells were then used for the scratch model described above.

Sham Cataract Surgery

An ECCE with lens capsule dissection was performed as previously established.[30,31] Residual LECs and cortical material were removed via gentle manual irrigation and aspiration with PBS, using a coaxial cannula, but no effort was made to polish the anterior and posterior lens capsule. Capsules were divided into three treatment groups ($n = 6$) and treated with either 2% HA viscoelastic (containing 20 mg/mL HA; Acri.Vet, Hennigsdorf, Germany), 1.2% HA viscoelastic (containing 12 mg/mL HA; Acri.Vet), or hydroxypropylmethylcellulose (HPMC) viscoelastic (contains 0 mg/mL HA; Acri.Vet). These surgical viscoelastics (with set HA concentrations) were chosen because they are commercially available and currently used during cataract surgery in veterinary ophthalmology. All viscoelastic material contained high-molecular weight HA. For the control group ($n = 6$), the lens capsules

were treated with PBS only. To approximately simulate the exposures during cataract surgery, the PBS control or various viscoelastic agents were left in the lens capsule for 5 minutes. Capsules were aspirated and irrigated with PBS to ensure removal of the viscoelastic material. Capsules were then excised from their zonular and vitreal attachments and placed in cell culture dishes with 5 mm of unsupplemented DMEM. Plates were then placed in a 37°C, 5% CO_2 incubator. Phase contrast photomicrographs were taken of the capsules immediately following mock cataract surgery and every 24 hours after the treatment to monitor LEC migration onto the posterior capsule. Posterior capsule percent confluence was calculated using ImageJ software analysis of the daily photomicrographs. Following 96 hours in culture, all lens capsules were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned. LECs were then manually counted from the anterior and posterior capsule from four different 5-μ sections.

Statistical Analysis

All statistical analyses were performed with Prism version 5 software (GraphPad Software Inc., San Diego, CA). A one-way ANOVA with a Bonferroni multiple comparison post hoc test was performed to evaluate differences in the scratch area between the four treatment groups at initial-SA and final-SA. The capsule cell counts were evaluated with a Kruskal-Wallis one-way analysis of variance with Dunn multiple comparison post hoc test. The level for statistical significance was set at $P < 0.05$ for all comparisons. All graphs were generated in Prism and displayed mean and standard errors of the mean (SEM), represented by error bars.

Results

Expression of CD44 and RHAMM in the Lens

As evaluated by immunohistochemistry, normal canine LEC exhibited minimal to no detectable expression of CD44 protein (Fig. 1A). All LEC within cataractous and PCO samples had pronounced immunoreactivity for CD44, and expression was predominantly cytoplasmic (Figs. 1B and 1C). Lens fiber cells were negative for CD44 expression. Western blots confirmed that LEC from clinical cataract samples had increased expression of CD44 protein compared to normal LEC (Fig. 1D). Ten normal LEC samples were evaluated for RHAMM expression, and of these, eight samples were faintly positive (Fig. 2A) and two were negative. All 10 cataractous LEC and all 4 PCO samples evaluated for RHAMM expression were positive, and the staining intensity varied from faint to moderate (Figs. 2B and 2C). All RHAMM immunoreactive LEC exhibited cytoplasmic staining and lens fiber cells were negative. Both normal and cataractous LEC were positive for RHAMM expression, as observed by Western blotting; cataractous LEC had a subjective increase in RHAMM expression comparatively (Fig. 2D).

Cataractous and normal LEC were evaluated for CD44 or RHAMM mRNA expression. Normal canine LEC had significantly ($P < 0.01$) lower expression of CD44 mRNA than cataractous LEC (Fig. 3A). In contrast, while normal LEC had reduced expression of RHAMM mRNA compared to cataractous LEC, the difference was not considered significant (Fig. 3A).

Effect of HA on LECs

Lens epithelial cells that were treated with various doses of HA for 8 hours were evaluated for expression of CD44 and RHAMM mRNA. Compared to confluent LEC that received vehicle treatment, all cultures with migrating LEC demonstrated increased expression of CD44 mRNA; this upregulation was significant ($P < 0.01$) when the 1.0 mg/mL HA treatment group was compared to confluent controls (Fig. 3B). Expression of RHAMM did not significantly increase in any treatment group compared to confluent LEC (Fig. 3B). No significant differences in either CD44 or RHAMM expression were noted

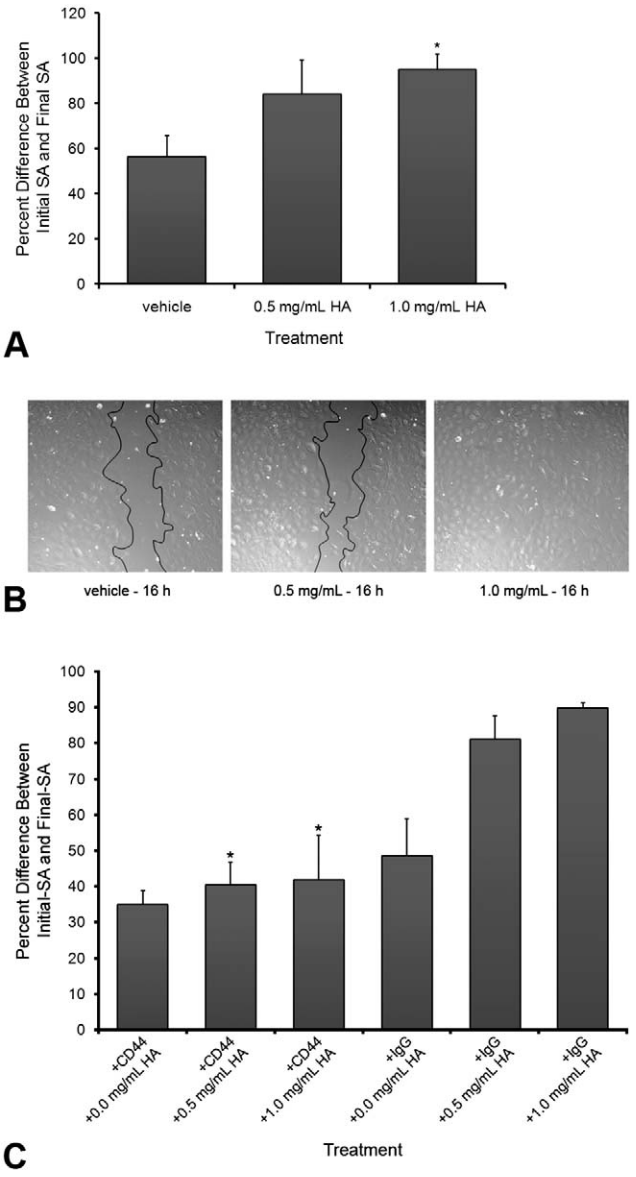

Figure 4. Changes in scratch area following treatment. Sixteen hours after initiation of the scratch (**A**) LEC treated with vehicle only has significantly larger scratch areas compared to LEC treated with 1.0 mg/mL HA. While not statistically significant, there was a trend of decreasing scratch area in LEC treated with 0.5 mg/mL HA. (**B**) Using inverted microscopy, we monitored the rate of cellular ingrowth into the initial scratch; the area devoid of LECs is outlined with dashed black lines. With higher concentrations of HA, the rate of cellular ingrowth increased. Many of the cultures treated with 1.0 mg/mL HA became completely confluent by 16 hours. (**C**) Change in scratch area following preincubation with either CD44 neutralizing antibodies or nonimmune IgG with subsequent stimulation by HA. Treatment with IgG did not affect the rate of cellular ingrowth into the initial scratch. When LEC were treated with 0.5 or 1.0 mg/mL HA after IgG pretreatment, a significant ($P < 0.001$) increase in the rate of cellular ingrowth was noted compared to controls. When LEC were treated with 0.5 or 1.0 mg/mL HA after pretreatment with CD44 neutralizing antibodies, a significant ($P < 0.001$) decrease in the rate of cellular ingrowth was found compared to controls. Error bars indicate SEM.

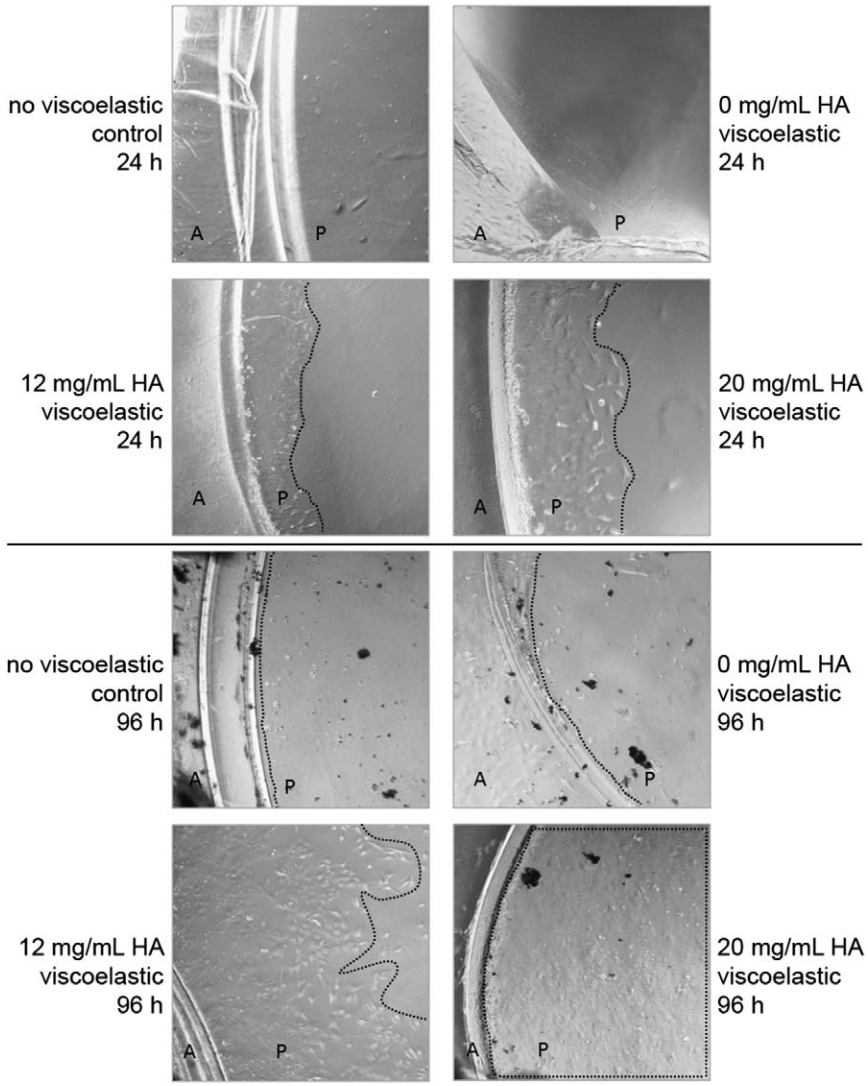

Figure 5. Ex vivo PCO formation after incubation with viscoelastic material. Using phase contract microscopy, LEC were located anteriorly (A) and equatorially in capsules treated with either no viscoelastic or 0 mg/mL HA viscoelastic; no cells were present on the posterior (P) capsule. As denoted by the dashed black line, LEC extended onto the posterior capsule and beyond the boundary of the capsulorhexis following treatment with either 12 mg/mL or 20 mg/mL HA viscoelastic. Following 96 hours of incubation with viscoelastic material, LEC were located anteriorly and equatorially in capsules treated with either no viscoelastic or 0 mg/mL HA viscoelastic; as denoted by the dashed black line very few cells were present on the posterior capsule. Capsules treated with 12 mg/mL HA viscoelastic had a marked increase in posteriorly located LEC. A large percentage of these posterior LEC were present beyond the boundary of the capsulorhexis. Capsules treated with 20 mg/mL HA viscoelastic had the greatest number of LEC located posteriorly; many of these capsules demonstrated complete cellular confluency on the posterior capsule.

following treatment with 0.5 mg/mL HA (Fig. 3B). In LEC treated with 1.0 mg/mL HA, CD44 expression was significantly ($P < 0.01$) higher than vehicle-treated cultures containing migrating LEC; RHAMM expression was slightly elevated but this increase was not found to be significant (Fig. 3B).

Scratched LEC cultures treated with various HA doses were evaluated over 16 hours, and the difference between initial-SA and final-SA was subsequently determined for each treatment group. There were no significant differences between any of the initial-SA measurement for all treatment groups (data not shown), indicating that the initial scratch was equal in size in all culture wells. Within each treatment group, when initial-SA was compared to final-SA, all cultures had a significant ($P < 0.001$) decrease in the area devoid of cells (data not shown). This indicated cellular migration occurred as expected in all treatment groups. When the percent difference between initial-SA and final-SA values were compared between treatment groups, a significant difference ($P < 0.001$) in the scratch area was noted; cultures receiving DMEM only had significantly larger scratch areas compared to cultures treated with 1.0 mg/mL of HA (Fig. 4A). As seen in Figure 4B, as the concentration of HA increased, the rate of cellular ingrowth into the defect increased; many of the cultures treated with a 1.0 mg/mL HA solution regained complete confluence by 16 hours (Fig. 4B).

Effect of CD44 Inhibition on HA Treatment

When primary cultures were pretreated with nonimmune IgG and cellular ingrowth into the scratched area was measured, as previously described, HA significantly ($P < 0.001$) increased the rate of cellular ingrowth compared to LEC that were not treated with HA (Fig. 4C). The role of CD44 in HA-induced cellular migration was confirmed as neutralization of CD44 resulted in significantly ($P < 0.001$) reduced LEC ingrowth

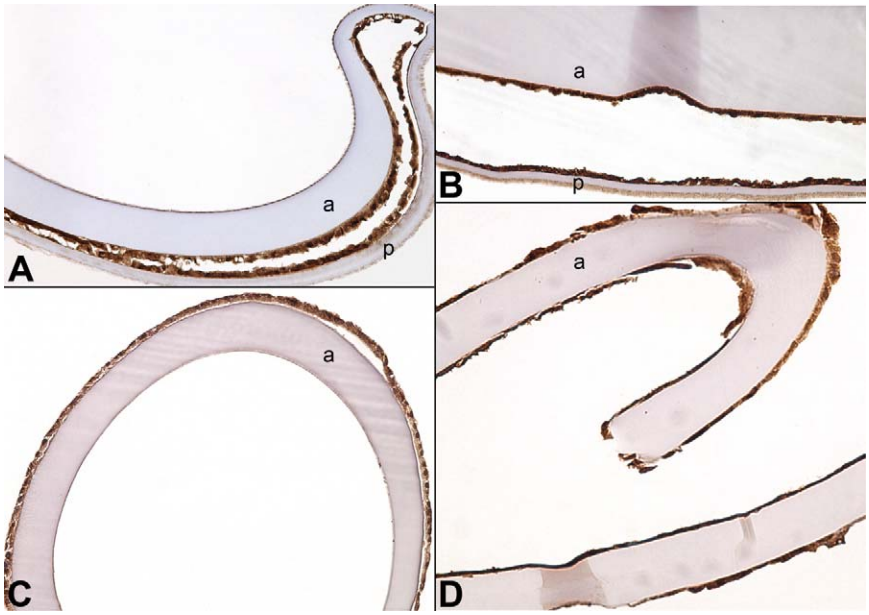

Figure 6. Expression of αSMA during ex vivo PCO formation. Following mock cataract surgery, canine lens capsules with residule LEC present on the anterior capsule were treated with various viscoelastic material for 96 hours. By the end of this period, LEC were present to various degrees on the anterior (a), equatorial, and posterior (p) capsule in samples that received (**A**) no viscoelastic; (**B**) 0 mg/mL HA viscoelastic; (**C**) 12 mg/mL HA viscoelastic; or (**D**) 20 mg/mL HA viscoelastic. All LEC were immunopositive for αSMA. This, in addition to previously published data,[26,30] provides evidence that this ex vivo model accurately models PCO formation.

following treatment with either 0.02 mg/mL HA or 1.0 mg/mL HA compared to controls (Fig. 4C6). There were no significant differences in the area devoid of cells when LEC were pretreated with either CD44 neutralizing antibodies or IgG in the absence of HA (Fig. 4C).

Effect of Surgical Viscoelastic on LECs<

Capsules were evaluated by phase contrast microscopy to determine the extent of LEC migration on the posterior lens capsule over time. Immediately following mock cataract surgery, LEC were located only on the anterior lens capsules in all treatment groups (data not shown). Within 24 hours, only lens capsules treated with either 12 mg/mL or 20 mg/mL HA viscoelastic had LEC present on the posterior capsule (Fig. 5). After 96 hours, capsules that were treated with PBS only predominantly had equatorial LEC, some of which extended onto the peripheral posterior capsule; however, no LEC were found beyond the boundary of the anterior capsulorhexis (Fig. 5). In capsules treated with HPMC viscoelastic (containing 0 mg/mL HA), LEC behaved similarly to the PBS treatment group; there were consistently no LEC present beyond the capsulorhexis after 96 hours of incubation (Fig. 5). Capsules treated with 12 mg/mL HA-containing viscoelastic contained numerous LEC extending past the region of the capsulorhexis. Lens epithelial cells in this treatment group extended into the central visual axis on the posterior capsule (Fig. 5). All capsules treated with viscoelastic containing 20 mg/mL HA demonstrated complete cellular confluency of the posterior capsule after 96 hours (Fig. 5). When LEC present in the treated lens capsules underwent immunohistochemistry for αSMA, all cells exhibited robust expression (Fig. 6). These data, in addition to previous reports,[26,30,31] provided evidence that lenticular migration does occur and that this ex vivo model accurately represents PCO formation.

Based on histologic sections, the total LEC counts for capsules treated 20 mg/mL HA viscoelastic were significantly higher ($P = 0.0109$) than those of the capsules treated with either PBS or 0 mg/mL HA viscoelastic (Fig. 7A). Although not statistically significant, there was a trend toward an increase in the total LEC number in capsules treated with the 12 mg/mL HA viscoelastic (Fig. 7A). Using inverted microscopy and image analysis, we calculated the rate of posterior capsule confluency (Fig. 7B). As the concentration of HA within the surgical viscoelastic increased, the rate of posterior capsule confluence increased. Capsules that received no viscoelastic material (control) or 0 mg/mL HA viscoelastic material, demonstrated similar rates of posterior capsule confluency (Fig. 7B).

Discussion

PCO is a universal complication that occurs in 20% to 60% of all surgical patients within 5 years postoperatively, depending on age, geographic location, and IOL type.[25,32–34] In pediatric patients, the varying incidence is greater and has been reported to be up to 100%, with recurrent PCO occurring in up to 57% of these patients.[23,35–38] PCO can typically be treated effectively with neodymium:yttrium-aluminum-garnet (Nd:YAG) laser capsulotomy. The cost of an Nd:YAG laser procedure can be substantial, and there can be significant morbidity due to postoperative complications that may include corneal endothelial damage, cystoid macular edema, retinal detachment, IOL damage or luxation, and exacerbation of localized endophthalmitis.[21,39,40] Unfortunately, PCO disproportionately affects the poor and uninsured and people living in underdeveloped nations. In an underdeveloped setting, cataract surgeries are often performed by surgeons who are undertrained in the latest surgical techniques and surgical IOLs and are overwhelmed by high patient numbers.[1,41,42] These differences cumulatively result in a high rate of PCO within 5 years postsurgically in underdeveloped areas.[1,43] Once PCO develops, low visual acuity persists and worsens due to the lack of availability of Nd:YAG laser surgery in these environ-

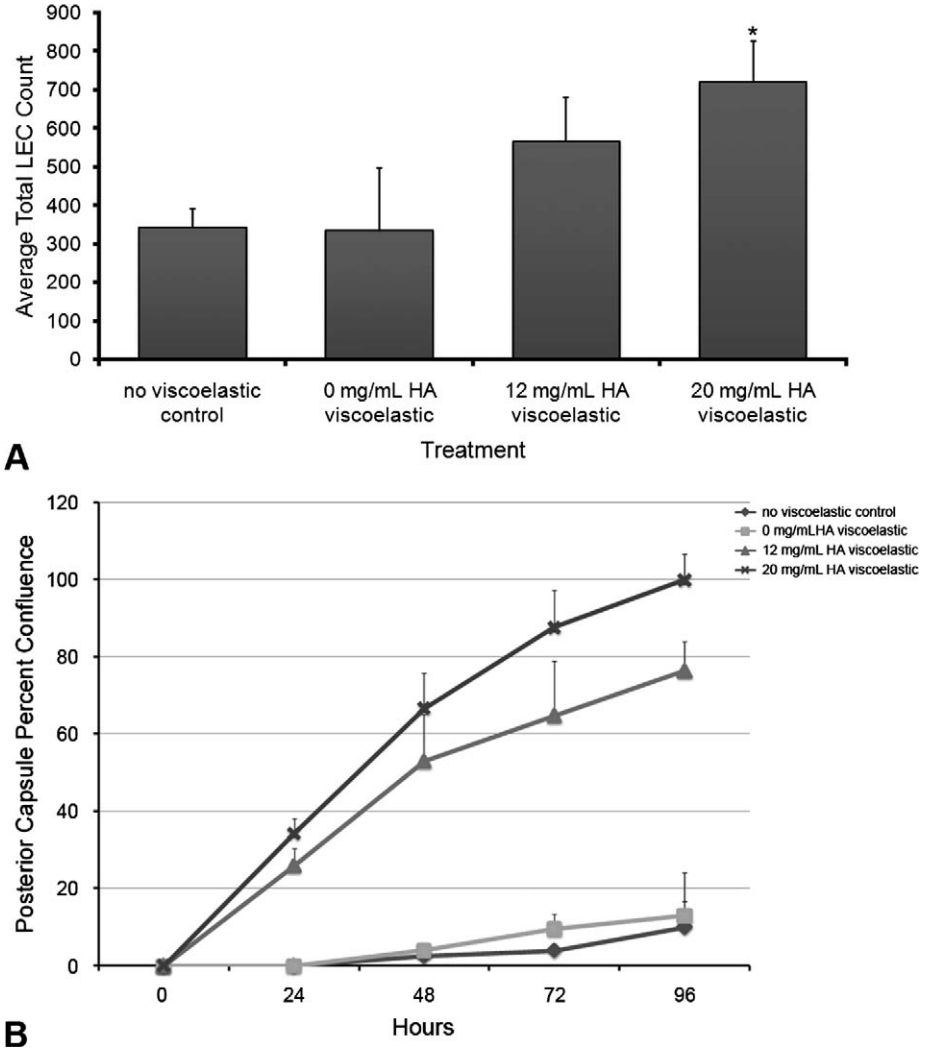

Figure 7. Number of LEC present and rate of posterior capsule confluency following treatment with viscoelastic material. (**A**) Capsules treated with no viscoelastic material or 0 mg/mL HA viscoelastic had similar numbers of LEC present. A significant ($P = 0.0109$) increase in LEC numbers was found in capsules treated with 20 mg/mL HA viscoelastic compared to controls. While not statistically significant, there was a trend of increasing LEC numbers in capsules treated with 12 mg/mL HA viscoelastic. (**B**) Percent confluency on the posterior capsule was calculated using inverted microscopy. Capsules treated with high concentrations on HA developed rapid confluence on the posterior capsule while samples treated with no viscoelastic or viscoelastic containing no HA had relatively few LEC present posteriorly. Error bars indicate SEM.

ments. Only 0.98% of PCO patients in underdeveloped areas have access to the Nd:YAG procedure.[1,44,45] It is clear that new surgical or medical treatment modalities are needed to decrease or prevent PCO.

There are fundamental ways in which HA can influence normal and abnormal cell behavior. The ability of HA to interact with water has a profound effect on the biomechanical properties of the extracellular and pericellular matrices in which cells reside. HA makes the surrounding cellular matrix conducive to the reorganization required for cell division, movement, and morphogenesis.[6] A separate function of HA is to interact with cell surface receptors that transduce intracellular signals and directly influence cellular form and behavior.[9,10,46–49] Loss of major hyaluronan synthases, which are responsible for cytoplasmic HA synthesis prior to extrusion into the pericellular matrix, is a lethal genetic mutation in mice as it results in a lack of migration during heart morphogenesis.[50,51] Further supporting the importance of HA in migration and cellular invasion, Zoltan-Jones, et al.[52] found that increased production of HA within epithelial cells was able to induce dispersion of cytokeratin, loss of adhesion proteins at intercellular boundaries, and upregulation of vimentin.

Expression of HA has been documented in adult lens fiber and epithelial cells, although the role this endogenous HA plays in lens homeostasis is still unknown.[18] The current study demonstrates that introduction of exogenous HA by means of surgical viscoelastics has the potential to influence lens behavior. Exposure to viscoelastic material with higher HA concentration induced LEC migration leading to increased rates of ex vivo PCO formation. The exposure time used in this study was acute (5 minutes) and is similar to the potential exposure time that LEC would have to surgical viscoelastics used during modern ECCE. This supports the hypothesis that many currently available surgical viscoelastics may impact PCO formation through HA signaling mechanisms. While the viscoelastic material used in this study is marketed for veterinary ophthalmology, the composition and nature of the materials are highly comparable to surgical viscoelastic used in human ophthalmology. The results of this study are not meant to discourage the use of HA-containing viscoelastic material;

rather, they illustrate the fact that the concentration of HA may be one factor for the surgeon to consider when selecting the appropriate viscoelastic agent. Additionally, these results suggest that surgeons should remove viscoelastic material from the anterior chamber and lens capsule when possible.

The hyaladherin RHAMM is capable of binding HA and appears to have both extracellular and intracellular functions.[10,53,54] While RHAMM is not essential for embryo viability, it has been found to play critical roles in several cellular events such as proliferation, migration, and tissue regeneration.[55–57] To date, expression of RHAMM has not been established within the crystalline lens. This study documents the fact that RHAMM protein and mRNA are expressed in canine lenses; however, no significant differences were found when normal lenses were compared to cataractous lenses. While others have found that RHAMM is an important mediator in cellular motility,[10,58,59] HA-promoted migration in LEC did not significantly alter RHAMM expression. These data suggests that RHAMM mediated signaling may not be the primary influence in lenticular migration and in vitro PCO formation.

Previously, CD44 expression has been detected in human samples from cataractous lenses and PCO material,[17] and function-blocking CD44 antibodies reduced in vitro migration of LEC.[16] A recent study by Desai et al.[18] characterized the distribution of CD44 in the murine lens during development. Here, we demonstrate that normal and cataractous canine lenses express CD44 mRNA and protein; a significant increase in CD44 was noted in clinical samples where LEC are undergoing migration compared to normal LEC. Similarly, others have found in mice that when LEC undergo migration following lens fiber removal, there was robust upregulation of CD44 protein.[18] This supports a role for CD44 in lenticular migration during cataract and PCO formation. Additionally, we observed a significant increase in CD44 expression in LEC undergoing migration following treatment with exogenous HA. Use of CD44 blocking antibodies was able to prevent the increased rate of cellular ingrowth following HA treatment; however, LEC were still able to migrate. This suggests that CD44 expression is not necessary for these events to occur in canine LEC. These results are in accordance with those obtained by Desai et al.[18], who found that CD44 expression is not essential for lenticular migration in the mouse. It is possible that in the absence of CD44, these functions are compensated for by alternate HA receptors. Recent work using CD44-deficient mice has demonstrated that loss of CD44 allows enhanced accumulation of the HA substrate, enabling augmented signaling through RHAMM.[60]

CD44 is involved in multiple signaling pathways known to play a role in cataract and PCO formation. For example, published data indicate that in pulmonary fibroblasts, CD44 is critical for migration and dependent on transforming growth factor-β (TGF-β) activation.[61] Additionally, in response to TGF-β, epicardial cells increase production of HA, resulting in increased migration and invasion in part through CD44 signaling.[62] While the exact molecular mechanism by which TGF-β induces lenticular migration is unclear, it is possible that CD44 plays a role.[24,27] Alternately, several studies have found that increased expression of CD44 or HA can stimulate several anti-apoptotic signaling events, including cyclooxygenase-2, Erk, and focal adhesion kinase activities.[52,63–67] Using various cell types, multiple studies have found that either CD44 or HA was capable of activating PI3K/Akt pathways, conferring resistance to apoptosis.[64,65] We have previously shown that phosphorylated Akt (pAkt) interacts with and phosphorylates telomerase in canine LEC that are undergoing migration; such an interaction did not occur in normal LEC.[26,30] While we have yet to investigate a potential interaction between CD44 and pAkt in the lens, it is possible that following cataract surgery, exogenous HA contained within surgical viscoelastic, protects remaining LEC from apoptosis. These events may be mediated by CD44 signaling.

Overall, this study demonstrates that both CD44 and RHAMM are expressed within the canine lens. We also demonstrate that CD44 expression increases during migration of LEC; treatment with HA further upregulates CD44. Use of surgical viscoelastics that contained HA resulted in increased rates of ex vivo PCO suggesting that judicious selection and use of viscoelastic material during cataract surgery is warranted. Regulation of gene expression and mRNA splicing for both HA receptors studied here, CD44 and RHAMM, is complex. This creates difficulty explicating true connections between lenticular migration and HA-mediated signaling through either CD44 or RHAMM. This study does help elucidate another step in understanding the process of migration within the lens. As such, it reveals future targets for intervention during such events and potential PCO prevention strategies.

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