Calpain May Contribute to Hereditary Cataract Formation in Sheep

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PURPOSE. To determine the involvement of calpain in ovine cataractogenesis by measuring calcium, calpain activity, proteolysis, and the effect of calpain inhibition.

METHODS. Sheep with genetic cataracts were examined for cataract severity. Calcium in normal and cataract lenses was measured. The presence of calpain was detected by casein zymography and immunoblotting. Calpain activity was assayed using BODIPY-casein as a substrate. Degradation of calpain substrates spectrin and vimentin was assessed by immunoblotting. The calpain inhibitor SJA6017 was applied to the left eye of cataract lambs, leaving the right eye as an untreated control. Both eyes were monitored by slit-lamp microscopy for cataract progression.

RESULTS. Cortical cataracts were first observed in lambs at 1 to 2 months of age. Lens calcium concentration increased in the early stages of cataract formation and was >10-fold higher in mature cataract than normal lenses. Three calpain isoforms were detected in young lamb lenses. Calpain activity decreased as cataracts progressed. Both spectrin and vimentin were degraded with cataract maturity, which could indicate calpain proteolysis. Cataract lambs treated with SJA6017 showed significantly smaller cataracts in the left treated eye over the right untreated eye.

CONCLUSIONS. The presence of calpains and calcium elevation during cataract formation suggests that proteolysis may play a role in opacification in ovine lens. This hypothesis is supported by the delay in opacification with SJA6017 treatment. The results also suggested that the ovine hereditary cataract is a useful nonrodent model to test the role of calpains in cataractogenesis. (Invest Ophthalmol Vis Sci. 2005;46: 4634–4640) DOI:10.1167/iosv.04-1291

Blindness caused by cataract formation is becoming increasingly more prevalent in the western world, mostly because of our increasing life expectancy. Although cataracts can be surgically removed, the operation is expensive and inconvenient. Cataracts may be prevented if the mechanisms of formation are known. Although the causes and the mechanisms of cataract formation are varied, 1 many researchers have implicated the involvement of the calpain proteolytic system in the pathology of cataractogenesis. 2–5 Cataracts have been studied in several animal models, including the guinea pig and the rabbit, 6,7 with rat and mouse being the most common models. 8–10 Both congenital and inducible cataract models exist in these species. At Lincoln University we have produced a cataract sheep flock as an alternative model for cataractogenesis. The ovine cataract, initially found in New Zealand Romney sheep, was first reported by Brooks et al. 11,12 These investigations showed evidence that the genetic defect was inherited as an autosomal dominant trait. The formation of ovine cataracts follows reproducible stages, therefore, these sheep provide a useful model to study cataract mechanisms, which may be relevant to human cataractogenesis. Experimental cataracts in rodents have been associated with a loss of calcium homeostasis, activation of the calcium dependent neutral proteases (calpains), and precipitation of partially degraded crystallins. 13 While calpains have also been demonstrated in human and bovine lenses, 14 evidence of their activation and participation in cataract formation in these species is lacking. This is because crystallins in human lenses become extremely heterogeneous with age, 15,16 which complicates detection of calpain-induced proteolysis in human cataract. Cortical cataracts are often associated with a rise in calcium levels. Although the genetic basis for the ovine cataract remains unknown, the cortical nature of the cataract may suggest a possible role for calpain in the opacification process. This article has several objectives: (1) determine whether total calcium concentrations in the ovine lens increase during cataract formation, (2) detect if calpains are present in the ovine lens, (3) examine if cataracts are associated with increased proteolysis, and (4) determine whether the calpain inhibitor SJA6017 slows the rate of cataract formation.

METHODS

Animals

For this study, all animal procedures were performed in accordance with Lincoln University Animal Ethics Protocol LU15/01, reviewed by the New Zealand Animal Ethics Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cataract Progression and Classification

To establish a cataract flock at Lincoln University, a cataractous Romney ram was obtained from Massey University 11 and was mated with normal-eyed, unrelated Coopworth ewes. To generate a time frame and a pattern of cataract formation in the Lincoln University Coopworth flock, the eyes of lambs born over three breeding seasons were carefully monitored. The number of progeny was recorded, together with information on how many lambs developed opacities and at what time after birth. The pattern of cataract formation and the time to maturity was noted.

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All sheep eyes were examined in a darkened room to determine cataract location and severity. Eye pupils were dilated with atropine (1% atropine sulfate; Sigma Pharmaceuticals Pty Ltd, South Croydon, Australia), and the lenses were inspected using a slit lamp microscope (Kowa Co. Ltd, Tokyo, Japan) and an ophthalmoscope (Vista Diagnostic Instruments, Kellar, UK). A description of each cataract, including the location of the opacity, and the degree of cortical involvement were recorded. Lenses were also photographed to provide a permanent visual record of each cataract stage (Fig. 1).

The cataracts typically exhibited an initial prominent suture line, which developed into a cortical opacity involving the anterior and posterior regions. A scoring system was devised at Lincoln University to follow the development of the ovine-inherited cataract. Cataract development was divided into discrete stages that could be used to aid the collection of samples for biochemical work and to follow cataract progression during treatment with inhibitors (Fig. 1). Early cataracts (stages 1 to 2) consisted of discrete anterior and posterior cortical opacities, usually centered at the lens suture lines. Mid-stage (stages 3 to 4) development cataracts formed spoke-like patterns radiating to the peripheral cortex and epithelial layer. By 10 months of age, most of the lambs had a mature cataract involving the whole lens and were effectively blind (stages 5 to 6). Assessment of affected breeding rams (4-years old) showed partial opacification from the lens cortex to the nucleus, leaving a fluid-filled lens remnant.

**Biochemistry.** For each experiment, the eyes of lambs were selected by cataract stage by using the above method. All animals were killed using captive bolt stunning and exsanguination. Whole-eye samples were collected immediately after slaughter, and the lenses were dissected into the globes by using a posterior approach. Globes were removed immediately after slaughter, and the lenses were killed using captive bolt stunning and exsanguination. Whole-eye selected by cataract stage by using the above method. All animals were deionized water acidified with HNO3. Data were analyzed by ANOVA, (1000 ppm; Spectrosol; BDH Lab Supplies, Poole, UK) and double digestion. The samples were mixed thoroughly before sonication for 45 minutes (Trasonic T460 sonic bath; Elma, Germany) and overnight digestion. The samples were analyzed without further dilution on an atomic absorption spectrophotometer (Avanta; GBC Scientific Equipment Ltd. [GBC], Victoria, Australia) using emission mode plus C2H2/N2O gas mixture. Standards were made from calcium nitrate AAS standard (1000 ppm; Spectrosol; BDH Lab Supplies, Poole, UK) and double deionized water acidified with HNO3. Data were analyzed by ANOVA, and then individual cataract stages were compared by the Student’s t-test.

**Plasma Glucose Concentration.** After an overnight fast, blood samples were collected by venipuncture from 12 one-year-old sheep with normal eyes and 16 one-year-old sheep that displayed mature cataracts. Plasma glucose concentration for each sample was determined by spectrophotometric analysis using the hexokinase method (Catalog No. 115-A; Sigma Diagnostics Inc, St Louis, MO).

**Casein Zymography.** Groups of lenses were collected immediately postmortem from lambs with normal lenses and lenses with cataracts at stages 1 to 2, 3 to 4, and 5 to 6. Lenses were dissected into anterior and posterior su-}

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**FIGURE 1.** Progression of the ovine inherited cataract. Each number depicts the stage of cataractogenesis used to describe cataracts for experiments. (1) Small opacity detected at either the anterior or posterior su-}

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Application of Calpain Inhibitor SJA6017

**SJA6017 Uptake into Sheep Lens.** Before initiating treatment of lambs with SJA6017, a single lamb was treated with SJA6017, and the treated eye was monitored closely for signs of irritation. The lamb (aged 2 months) was housed indoors for 8 hours. A single eyedrop containing 0.5% SJA6017 in a liposome preparation (3.5% egg yolk lethicin; 1.5% cholesterol; PBS, pH 7.0) was applied topically to the left eye only, hourly for 4 hours. Penetration of SJA6017 into ovine lens was measured by applying SJA6017 eyedrops to lambs in two dosing regimes. Six young lambs (6 to 8 weeks old) received a single eyedrop in the left eye every 15 minutes for 4 hours. A further six lambs of the same age were treated with a single eyedrop three times (3 hourly intervals) per day for 7 days. In both cases, the right untreated eye served as a control.

Fifteen minutes after the last eyedrop, lambs were killed and the eye globes were removed. Aqueous humor was collected by corneal puncture with an insulin syringe and was stored in 1.5 mL Eppendorf tubes, while lenses were dissected. Aqueous humor samples were homogenized with 1 mL distilled water and SJA6017 was extracted with 20 mL ethyl acetate by using a separating funnel. Whole lenses were dehydrated with an additional 10 mL ethyl acetate by using a separating funnel. Ethyl acetate extracts were dehydrated under vacuum for analysis.

All SJA6017 extracts were dissolved with 0.5-mL solution of water/acetonitrile/formic acid (50:50:0.5 volume ratio), then filtered through a filter of 0.45-μm pore size. Forty μL volumes of the filtrates were injected into an API-4000 LC/MS/MS system (Applied Biosystems, Foster City, CA) for detection of SJA6017. An SJA6017 recovery experiment was performed where control lens and aqueous humor samples were spiked with 26.5 ng and 13.25 ng SJA6017, respectively, before the extraction procedure above.

**Topical Application of SJA6017 Treatment to Sheep Eyes In Vivo.** Eighty-two lambs (2 to 3 months old), bred from two cataract sires and normal-eyed unrelated ewes, were housed in grass paddocks at Lincoln University. For four consecutive months, the lambs were treated three times daily to receive SJA6017 eyedrops in one eye only, hourly for 8 hours. A further six lambs of the same age were treated with a single eyedrop in the left eye hourly for 4 hours. A further six lambs of the same age were treated with a single eyedrop three times (3 hourly intervals) per day for 7 days. In both cases, the right untreated eye served as a control.

Eyes In Vivo. The left eyes of all lambs were treated with a single eyedrop containing 0.5% SJA6017, while the right eye served as an untreated control. The dose rate during the third month was raised to one eyedrop four times per day. No other intervention was imposed on the lambs other than tailing, weaning, and yarning for veterinary assessment of cataract score. Although less than half of the lambs formed cataracts, all 82 lambs were treated, because the lambs forming cataracts could not be predicted. The control group receiving no eyedrops consisted of an additional 41 lambs. All lambs were yatted monthly to assess cataract formation by using the technique described above. Cataract scores were recorded for both left and right eyes of each lamb. The Wilcoxon signed rank test was used to detect differ-
ences between the cataract progression of the left and right eyes of all sheep.

RESULTS

Heritable Ovine Cataract

Of 255 lambs born after matings of heterozygous cataract rams, 102 (40%) developed cataracts. Six of these had mature cataracts at birth, while most of the remainder had visible opacities at 6 weeks. Only nine lambs, whose eyes appeared normal at 6 weeks subsequently developed cataracts.

Mechanism of Cataract Formation

To assess if cataract formation was a result of diabetes, the plasma glucose levels of normal and cataract lambs were measured but were not significantly different. Plasma glucose of lambs with cataracts (stage 6) was 56 mg/dL compared with 51 mg/dL from lambs with normal eyes. However, analysis of normal and cataract lenses revealed that cataract formation was associated with a highly significant rise in total lens calcium concentration ($P < 0.001$). Total calcium content of the cataract lenses at stage 1, stage 3, and stage 6 were all significantly higher ($P < 0.05$) than in normal lamb lenses (Table 1). The rise in calcium concentration as the cataract progresses from stage 1 (early cataract) to a stage 6 (mature cataract) is also significant ($P < 0.05$).

Calpain Detection

With evidence of a rise in calcium, activation of the calcium-dependent proteases (calpains) was investigated as a possible mechanism of cataract development. The presence of calpain in ovine lens was first investigated by using zymograms and Western blotting. Three calpain isoforms were detected in zymograms of young lamb lens (7 weeks old) cortex and nucleus (Fig. 2a). Bands corresponding to the position of ubiquitous calpain I and II, and the lens-specific calpain, Lp82, were observed. The most prominent band in both cortex and nucleus was calpain II in its native and autolyzed forms. The identity of the three bands was confirmed by Western blotting of native electrophoresis gels using specific antibodies against the three calpain isoforms (Fig. 2b).

Detection of Calpain Substrates by SDS PAGE and Immunoblotting

A number of bands disappeared with cataract formation in both the soluble and insoluble lens fractions. This was associated with the appearance of other bands at lower molecular weight, indicating proteolysis. Changes between normal and cataract lenses were observed in the insoluble fraction where the 280 kDa band for αII-spectrin was completely degraded, as was the vimentin band at approximately 56 kDa (Fig. 3).

These results were confirmed by immunoblotting. For αII-spectrin, the 280 kDa intact band was missing in the cataract

![Figure 3](image_url)

**Figure 3.** SDS PAGE (12%) of normal and cataract (stage 5) soluble and insoluble ovine lens proteins. Bands appearing and disappearing in each fraction are indicated by arrows. Cytoskeletal proteins αII-spectrin and vimentin disappear from the insoluble protein fraction with cataract formation.

![Image](image_url)

**Figure 4.** Immunoblot for αII-spectrin in the insoluble protein fraction (15 μg/lane) of normal and cataract (stage 5) ovine lenses. Lanes 1 to 3 are normal, lanes 4 to 6 are cataract. The intact 280 kDa band is degraded in cataract lenses to two different products of 150 kDa and 145 kDa. The 145 kDa product is a specific calpain II proteolysis product for αII-spectrin.

![Image](image_url)

**Figure 4.** Immunoblot for αII-spectrin in the insoluble protein fraction (15 μg/lane) of normal and cataract (stage 5) ovine lenses. Lanes 1 to 3 are normal, lanes 4 to 6 are cataract. The intact 280 kDa band is degraded in cataract lenses to two different products of 150 kDa and 145 kDa. The 145 kDa product is a specific calpain II proteolysis product for αII-spectrin.
lens. A 150 kDa breakdown product was observed in both the normal and cataract lens, but this band had almost disappeared with cataract development. The cataract lens had a lower 145 kDa breakdown product, which is often attributable to calpain (Fig. 4). The intact band for vimentin decreased dramatically with cataract formation (Fig. 5).

**Calpain Activity**

Because calpain II was the major calpain isoenzyme present in the ovine lens, the activity was determined. The assay of calpain II, from chromatographically separated lens samples, using BODIPY-casein as a substrate, showed liberation of fluorescence from casein within the first 10 minutes of incubation. After 10 minutes the increase in the released fluorescence was no longer linear. The relative calpain II activities (units/g lens) of lamb lenses at different stages of cataract development were compared with lenses from age-matched control lambs. A significant decrease in the relative calpain II activity was associated with increasing cataract formation (Fig. 5).

Post-hoc analysis revealed that the calpain II activity decreased significantly between normal and mature cataracts (P < 0.05) (Fig. 6). Values are expressed as means ± SD.

**SJA6017 Uptake into Sheep Lens**

The uptake of SJA6017 into aqueous humor and lenses was measured by following two dosing regimes. In each case, the SJA6017 was applied to only one eye. There was a significantly higher concentration (P < 0.05) of SJA6017 in lenses dosed at 15-minute intervals for 4 hours (24.4 nM) than in lenses from lambs dosed three times a day for 7 days (6.6 nM). For both dosing regimes, the SJA6017 concentration was numerically higher in the aqueous humor than in the lens, although the difference was not significant. SJA6017 was detected at lower levels in the lens from untreated eyes (Fig. 7). The recovery of SJA6017 from spiked lens and aqueous humor samples was 14% and 8.9%, respectively.

The experimental design for testing the efficacy of the SJA6017 treatment involved instillation of the left eye only with SJA6017, using the right eye as a control. It was necessary to ensure in this evaluation that the normal cataract showed no bias between the left and right eyes. Cataract progression in 15 untreated lambs was observed over 4 months. Cataract progression in these lambs was bilaterally uniform. There were small left and right eye differences but no significant eye bias. Thirty-six of the 82 lambs treated with SJA6017 developed cataracts. The eyes of the treated lambs were scored each month, and the mean cataract stage for the left treated eye was compared with the right untreated eye (Fig. 8). After month 1 of SJA6017 application, the left eye of the cataract lambs had a significantly lower mean cataract score that the right eye (2.3 and 2.6, respectively, P < 0.01). This trend was observed in each of the following 3 months, although the response was not significant (P = 0.107, 0.057, and 0.134, respectively). For most of the cataract lambs that displayed a smaller cataract in the right eye compared with the treated left eye, the difference was only by one stage. In the third month of SJA6017 application, two lambs had cataracts, which had two cataract stages less in their left eye compared with the right eye. A breakdown of the cataract stage observed at each month of treatment for the treated and untreated eyes is shown in Figure 9. The cataracts progressed naturally in the untreated eye as the lambs were a month older at each observation point. In the final month there was a higher proportion of eyes with mature cataracts compared with the beginning of the trial.

**Discussion**

The lack of a natural large animal model of cataractogenesis has limited our ability to investigate the development of opacity. The ovine cataract is a cortical cataract, which is inherited as a dominant trait, appears at an early age and proceeds in a reproducible fashion over several months. This inherited ovine cataract provides a convenient model to research the biochemical changes in the lens as it becomes opaque, because a single lens is of sufficient size to carry out a whole range of biochemical and chemical tests.

Cortical cataracts in many species, including humans, have been associated with imbalances in ions and, in particular, calcium levels. Further work is required to determine whether the high calcium concentrations found in the ovine cataract are generalized or localized to a particular part of the lens. The ovine cataract has also displayed increased degradation of lens proteins, which is a characteristic feature of corti-
cal cataracts. The production of degraded proteins suggests there is a possible role for the calcium-dependent proteases or calpains in cataract development. Calpains have been found in the lenses from many species. The ovine lens appears similar to the lenses of other species in that calpain II is the dominant calpain isoform followed by lower levels of calpain I activity. The lens-specific calpain isoform, Lp82, has been reported in young rats, mice, and cattle. Calpain is involved in regulating the proteolysis of several proteins that are required for the normal development of a lens, including crystallins, connexins, and major intrinsic protein. The rise in calcium levels observed in cataractogenesis may lead to the overactivation of calpain and the degradation of lens proteins. For example, the profile of spectrin breakdown in the lenses with cataracts, particularly bands at 145 and 150 kDa, are indicative of calpain proteolysis compared with normal lenses, which have an abundant 120kDa breakdown typical of caspase 3 proteolytic activity. The decline in extractable calpain activity with progression of the cataract is an indication of its activation and subsequent autolysis. A similar, although more pronounced pattern is seen for calpain I in postmortem meat.

If calpain proteolysis is a cause of ovine cataract, then inhibition of calpain may prevent or retard any cataract progression. SJA6017, a cell-permeable peptide aldehyde, has been reported to be effective in preventing opacities in cultured rat and pig lenses, and in slowing induced cataracts in rats. In the present study, SJA6017 was applied as an eyedrop to the left eye of sheep over a 4-month period. The inhibitor appeared to slow the progression of cataractogenesis in the first month (Wilcoxon signed rank test, $P < 0.01$), but, after this period, both lenses progressed at similar rates (Fig. 9). This is consistent with in vitro studies in which SJA6017 was only partially effective in reducing cataract formation in lens culture systems. In vivo studies have shown the systemic uptake of SJA6017 concentrations (nM) postextraction from treated and control lenses and aqueous humor (AqH) of lambs. Values are expressed as means ± SD.

**Figure 7.** SJA6017 treatment of cataract ovine lenses. Cataract stage was evaluated on the 1 to 6 scale and the mean cataract stage calculated (stage 0, normal eyes, was not included). Mean cataract stage plotted for treated (left $\bullet$) and untreated (right $\bigcirc$) eyes during the 4-month treatment period. Lambs were approximately 7-weeks old at beginning of trial and cataracts progressed naturally as the lambs aged. The treated eye progressed at a slower rate compared with the untreated eye. Different italicized letters within month 1 indicates significant difference (Wilcoxon signed rank test, $P < 0.01$).

**Figure 8.** Frequency of cataract stage of SJA6017 treated ($L$) and untreated ($R$) eyes for all treatment animals that had cataracts (stage 0, normal eye, not included) during the 4-month trial period.
SJA6017 was initially able to slow cataract formation in selenite-induced cataracts in rats.\textsuperscript{37} The SJA6017 effect was temporary, and the cataracts progressed to the mature stage. The limited effectiveness of SJA6017 may be because of its low concentration in lens. The levels obtained in these reported experiments were <10 nM, and are well below the IC\textsubscript{50} of 80 nM.\textsuperscript{37} Similar results were obtained in the lens of SJA6017 peritoneally injected rats, where SJA6017 concentrations were measured at 30 nM.\textsuperscript{37} It is possible that any SJA6017 reaching the lens becomes localized in the lens epithelium and the cortex, where most of the calpain II activity resides in rat lens.\textsuperscript{16}

In conclusion, this study has characterized the calpains in ovine lens and provided evidence that calpains have a role in the inherited ovine cataract. Topical application of a calpain inhibitor has been shown to slow but not prevent cataractogenesis in sheep, which inherit cataracts. Further research is continuing to determine the suitability of this naturally inherited ovine cataract as a model for cataract formation in humans.

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