Loss of Calpastatin Leads to Activation of Calpain in Human Lens Epithelial Cells

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Purpose. Activation of calpains (calpain 2 and Lp82) in rodent lenses readily causes proteolysis and cataract formation. In contrast, primate lenses are quite resistant to activation of calpains. The hypothesis is that high levels of human endogenous calpain inhibitor, calpastatin (CS), prevent calpain activation in human lenses. The purpose of the present study was to directly test if CS is a major inhibitory factor in a human lens epithelial cell line, HLE B-3.

Methods. Small interfering RNAs (siRNAs) were used to knock down expression of CS in HLE B-3. The cells then were cultured with the calcium ionophore, ionomycin, with or without a calpain inhibitor SNJ-1945. Transcripts for calpain 2 and CS were measured by quantitative PCR (qPCR). Calpain 2 activity was detected by immunoblotting for the calpain-specific, α-spectrin breakdown product and for activation-associated, fragments of calpain 2.

Results. Expression of CS in HLE B-3 was remarkably higher than in α-TN4 (mouse comparator cell line). Proteolysis of α-spectrin was observed in the soluble proteins from α-TN4 incubated with Ca^{2+}, but not in the human HLE B-3. When CS-reduced HLE B-3 cells (transfected with CS siRNA) were cultured with ionomycin, calpain 2 was activated, specific proteolysis of α-spectrin occurred, and cell death ensued; SNJ-1945 inhibited these changes.

Conclusions. Our data demonstrated that the high levels of endogenous CS do, indeed, inhibit calpain activity in normal human lens epithelial cells. We speculate that age-related oxidation might cause loss of CS activity in human lens epithelial cells, allowing activation of long-dormant calpain 2, proteolysis of critical cytoskeletal proteins, and cataract formation.

Keywords: calpain, calpastatin, human lens epithelial cells, SNJ-1945, proteolysis

Calpains are a family of 14 nonlysosomal, cysteine proteases, activated by calcium.[1] They are comprised of ubiquitous calpains, such as calpain 1 (μ-calpain), calpain 2 (m-calpain), and calpain 10. Tissue-preferred calpains also exist; such as lens-specific (nonhuman) Lp82 and Lp85, which are splice variants of muscle calpain 3 (p94). Atypical and possibly calcium-independent calpains are part of this widespread protease family.

In rodent lenses, calpain 2 and Lp82 are the major calpains,[2] and they are known to cause proteolysis that causes subsequent lens opacities. The anatomical localization of calpain 2 and Lp82 is different. Calpain 2 is dominant in rodent lens epithelium, while Lp82 shows the highest activity in the lens nucleus and fibers.[3,4] The lens epithelium is particularly important because it is the origin of all the inner differentiated lens fibers. The inner lens fibers lose all organelles during the differentiation, so major metabolic processes occur in the epithelium. The lens epithelium also regulates intracellular ion concentrations and hydration by Na^+-, K^+-, and Ca^{2+}-adenosine triphosphatases (ATPases).

In contrast to rodents, to our knowledge no typical calpain cleavage sites have been detected in crystallins from aged human lens, although calcium levels are elevated in many human cataracts.[5] Further, monkey lenses are quite resistant to activation of calpain.[6] One reasonable explanation for marked difference in calpain activation between primate and rodent lenses is the lack of lens-specific Lp82 and Lp85, which are not expressed in human and primate lenses due to a nonsense mutation that produces a stop codon.

Another reason for the apparent lack of calpain activity in primate lenses is the presumably high level of calpastatin (CS) in primate lenses. The activity of calpains 1 and 2 is tightly regulated by this specific endogenous inhibitor.[7] Calpastatin is an intrinsically unstructured protein that can reversibly bind and inhibit up to four molecules of calpain in the presence of calcium. We hypothesize that initial damage to human lens epithelium, such as from age-related ultraviolet (UV) oxidation, may lead to loss of CS inhibitory function, activation of calpain 2, proteolysis, and cataract.[8,9] We tested this in the experiments below by determining if CS knockdown allows calpain-induced proteolysis to occur in human lens epithelial cells.

Materials and Methods

Cell Culture

Immortalized human lens epithelial cells (HLE B-3)[10] were purchased from American Type Culture Collection (ATCC,

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Catalog No. CRL-11421; Manassas, VA, USA) and maintained in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and antibiotics (Kurabo, Osaka, Japan) at 37°C under 5% CO_2. Note that the basal level of calcium in Dulbecco's modified Eagle's medium (DMEM) medium is 1.8 mM Ca^{2+} pertinent to the present experiments (Life Technologies).

Immortalized mouse lens epithelial cells (α-TN4)[11] were derived originally from a transgenic animal expressing the SV40 large-T antigen, driven by the promoter for the αA-crystallin gene.[12] The α-TN4 cells also were cultured in the medium described above.

Preparation Soluble Proteins and In Vitro Proteolysis

The HLE B-3 and α-TN4 cells were homogenized in buffer containing 50 mM Tris (pH7.5), 1 mM EGTA, and 3 mM dithiothreitol (DTT), and the supernatant was collected by centrifugation at 13,000*g* for 20 minutes at 4°C. Protein concentrations were measured with the BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA), using BSA as standard. All steps were performed at 4°C. Immunoblotting confirmed that most of the α-spectrin was present in the soluble fraction.

To activate endogenous calpains, soluble proteins (1 μg/μL) were incubated with additional 2.5 mM calcium for 90 minutes at 37°C. The reaction was stopped by the addition of excess EDTA to 5 mM final concentration. To inhibit calpains, 100 μM SNJ-1945[13,14] was added just before addition of calcium. The SNJ-1945 is a membrane permeable calpain inhibitor, and, unlike previous peptidyl inhibitors,[15] SNJ-1945 is less prone to react nonspecifically with various biological proteins. The incubation mixtures then were subjected to immunoblotting to detect proteolysis of α-spectrin and activation of calpain 2. The experiments were performed in duplicate.

Immunoblotting for α-Spectrin, Calpain, and CS

We performed SDS-PAGE of soluble proteins on 4% to 12% NuPAGE Bis-Tris gels with MOPS (for CS and calpain 2) or MES (for α-spectrin) buffers (Life Technologies). Immunoblotting was performed by electro-transferring proteins from NuPAGE gels onto polyvinylidine fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) with TG buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing 20% ethanol. After blocking with 0.5% skim milk in Tris-buffered saline (Bio-Rad Laboratories) containing 0.05% Tween 20, the PVDF membranes were incubated overnight at 4°C with primary antibodies diluted at 1:500 for α-spectrin (Enzo Life Sciences, Plymouth Meeting, PA, USA), CS (Cell Signaling, Danvers, MA, USA), or calpain 2 (Sigma-Aldrich Corp., St. Louis, MO, USA). Immunoreactivity was visualized with horseradish peroxidase (HRP)–conjugated to secondary antibody and ECL Plus detection reagents (GE Heath Care, Little Chalfont, Buckinghamshire, UK). Images of membranes were digitized on an image analyzer with a CCD camera (ImageQuant LAS4000; GE Healthcare), and the intensity of the bands was quantified by use of ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA; available in the public domain at http://imagej.nih.gov/ij/).

RNA Extraction and Reverse Transcription

The cells were dissolved with Trizol reagent (Life Technologies), and total RNA was extracted with 200 μL of chloroform per mL Trizol with vigorous shaking for 1 minute. The aqueous layer was mixed well with 0.5 mL of 2-propanol per mL Trizol, and the total RNA was precipitated by centrifugation at 20,800*g* for 10 minutes at 4°C. After washing with 75% ethanol, total RNA was dissolved in water containing SUPERase-In (Life Technologies) to inhibit degradation by RNase and then treated with DNase using DNA-*free* (Life Technologies).

Total RNA samples (1 μg) were treated with SuperScript II RNase H^- (Life Technologies). The reverse transcription reaction, consisting of 1 × first strand buffer, 10 mM DTT, 500 μM each dNTP, 2.5 μM random decamers, and 200U Superscript II enzyme (Life Technologies), was incubated at 42°C for 50 minutes and then at 70°C for 15 minutes.

Quantitative PCR (qPCR) for Calpain 2 and CS

Quantification of gene transcripts in lens epithelium was determined by qPCR using standard dilutions of PCR products. Primers and probes were developed by the TaqMan Gene Expression Assays (Human calpain 2, Hs00156251_m1; Human CS, Hs00156280_m1; Mouse calpain 2, Mm00486669_m1; Mouse CS, Mm_01345276_mH; Life Technologies). The standard PCR products harboring the sequence for qPCR were amplified by the following primers: human calpain 2, forward 5′-AGT CGC CCC GAC CTT TCT-3′ and reverse 5′-ATA GTT TTC CTG GAA GCT CTG GT-3′; human CS, forward 5′-GGT GGA GAA GGA TAC AAT GAG TG-3′ and reverse 5′-AGC TTT TCA CGG TCT TCT TCT TT-3′; mouse calpain 2, forward 5′-TGG GGT CGC ATG AGA GAG-3′ and reverse 5′-ACT CCA GAA CTC ACT CCC TTC AG-3′; and mouse CS, forward 5′-ATT GAT GCC CTC TCA GAA GAT TT-3′ and reverse 5′-ACA GGT GAA GCA GAC AAA TTC AT-3′. The amplified standard PCR products were purified with the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands). The PCR reaction was run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute following an initial activation at 95°C for 10 minutes (7300 and 7500; Applied Biosystems, Foster City, CA, USA). Fluorescence measurements were taken every cycle following extension.

Transfection of Small Interfering RNAs (siRNAs) Into Human Lens Epithelial Cells and Treatment With Ionomycin

The siRNAs were obtained from Life Technologies (CS siRNA ID No. s2398, ID No. s2399, ID No. s2400, and negative control siRNA # 5). The HLE B-3 cells were plated at 1.5 or 3.4×10^5 cells on collagen type IV (5 μg/cm^2)–coated, 3.5 or 6 cm dishes, respectively, and cultured in DMEM (Life Technologies) supplemented with 10% FBS (Life Technologies) and antibiotics (Kurabo) at 37°C under 5% CO_2. When cell growth was sub confluent, the cells were rinsed with fresh DMEM medium with 10% FBS, and then the siRNA duplexes at 10 nM final concentration were transfected into cells using Lipofectamin RNAiMax (Life Technologies) in OptiMEM I (Life Technologies), according to the suggested protocol. After 3 days of culture, the medium was replaced with DMEM medium with 1% FBS and the cells were incubated for 24 minutes with 4 μM calcium ionophore, ionomycin (Merck Millipore, Darmstadt, Germany), to promote calcium influx. When tested, SNJ-1945 was added 1 hour before treatment with ionomycin.

After incubation with ionomycin, HLE B-3 cells were detached with a cell scraper and collected by centrifugation at 400*g* for 5 minutes. The collected cells were immersed in RIPA buffer (Pierce Biotechnology, Rockford, IL, USA) containing protease inhibitors (Complete Mini-EDTA free; Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitor (PhosSTOP Phosphatase Inhibitor Cocktail; Roche Diagnostics), and the samples were subject to immunoblotting. The experiments were performed in triplicate.

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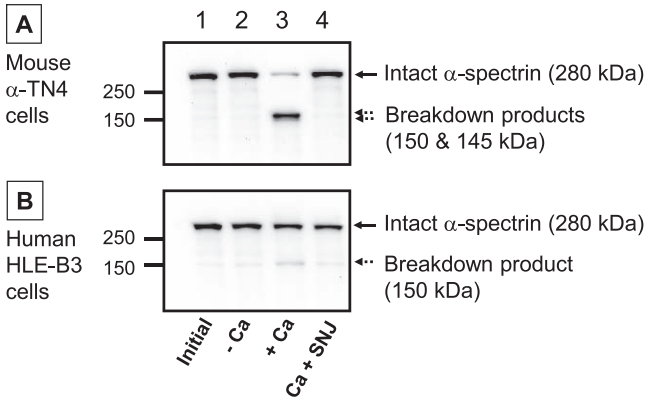

FIGURE 1. Immunoblots for α-spectrin and breakdown products in the soluble proteins from α-TN4 cells (**A**) and HLE B-3 cells (**B**) after incubation in calcium. *Lane 1*: initial t_0. *Lane 2*: minus calcium. *Lane 3*: plus 2.5 mM calcium. *Lane 4*: calcium plus 100 μM SNJ-1945. *Solid arrows* indicate the intact α-spectrin band at 280 kDa, and *dotted arrows* indicate 150 and 145 kDa breakdown products.

Measurement of Lactate Dehydrogenase (LDH) Activity

Membrane breakage and cell death were quantified using release of LDH into the culture medium.[16] Cultured cells were collected and homogenized by addition of medium containing 1% Triton X-100. The LDH activities in the supernatant of cell homogenates and in the culture medium were measured with the LDH Cytotoxicity Detection Kit (Takara Bio, Shiga, Japan). Percent leakage of LDH was calculated as (LDH activity in medium/[LDH activity in medium + LDH activity in cells]) × 100. Statistical analysis of data was performed by Dunnett's test.

RESULTS

Comparison of Calpain Activation in Mouse and Human Lens Epithelial Cells

After incubating the soluble proteins from mouse-derived α-TN4 cells with calcium, the intact band of endogenous α-spectrin at 280 kDa (Fig. 1A, lane 1) was hydrolyzed to breakdown products at 150 and 145 kDa (lane 3). This degradation of α-spectrin was completely inhibited by a calpain inhibitor, SNJ-1945 (lane 4). This demonstrated that the endogenous calpains in α-TN4 cells are readily activated by calcium.

Human-derived HLE B-3 cells also contained large amounts of intact 280 kDa α-spectrin in their soluble proteins (Fig. 1B, lane 1). The α-spectrin was an obvious substrate for calpains (above). However, in contrast to mouse, incubation of human HLE B-3 soluble proteins with calcium produced only very limited amounts of 150 and 145 kDa spectrin breakdown products (Fig. 1B, lane 3). Thus, human lens epithelial cells in vitro showed a marked resistance to calpain activation.

The expression of mRNA transcripts for calpain 2 was similar in HLE B-3 and α-TN4 cells (Fig. 2, first and third bars). Note, however, that the expression level of mRNA for the endogenous inhibitor CS was significantly higher in human lens epithelial cells compared to mouse α-TN4 cells (second and fourth bars). This suggested that the higher ratio of CS to calpain could be a major inhibitor of calpain-induced proteolysis in human lens epithelial cells.

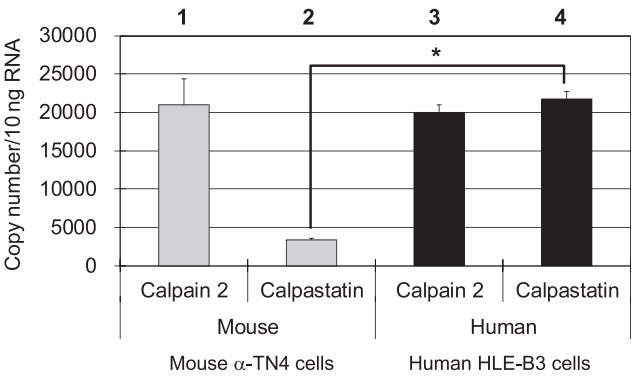

FIGURE 2. Transcript copy numbers for calpain 2 and CS in α-TN4 and HLE B-3 cells. Data are means ± SD ($n = 3$). *$P < 0.05$ with Student's *t*-test.

Calpain-Induced Proteolysis in CS Knockdown-HLE B-3 Cells: Effect of Ca^{2+} Added to Cell Lysates

We knocked down CS transcripts in HLE B-3 cells by transfecting separately with 3 different siRNAs against CS (Fig. 3A, lanes 2–4). After 72 hours, protein levels for CS also were depressed in the siRNA-treated cells (Fig. 3B, lanes 2–4). Note that none of these siRNAs affected calpain 2 protein expression (Fig. 3C, lanes 2–4). Thus, the siRNAs were specific for CS, and they did not affect expression of calpain 2 protein in our transfected HLE B-3 cells.

In nontransfected HLE B-3 cells (Fig. 4A, lane 3), or in cells transfected with nonsense control siRNA (Fig. 4B, lane 3), incubation with calcium did not increase proteolysis of α-spectrin. However, in CS siRNA-transfected HLE B-3 cells, addition of calcium to the cell lysate led to proteolysis of α-spectrin (Fig. 4C, lane 3). The proteolysis of α-spectrin was inhibited by SNJ-1945 (Fig. 4C, lane 4). These knockdown data demonstrated that even addition of large amounts of calcium to the cell lysate were not able to activate calpains because of the overwhelming amounts of endogenous CS in human lens epithelial cells.

Calpain-Induced Proteolysis in CS Knockdown-HLE B-3 Cells: Effect of Ionomycin Added to the Culture Medium to Elevate Cytoplasmic Ca^{2+}

Since calpains are intracellular enzymes,[7] the lack of effect of calcium added to the cell lysate in the nontransfected cells above might have been due to failure to sufficiently elevate intracellular calcium. Therefore, we repeated the experiments, but we used the calcium ionophore, ionomycin, instead of external calcium. Ionomycin elevates cytoplasmic calcium by promoting Ca^{2+} entry through the plasma membrane[17] and from the internal organelle stores.[18] Note that the basal level of calcium in DMEM/FBS medium was approximately 1.8 mM Ca^{2+}. Thus, although no external calcium was added, the medium-to-cytoplasmic Ca^{2+} ratio was >1000:1 at the beginning of the experiment.

When CS siRNA-transfected HLE B-3 cells were exposed to ionomycin, intact α-spectrin was markedly proteolyzed to the145 kDa breakdown product (Fig. 5A, lane 2). Ionomycin-treated cells plus 1 μM SNJ-1945 showed an additional breakdown product at 150 kDa along with the145 kDa product (lane 3). Since the α-spectrin breakdown products are produced sequentially (280 → 150 → 145 kDa → undetected fragments),[19] the data suggested that calpains were not fully inhibited by 1 μM SNJ-1945. Indeed, 10 μM SNJ-

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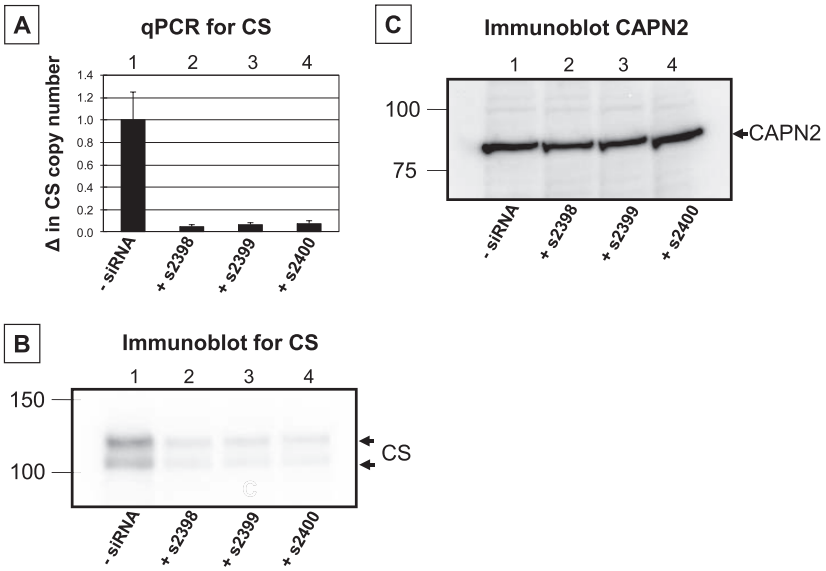

Figure 3. Depression of CS mRNA and protein in HLE B-3 cells after transfection with CS siRNAs. Three different CS siRNAs each reduced expression of CS mRNA (**A**) and protein (**B**). Data are means ± SD ($n = 3$). Calpain 2 protein levels were not changed (**C**). See Methods for description of siRNA numbering.

1945 inhibited proteolysis of almost all the α-spectrin at 280 kDa and allowed only minor production of the 145 kDa breakdown product (lane 4). A total of 100 μM SNJ-1945 caused nearly complete inhibition of α-spectrin breakdown (lane 5). Quantitative analysis confirmed loss of intact α-spectrin and significant inhibition by SNJ-1945 (graph insert). No proteolysis of α-spectrin was induced in HLE B-3 cells transfected with control siRNA (data not shown), indicating that the overwhelming amounts of endogenous CS caused no calpain activity against α-spectrin.

The presence of degraded calpains is interpreted as indirect evidence for calpain activation because activation is followed by auto degradation of calpain.[20] Indeed, this was observed in the CS knockdown-HLE B-3 cells treated with ionophore (Fig. 5B, lane 2). The intact 80 kDa calpain 2 band was decreased and degraded to a fragment at 45 kDa. This activation of calpain 2 was inhibited almost completely by 10 or 100 μM SNJ-1945 (Fig. 5B, lanes 4 and 5). Along with calpain activation, ionomycin caused a concomitant leakage of LDH into the medium (Fig. 6, bar 2). Ten or 100 μM SNJ-1945 significantly inhibited LDH leakage (Fig. 6; bars 4, 5). These data suggested that calpain-induced proteolysis, cell leakage and death can, in fact, occur in human lens epithelial cells under conditions where cytoplasmic calcium is increased along with depressed CS levels.

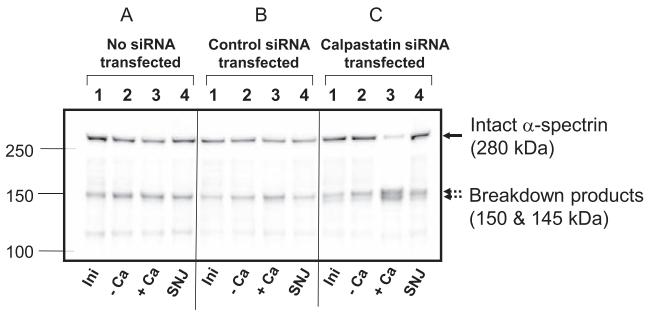

Figure 4. Immunoblot for α-spectrin in the soluble proteins from HLE B-3 cells transfected with CS siRNA and incubated with calcium. Multiple lane designations: 1s, initial t_0; 2s, minus calcium; 3s, plus 2.5 mM Ca^{2+} added to the cell lysate; 4s, calcium plus 100 μM SNJ-1945. *Solid arrows* indicate the intact α-spectrin band at 280 kDa, and *dotted arrows* indicate 150 and 145 kDa breakdown products. Cells in (**C**) were transfected with siRNA ID No. s2398 shown in Figure 3.

Discussion

The present data showed that the reason why human lens epithelial cells are resistant to calcium/calpain-induced proteolysis is because of the high ratio of CS to calpain. This conclusion was supported by the following observations: Expression of CS in human HLE B-3 cells was much higher than mouse α-TN4 cells (Fig. 2). Calpain-specific proteolysis of α-spectrin was observed in soluble proteins from human HLE B-3 cells only when CS mRNA and protein were knocked down (Fig. 4). When the CS knockdown-HLE B-3 were cultured with ionomycin, calpain 2 was activated, calpain-specific proteolysis of α-spectrin was induced, and cell death ensued (Figs. 5, 6). The calpain-preferred inhibitor SNJ-1945 reduced calpain-induced proteolysis in the CS knockdown-HLE B-3 cells.

These data are important in the search for molecular mechanisms for cataractogenesis because they help explain the disparity in the ease of producing calpain-induced proteolysis in rodents[21] and the presence of calpain in human lenses,[22] but the lack of calpain cleavage sites on proteins from human lenses.[6]

We now must identify those patients in which environmental factors or human conditions reduce CS activity in their lens epithelium. Numerous factors have been shown to reduce CS in other tissues: Animal experiments support that aging could be such a factor. For example, the klotho knockout mouse shows multiple phenotypes resembling human aging.[23] The deficiency in the klotho protein caused degradation of CS leading to over activation of calpain 1.[24] Age-related fluctuations for the CS gene expression were found in chicken breast muscle and liver, with highest at 2 weeks and lowest at 8 weeks.[25] Hypertension increases in human aging,[26] and the Milan strain of hypertensive rats show a significant decrease in CS activity.[27] PMA-induced phosphorylation of cytoplasmic CS causes redistribution to aggregated CS, which may allow

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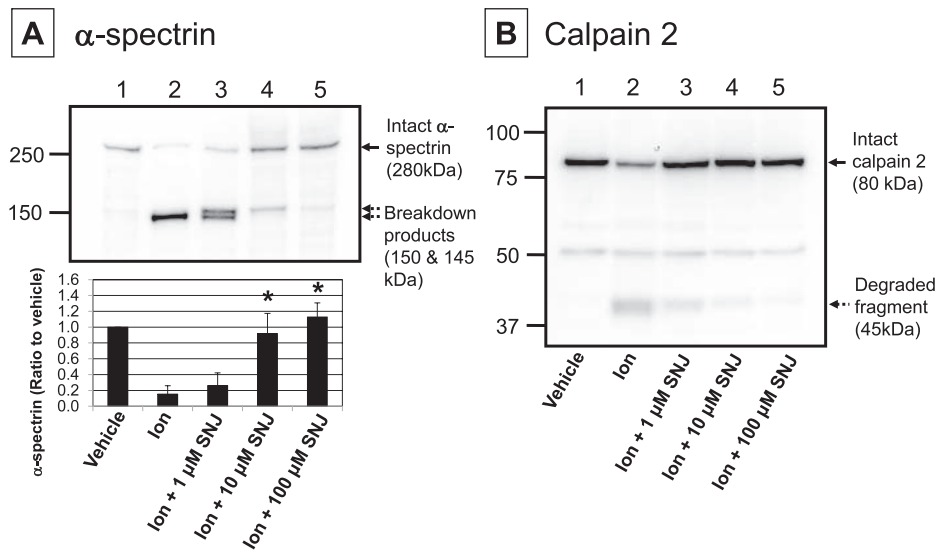

Figure 5. Immunoblots for α-spectrin (**A**) and calpain 2 (**B**) in HLE B-3 cells transfected with CS siRNA and incubated with ionomycin. *Lane 1*: vehicle (DMSO). *Lane 2*: 4 μM ionomycin (ION). *Lane 3*: ionomycin plus 1 μM SNJ-1945. *Lane 4*: ionomycin plus 10 μM SNJ-1945. *Lane 5*: ionomycin plus 100 μM SNJ-1945. Quantitative measurements of the intact α-spectrin band at 280 kDa (inserted graph). Data are means ± SD ($n = 3$). *$P < 0.05$ relative to Ion (Dunnett's test).

calpain to escape CS inhibition.[28] This implicates cytokines in the regulation of CS. Primary human meningioma cells cultured with hydrogen peroxide showed reduction in CS and calpain-dependent proteolysis. Proteolysis was blocked by calpain inhibitor Z-LLal.[29] Thus, oxidation, an important risk factor for cataract formation has been linked to loss of CS. These data supported our hypothesis that aging and continual exposure to ambient UV-induced oxidation might causes loss of CS activity in human lens epithelial cells; allowing activation of long-dormant calpains, proteolysis of critical cytoskeletal proteins, and cataract formation. The CS activity in the soluble proteins was reported to be similar in a 1.5-month-old and 50-year-old donors.[22] However, these samples were prepared from decapsulated human lenses without epithelium. Thus, a mass spectrometry-based search for changes in CS levels in aging human epithelium could be conducted to test this hypothesis.

A limitation of the present studies was the use of the HLE B-3 cell line that had been immortalized with the adenovirus 12-SV40 virus.[10] These cells theoretically could have reacted differently than primary cells to siRNAs and calpain activation.

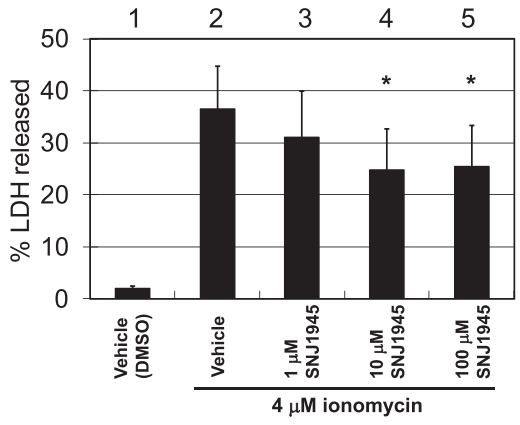

Figure 6. Leakage of LDH from HLE B-3 cells transfected with CS siRNA and incubated with ionomycin. Data are means ± SD ($n = 6$). *$P < 0.05$ relative to ionomycin in vehicle (Dunnett's test). % LDH released = LDH in medium/(LDH in medium + LDH in cells).

However, the cell line is well characterized and shows stable epithelial morphology, continues to produce lens β-crystallin, a protein characteristic of lens cell differentiation in vivo, and shows tight cell-to-cell relationships, conditions that may be similar to that found in vivo. The HLE B-3 cells also are useful as an alternative to the more difficult to culture primary human lens epithelial cells because HLE B-3 cells showed tight regulation of calpain activity by CS (Figs. 1, 2), which is similar to primate lens.[6] The HLE B-3 cells also express transcripts for vimentin and αB-crystallin, which are substrates for calpain.[30,31] Although this cell line has been used by investigators for a number of years,[32] the experiments above will need confirmation in primary human lens epithelial cell culture.

Acknowledgments

Disclosure: **T. Nakajima**, Senju Pharmaceutical Co., Ltd. (E); **T.R. Shearer**, Senju Pharmaceutical Co., Ltd. (C); **M. Azuma**, Senju Pharmaceutical Co., Ltd. (E)

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