Role of Cys^{41} in the N-terminal domain of lumican in *ex vivo* collagen fibrillogenesis by cultured corneal stromal cells

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The keratan sulphate proteoglycan lumican regulates collagen fibrillogenesis to maintain the integrity and function of connective tissues such as cornea. We examined the role of a highly conserved cysteine-containing domain proximal to the N-terminus of lumican in collagen fibrillogenesis using site-specific mutagenesis to prepare plasmid DNA encoding wild-type murine lumican (Cys^{37}-Xaa$_3$-Cys^{41}-Xaa-Cys-Xaa$_9$-Cys) and a Cys → Ser (C/S) mutant (Cys^{37}-Xaa$_3$-Ser^{41}-Xaa-Cys-Xaa$_9$-Cys). cDNAs were cloned into the pSecTag2A vector, and cultures of MK/T-1 cells (an immortalized cell line from mouse keratocytes) were transfected with the cDNAs. Stable transformants were selected and cloned in the presence of Zeocin. All stable transformants maintained a dendritic morphology and growth rate similar to those of parental MK/T-1 cells. Western blot analysis with anti-lumican antibody detected a 42 kDa lumican protein secreted into the culture medium of both wild-type and C/S mutant lumican cell lines. Ultrastructural analyses by transmission electron microscopy showed both cell lines to form a multi-layered stroma *ex vivo*, but the matrix assembled by the two cell lines differed. Compared with the mutant cell line, the wild-type cells assembled a more organized matrix with regions containing orthogonal collagen fibrils. In addition, the fibrils in the extracellular matrix formed by the mutant cell line exhibited alterations in fibril packing and structure. Immunostaining analysed by confocal microscopy showed a further difference in this matrix, with the marked occurrence of lumican and collagen I co-localization in the lumican wild-type cells, but a lack thereof in the lumican C/S mutant cells. The results indicate that the cysteine-rich domain of lumican is important in collagen fibrillogenesis and stromal matrix assembly.

Key words: cornea, fibril, keratan sulphate proteoglycan.

INTRODUCTION

Corneal strength and transparency depend upon the development and maintenance of a well organized stromal extracellular matrix (ECM) characterized by a uniformly small collagen fibril diameter and a uniform fibril density facilitated by critical stromal hydration. In corneal stroma, collagen is organized further into lamellae, with the orientation of fibrils in adjacent layers approximately perpendicular to one another [1,2]. The mechanisms that govern the assembly of these complex levels of stromal architecture are not well understood. However, proteoglycan–collagen and collagen–collagen interactions have been implicated. It has been suggested that the stoichiometry and interactions of different collagen types play important roles in modulating collagen fibril diameter [1,3–5]. The mature corneal stroma contains small uniform heterotypic collagen fibrils ($\sim$ 25 nm in diameter) consisting of type I and type V collagen [1,6]. The high percentage of type V collagen relative to that of type I [7] has been suggested to be important in determining fibril diameter [6,8]. However, a percentage of type V collagen comparable with that found in the mature corneal stroma resulted in fibrils with larger diameters than those seen *in situ* [6], raising the possibility that other factors may be important in the regulation of corneal fibril diameter.

The small leucine-rich proteoglycans (SLRPs) are thought to regulate collagenous matrix assembly in connective tissues [1,2,9–13]. The major proteoglycans in the corneal stroma, i.e. lumican, keratocan, mimecan and decorin, are members of this SLRP family. Lumican and keratocan contain keratan sulphate glycosaminoglycan chains which are uniquely abundant in the cornea. These highly charged proteoglycans contribute to the hydrophilic properties of the stroma [14–19] and are considered to be important in fibrillogenesis. The role of SLRPs in regulating collagen fibrillogenesis is demonstrated in decorin- and lumican-null mice. The ablation of either gene in mice via gene targeting results in pathology due to alterations in collagen fibril diameter as well as collagen fibril density. Both knockout mice exhibit fragile skin, but the decorin$^{-/-}$ mice have transparent corneas, whereas the lumican$^{-/-}$ mice have cloudy corneas [20,21]. The molecular mechanism(s) by which SLRPs modulate collagen fibril diameter and density remains elusive.

SLRP proteins consist of four major domains: (1) the signal peptide, (2) a negatively charged N-terminal peptide, (3) tandem leucine-rich repeats (LRRs) and (4) the C-terminal peptide. Rotary shadowing electron microscopy and molecular modelling have shown that the LRR cores of SLRPs are horseshoe-shaped, with the arch having approximately the diameter of a collagen molecule [10,22]. This LRR domain appears to be the region of the protein that mediates binding between the SLRP and the collagen [9,10]. SLRPs are also post-translationally modified by O-linked and/or N-linked sulphated glycosaminoglycan. Mod-

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; KSPG, keratan sulphate proteoglycan; LRR, tandem leucine-rich repeat; lumC/S, lumican Cys^{41} → Ser mutant construct; lumWT, lumican wild-type construct; SLRP, small leucine-rich proteoglycan; TGF, transforming growth factor.

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elling and electron micrography studies suggest that the highly charged glycosaminoglycan chains extend out from the SLRP–collagen complex in a manner that regulates fibril density, thereby implicating roles for the SLRPs in both fibrillogenesis and interfibrillar spacing.

Variations in the properties of individual proteoglycans derive from amino acid substitution in less conserved N- and C-terminal peptides, surface residues, changes in the number and length of LRRs, and variations in glycosylation. The functional roles of these N- and C-terminal peptides in collagen fibrillogenesis remain elusive. The SLRP N-terminal domains contain a cluster of highly conserved cysteine residues with consensus sequence Cys-Xaa$_{2-3}$-Cys-Xaa-Cys-Xaa$_{6-9}$-Cys (where Xaa is any amino acid and the subscripts denote the number of intervening residues). These cysteine residues are involved in the intrachain disulphide-bond formation at the N-terminal region of the core proteins. It has been suggested that this N-terminal disulphide bond is essential for the core proteins to bind collagen molecules and regulate fibril diameter [5]. To investigate the role of this cysteine domain, a mutant lumican minigene encoding Cys-Xaa$_3$-**Ser**-Xaa-Cys-Xaa$_9$-Cys instead of Cys-Xaa$_3$-**Cys**-Xaa-Cys-Xaa$_9$-Cys was overexpressed by stable transformants of MK/T-1 cells [23], an immortalized cell line from cultured mouse corneal stroma cells. In the presence of ascorbic acid 5-phosphate and transforming growth factor-β1 (TGF-β1), these stable transformants formed a three-dimensional ECM that was shown by transmission electron microscopy to be significantly altered due to modification of the lumican cysteine domain.

MATERIALS AND METHODS

Preparation of plasmid DNA for wild-type lumican and mutant C/S lumican

Lumican wild-type cDNA (985 bp) was first cloned into the *Sfi*I and *Not*I sites of the pSecTag2A vector (Invitrogen, Carlsbad, CA, U.S.A.). Two oligonucleotides for PCR were generated to add a 5′ *Sfi*I restriction enzyme site (LumSfi+ primer) at the 5′ end of lumican and a *Not*I restriction enzyme site (LumNot− primer) at the 3′ end (LumSfi+, 5′ GCG GCC CAG CCG GCC AGT GGC CAA TAC TAC GAT T-3′; LumNot−, 5′ ATA AGA ATG CGG CCG CGT TAA CGG TGA TTT CAT T-3′). PCR was performed using 100 ng of lumican cDNA [14] as a template in a standard PCR reaction mixture with the following cycling conditions: 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min for 35 cycles, followed by one 72 °C cycle for 10 min. The PCR product was then gel purified, phenol extracted, and ethanol precipitated. A restriction enzyme digest using *Sfi*I and *Not*I and buffer 2 according to the manufacturer's recommendations (New England Biolabs Inc., Beverly, MA, U.S.A.) was performed on the PCR product and the pSecTag2A vector by incubation at 37 °C for 2 h followed by 50 °C for 2 h. The cDNA was cloned into the pSecTag2 vector (lumWT). The fidelity of plasmid DNA was verified by nucleotide sequencing in both strands.

PCR-based mutagenesis was performed in order to change the cysteine residue (emboldened) of Cys-Xaa$_3$-**Cys**-Xaa-Cys-Xaa$_9$-Cys into serine, giving Cys-Xaa$_3$-**Ser**-Xaa-Cys-Xaa$_9$-Cys (C/S mutant). To generate a Cys → Ser mutation in the N-terminal region of lumican, lumWT was used as the template DNA for PCR, using T7 and a C/S mutant oligonucleotide LumC/S 2720− (5′ GTT A**G**A TTC TGG TGC ACA GTT GGG-3′). The PCR cycles were as described above for the lumWT minigene. The resulting PCR product (194 bp) was then used as a primer in combination with the LumNot− primer for the second PCR. The product of this PCR was digested and cloned into the pSecTag2 vector also as described above to generate a lumC/S minigene.

Cell cultures, and three-dimensional cell culture with phosphoascorbate and TGF-β1

Either the lumican wild-type (lumWT) or lumican C/S mutant (lumC/S) construct was transfected into MK/T-1 cells using calcium phosphate [23]. Cells were cultured in medium containing 100 μg/ml Zeocin (Invitrogen) and 10 % (v/v) fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) in 5 % CO_2 at 37 °C for 14 days, and individual Zeocin-resistant clones were isolated.

To construct a three-dimensional cell culture, 2.5×10^4 cells per well were seeded into 0.4 μm-pore-size Falcon® cell culture inserts (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) that were placed in the wells of 24-well culture plates and cultured for 4–6 weeks in 5 % CO_2/95 % air in the presence of 100 μg/ml Zeocin, 0.1 mM phosphoascorbate, 1 ng/ml recombinant TGF-β1 (R&D, Minneapolis, MN, U.S.A.) and 10 % (v/v) FBS in DMEM. The medium was changed twice weekly.

Transmission electron microscopy

Cells were grown in 24-well Transwell culture dishes (Costar; Corning Inc., Corning, NY, U.S.A.) for 4 and 6 weeks, and fixed for transmission electron microscopy. The cell cultures were fixed in 4 % paraformaldehyde, 2.5 % glutaraldehyde and 0.1 M sodium cacodylate, pH 7.4, with 8.0 mM $CaCl_2$ for 2 h at 4 °C and processed as described previously [24,25]. Briefly, the cultures were post-fixed with 1 % osmium tetroxide and stained *en bloc* with 2 % uranyl acetate/50 % ethanol. After dehydration in an ethanol series followed by propylene oxide, the inserts were infiltrated and embedded in a mixture of Embed 812, nadic methyl anhydride, dodecenyl succinic anhydride and DMP-30 (Electron Microscope Sciences, Fort Washington, PA, U.S.A.). Thick sections (1 μm) were cut and stained with Methylene Blue/Azur Blue for examination and selection of comparable stratified regions for analysis. Thin sections were then prepared using a Reichert UCT ultramicrotome and a diamond knife. Staining was with 2 % aqueous uranyl acetate followed by 1 % phosphotungstic acid, pH 3.2. Sections were examined and photographed at 75 kV using a Hitachi 7000 transmission electron microscope.

Analysis of fibril diameter

Transfected cells were examined in at least two independent experiments for each time point. In each experiment, three different cultures were analysed. At least five different regions were selected from thick sections in which the cells were multilayered and comparable. Micrographs of non-overlapping regions were taken at a magnification of × 31680. The microscope was calibrated using a line grating. For measurements of fibril diameter, micrographs were chosen randomly in a masked manner from the different groups, digitized, and diameters were measured using an RM Biometrics-Bioquant Image Analysis System (Memphis, TN, U.S.A.). A Mann–Whitney test was performed on the non-parametric data obtained from the fibril diameter analysis with a probability level of 0.05 on 241 measurements for either lumWT or lumC/S fibril diameters cultured for 4 weeks. $P < 0.05$ indicated a statistically significant

difference between the diameters of the lumWT and lumC/S fibrils.

Lumican Western blotting

Confluent cultures of transformed cell lines were transferred to medium containing 1% (v/v) horse serum in DMEM/F12 medium and incubated at 37 °C for 48 h. Lumican was recovered from the conditioned media by passing the culture medium and rinse through SPEC 3 ml NH_2 microcolumns (Ansys Diagnostics, Lake Forest, CA, U.S.A.) preactivated with methanol and distilled water as per the manufacturer's instructions. The columns were rinsed with 3 ml of 6 M urea/0.1 M NaCl/0.02 M Tris/HCl, pH 8, and lumican was eluted from the column with 0.4 ml of 4 M guanidine HCl/0.02 M Tris/HCl, pH 8 [26]. After dialysis and freeze drying, lumican from the medium was separated by SDS/PAGE on 10% (w/v) acrylamide gels and detected by immunoblotting as described previously [14].

Confocal microscopy

Stable transformants grown on 24-well plate inserts using the same conditions as described above were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 1 h. Following a brief wash with PBS, samples were blocked with 5% (w/v) non-fat milk in PBS for 45 min at room temperature. Either a rabbit anti-lumican antibody (8 μg/ml) (as described previously [27]) or a goat anti-(collagen I) antibody (Santa Cruz Biotechnology, Carlsbad, CA, U.S.A.) was diluted in a 5% (w/v) non-fat milk solution and incubated with the samples overnight at 4 °C. After the incubation, unbound antibodies were removed by washing with PBS for 3×10 min. Samples were then incubated for 60 min with a mixture of secondary antibodies: donkey anti-rabbit IgG (H+L) Alexa Fluor® 488 and Alexa Fluor® 546 conjugates (Molecular Probes, Inc., Eugene, OR, U.S.A.) diluted in 5% non-fat milk/PBS. After washing the samples for 3×10 min in PBS, the cells were either stained with propidium iodide (Molecular Probes) and washed with PBS or mounted directly on to glass slides with a SlowFade® Light Antifade Kit (Molecular Probes) and the coverslips sealed.

Confocal microscopy was performed on an LSM 510 upright confocal microscope (Zeiss, Oberkochen, Germany). The fluorochromes were excited with argon laser light of the appropriate wavelength, and images were captured using the manufacturer's software. Images captured with Zeiss LSM 510 software were exported to Adobe Photoshop (Adobe Systems Inc., San Jose, CA, U.S.A.) for fluorescent quantification. Quantification was performed as described in Lehr et al. [28]. Briefly, four images were analysed for the three stable transformant clones of each lumican minigene. A positive red, green or yellow fluorescent region was selected first, and the program was then used to select all similar areas. Total pixels selected were recorded and plotted on a histogram for each fluorochrome. Co-localized regions were identified by selecting a yellow region and then using the program to select corresponding pixels. This allowed calculation of co-localized pixels and subsequent histogram plotting.

RESULTS

Figure 1 (upper panel) is a schematic of the pSecLum plasmid DNA generated in the pSecTag2A vector. The lower panel shows alignment of the oligonucleotide and amino acid sequences of the lumWT and lumC/S constructs. Cys^{41} is shown in bold in the lumWT sequence, and the generated mutation to serine is shown in bold in the lumC/S sequence.

Zeocin-resistant transfected MK/T-1 clones were characterized by PCR genotyping using T7 and BGH− primers to identify stable transformants carrying the lumWT or lumC/S minigene, or the control empty vector. DNA was extracted from stable transformants that were resistant to 100 μg/ml Zeocin, PCR was performed, and the PCR products were analysed on a 1% (w/v) agarose gel with ethidium bromide (Figure 2). A positive PCR reaction was the presence of a 1.3 kb PCR product for lumWT or lumC/S, while a positive reaction for the empty vector control was a 0.3 kb PCR product.

Morphology and growth analysis was then performed on the stable transformants to determine any impact of the C/S mutation on these variables (Figure 3). The morphology of all the stable transformants was dendritic, independent of the transgene (results not shown). The growth results were used to perform an ANOVA ($\alpha = 0.05$), which indicated no significant difference in growth rate between the stable transformants (Figure 3).

Western blot analysis performed using an antibody against keratan sulphate proteoglycan (KSPG) detected lumican proteins secreted into the conditioned media from lumWT and lumC/S cells, but not those from parental MK/T-1 cells or cells transfected with empty vector. The blot in Figure 4 was probed with anti-KSPG antibody. WT2, WT5 and WT6 are three separate lumWT clones, and the conditioned media of these clones showed the presence of a 43 kDa band that was positive for secreted lumican protein. The conditioned media from lumC/S mutant clones C/S2, C/S12 and C/S39 were also all positive for secreted lumican mutant protein. The final lane in Figure 4 contains conditioned medium from empty vector control cells, and is negative for the presence of secreted lumican. An attempt was made to detect keratan sulphate in the parental cell line and in the lumWT protein via sulphate labelling analysis and antibodies to keratan sulphate, but to no avail (results not shown). For this reason, we did not test the lumC/S mutant for the presence of keratan sulphate, as we believe that these cells are unable to perform this post-translational modification of the recombinant protein. Secondly, we did not analyse the recombinant lumican proteins under non-reducing conditions, as this has been performed extensively with wild-type lumican protein with no change in electrophoretic mobility.

Cultures of lumWT and lumC/S cells were analysed to determine the effects of the C/S mutation on ECM deposition and fibril structure. Ultrastructural analyses demonstrated that both cell lines generated a multi-layered stroma *ex vivo* (Figure 5). In both cases, striated collagen fibrils were assembled between cell layers and demonstrated the characteristic 67 nm banding pattern. However, the two cell types demonstrated differences in the organization of the deposited matrix (Figure 6). The lumWT cells had a more organized ECM, with fibrils organized into lamellae with an orthogonal orientation (as seen in normal corneal stroma). In contrast, the lumC/S cells demonstrated a less organized ECM, with poorly defined lamellae and less orthogonal organization. These differences were apparent at both time points, but were more marked in the 4-week cultures. The fibrils assembled in cultures of lumWT cells were smaller and more homogeneous in diameter than fibrils assembled by lumC/S cells (Figure 6). To evaluate this observation further, diameters of fibrils in lumWT and lumC/S cells were measured, and the fibril diameter distributions were significantly different in the two different cultures. The mean ($\pm$S.D.) fibril diameter in lumWT cultures was 42 ± 6 nm and 44 ± 7 nm, and that in the lumC/S cultures was 44 ± 8 nm and 47 ± 8 nm, after 4 and 6 weeks of culture respectively. The lumC/S fibril diameters were significantly ($P < 0.05$) different from those in the lumWT

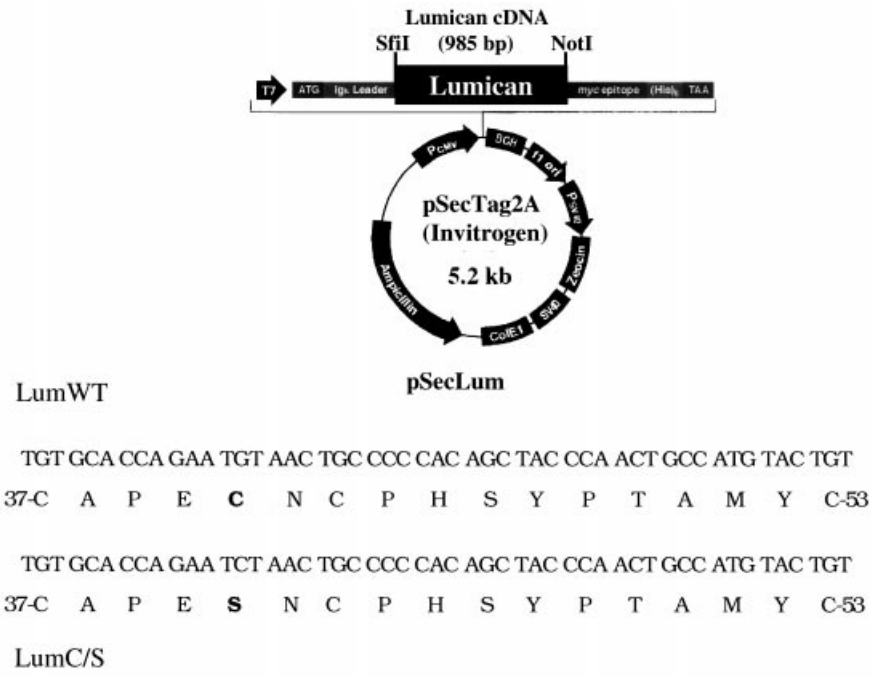

Figure 1 Schematic of lumican minigenes

The upper panel is a schematic of the lumican minigene created with a pSecTag2A vector (Invitrogen) backbone and a 985 bp lumican wild-type or C/S mutant cDNA between the *Sfi*I and *Not*I cloning sites. A c-*myc* epitope tag immediately follows the lumican minigene for protein identification purposes. The lower panel shows an alignment of the lumWT and lumC/S nucleotide and amino acid sequences. Cys[41] (shown in bold type) in lumWT is mutated in the lumC/S construct.

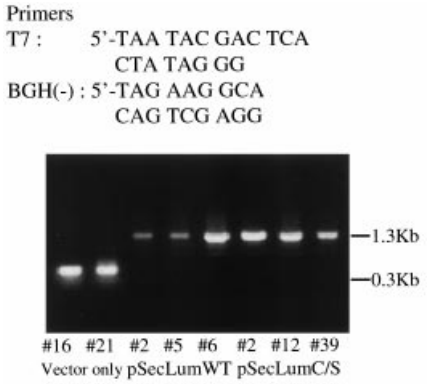

Figure 2 Identification of stable transformants by PCR

Genomic DNA was extracted from stable transformants and PCR was performed using 5′ T7 and 3′ BGH— primers. The PCR product underwent electrophoresis in a 0.8% (w/v) agarose gel and was stained with ethidium bromide. Stable transformants (lumWT clones 2, 5 and 6, and lumC/S clones 2, 12 and 39) were identified by a ~ 1.3 kb PCR product, and empty vector clones had a ~ 300 bp PCR product due to the absence of the lumican minigene.

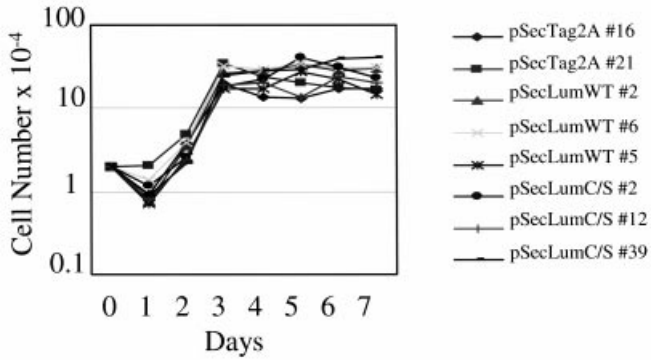

Figure 3 Growth of stable transformants

Stable transformants were cultured in DMEM containing 10% (v/v) FBS and 100 μg/ml Zeocin in 5% CO_2 at 37 °C for 0–7 days. Cell number was calculated at 24 h intervals for a period of 1 week and the values were plotted. The growth curve generated shows no significant difference in cell growth between the various clones, regardless of the minigene (ANOVA $\alpha = 0.05$).

cultures. The analysis of fibril diameter distributions at both time points (Figure 7) showed differences between the lum WT and lumC/S cultures. At 4 weeks the lumWT fibril diameters were distributed normally, whereas the lumC/S distribution was skewed toward larger-diameter values. At 6 weeks the lumWT fibril diameter distribution showed a slight deviation from normal; however, the lumC/S distribution was markedly skewed toward larger-diameter values. These data indicate the presence of a significant population of larger-diameter fibrils in the lumC/S compared with lumWT cultures, as well as more heterogeneity in fibril diameter. At least two populations of collagen fibrils were present in the lumC/S cultures: a normal distribution of relatively small-diameter fibrils and a heterogeneous population of abnormally large-diameter fibrils. These data indicate that the lumican C/S mutant molecule is not capable of regulating fibril lateral growth of collagen fibrillogenesis.

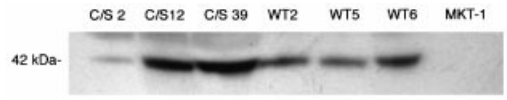

Figure 4 Western blot analysis of lumican secreted by stable transformants

In order to confirm that the stable transformants secreted lumican, conditioned media were collected and lumican protein extracted. This immunoblot of conditioned culture media from lumC/S mutant (C/S2, C/S12, C/S39), lumWT (WT2, WT5, WT6) and non-transfected parental (MK/T-1) stable transformants probed for lumican with anti-KSPG antibody shows that the lumican stable transformants expressed and secreted lumican protein into the culture medium, whereas the parental MK/T-1 cells did not.

Perturbation of fibrillogenesis by the lumC/S mutant was evaluated further by studying in more detail the ECM formed by these cultures. Immunofluorescent staining of the recombinant lumican protein and collagen I in conjunction with propidium iodide staining confirmed secretion of these two proteins into the ECM (Figure 8). The cells were not treated with RNase prior to propidium iodide staining so that the cytoplasm (RNA) and nucleus (DNA) could be stained in order to establish cellular boundaries. Figure 8 (top panels) shows positive reactions for both recombinant lumican and collagen I in the ECM of representative clones. All clones analysed displayed a similar phenotype (results not shown). The interaction of the recombinant lumican proteins with collagen I in the ECM was visualized

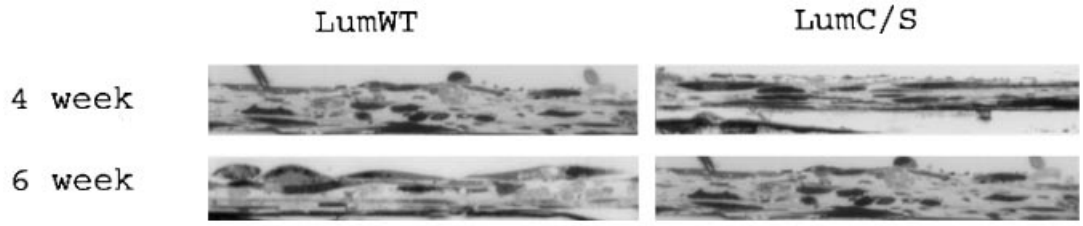

Figure 5 Light microscopy of semi-thin sections from Transwell cultures of stable transformants

Light microscopic analysis was carried out of 4-week or 6-week Transwell cultures of lumWT and lumC/S stable transformants cultured in the presence of 5-phosphoascorbic acid and TGF-β1. Ultrastructural analysis confirms the piling up of cells and the formation of ECM prior to analysis.

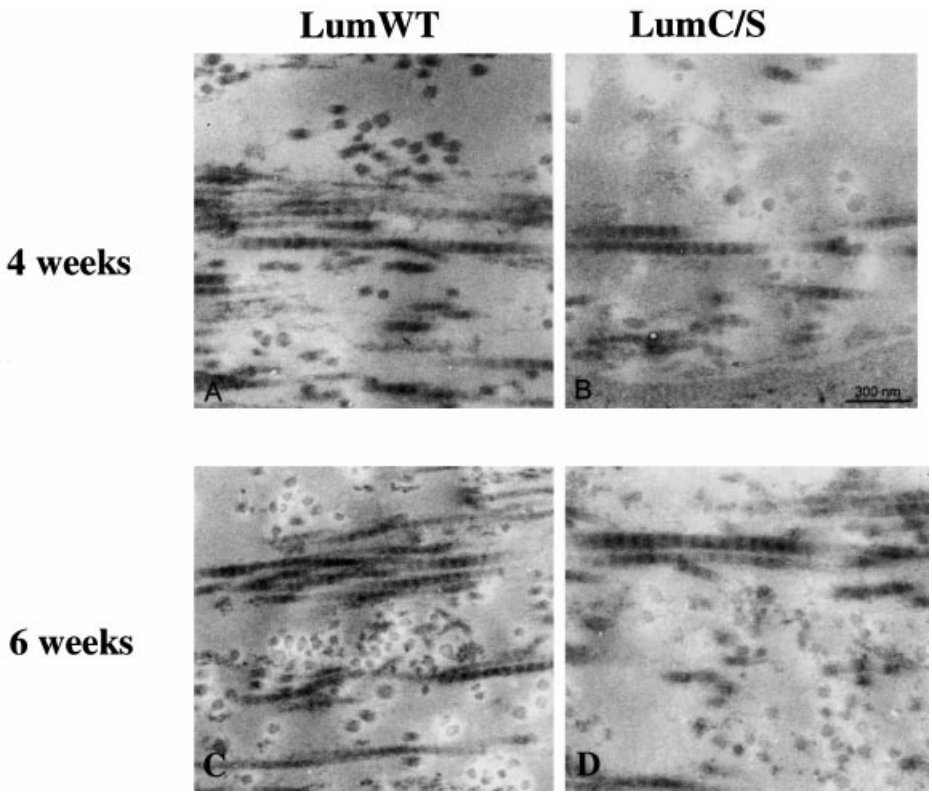

Figure 6 Collagen fibrils synthesized by stable transformants

Shown are transmission electron micrographs of ECM formed by lumWT (left panels) and lumC/S (right panels) stable transformants after 4 weeks (upper panels) and 6 weeks (lower panels). The lumWT and lumC/S transformants both produced a collagen matrix, but more uniform-diameter and orthogonal fibrils are seen in lumWT samples as compared with the lumC/S mutant, which perturbed collagen fibrillogenesis.

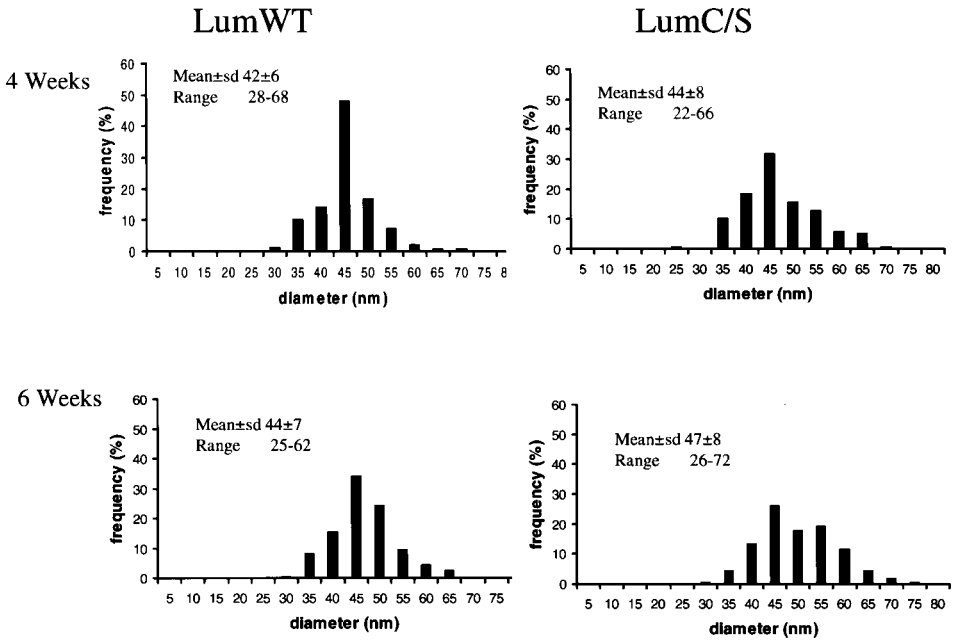

Figure 7 Histograms of the diameter of collagen fibrils synthesized by the stable transformants

Fibril diameter was measured in lumWT and lumC/S three-dimensional cell cultures at 4 and 6 weeks. The lumWT cell culture showed uniform-diameter collagen fibrils and orthogonal products, whereas lumC/S cultures exhibited a statistically significant ($P < 0.05$) difference in collagen fibril diameter, with a greater fibril diameter distribution as compared with lumWT cultures.

via immunofluorescent staining followed by confocal microscopy. LumWT (Figure 8, middle panels) and lumC/S (bottom panels) cells were grown on 24-well plate inserts for 5 weeks and then immunostained for lumican and collagen I followed by laser scanning confocal microscopy. The left panels of Figure 8 show the collagen I matrix formed by each cell type (in red). Both the lumWT and lumC/S cells generated an established ECM rich in collagen I. Lumican immunostaining is shown in the middle panels (in green). As shown by both Western-blot analysis (Figure 4) and confocal microscopy (Figure 8), lumican is secreted by both the lumWT and the lumC/S cells into the ECM. The right panels of Figure 8 are overlays of the collagen I and lumican immunofluorescence. Co-localization of collagen I and lumican is apparent for the lumWT cells, as shown by the large area of yellow fluorescence in the ECM. However, in the lumC/S overlay panel, very little yellow fluorescence is seen, demonstrating the lack of a lumican–collagen I interaction. Figure 9 shows a histogram of quantified fluorescent signal pixel area for lumican, collagen I and co-localization. The grey bars are representative of the amounts of lumican, collagen I and co-localized signal detected in the lumWT cells, and the black bars represent expression by the lumC/S clones. Superimposing the two images clearly shows a significant amount of co-localization of lumican and collagen I in lumWT cells, but no co-localization in lumC/S cells. Also significant is the increased amount of collagen I in the matrix of the lumWT as compared with the lumC/S clones.

DISCUSSION

The corneal stroma is a highly organized matrix rich in KSPGs and collagen, which contribute to corneal curvature, hydration pressure, strength and transparency. Corneal transparency is due largely to a well organized collagen matrix consisting of fibrils of uniform diameter and fibril density. A large amount of evidence indicates that the corneal KSPGs lumican, keratocan and mimecan play a critical role in the development and maintenance of this collagen matrix. For example, lumican knockout mice develop corneal opacity, due to a disorganized corneal stroma ECM consisting of collagen fibrils of various diameters and disproportionate spacing between these fibrils [27]. However, the precise role of KSPGs in the modulation of collagen fibrillogenesis and the maintenance of corneal transparency remains unknown.

In the present study we used an immortalized cell line, MK/T-1, derived from cultured corneal stromal cells to examine the effects of wild-type and mutant lumican on collagen fibrillogenesis *ex vivo*. It is of interest to note that MK/T-1 cells, and derivative stable transformants that express recombinant lumican molecules, are capable of synthesizing a three-dimensional ECM in the presence of ascorbic acid and TGF-β1. This immortalized cell line has changed some of its characteristics of KSPG expression. Thus MK/T-1 cells at high passage number (> 70), as used in the present study, no longer synthesize and secrete keratocan or lumican. The reasons for the suppression of KSPG core proteins by these tumour cells are not known. However, it provides an ideal cell culture system in which to examine the roles of lumican and/or other core proteins in collagen fibrillogenesis *ex vivo*. Interestingly, the expression of wild-type and mutant lumican core protein does not significantly alter the characteristics of the MK/T-1 cells, e.g. dendritic morphology, growth rate, and the ability to synthesize a three-dimensional ECM in the presence of ascorbic acid and TGF-β1. As shown in the immunoblots (Figure 4), the lumWT and lumC/S clones both express and secrete lumican protein.

Utilizing this MK/T-1 cell culture system, we have tested the hypothesis that the intrachain disulphide bond at the N-terminus of lumican is essential in order to maintain the functions of lumican in modulating collagen fibrillogenesis. It has been suggested that the disulphide loop between Cys^{41} and ^{53}Cys of

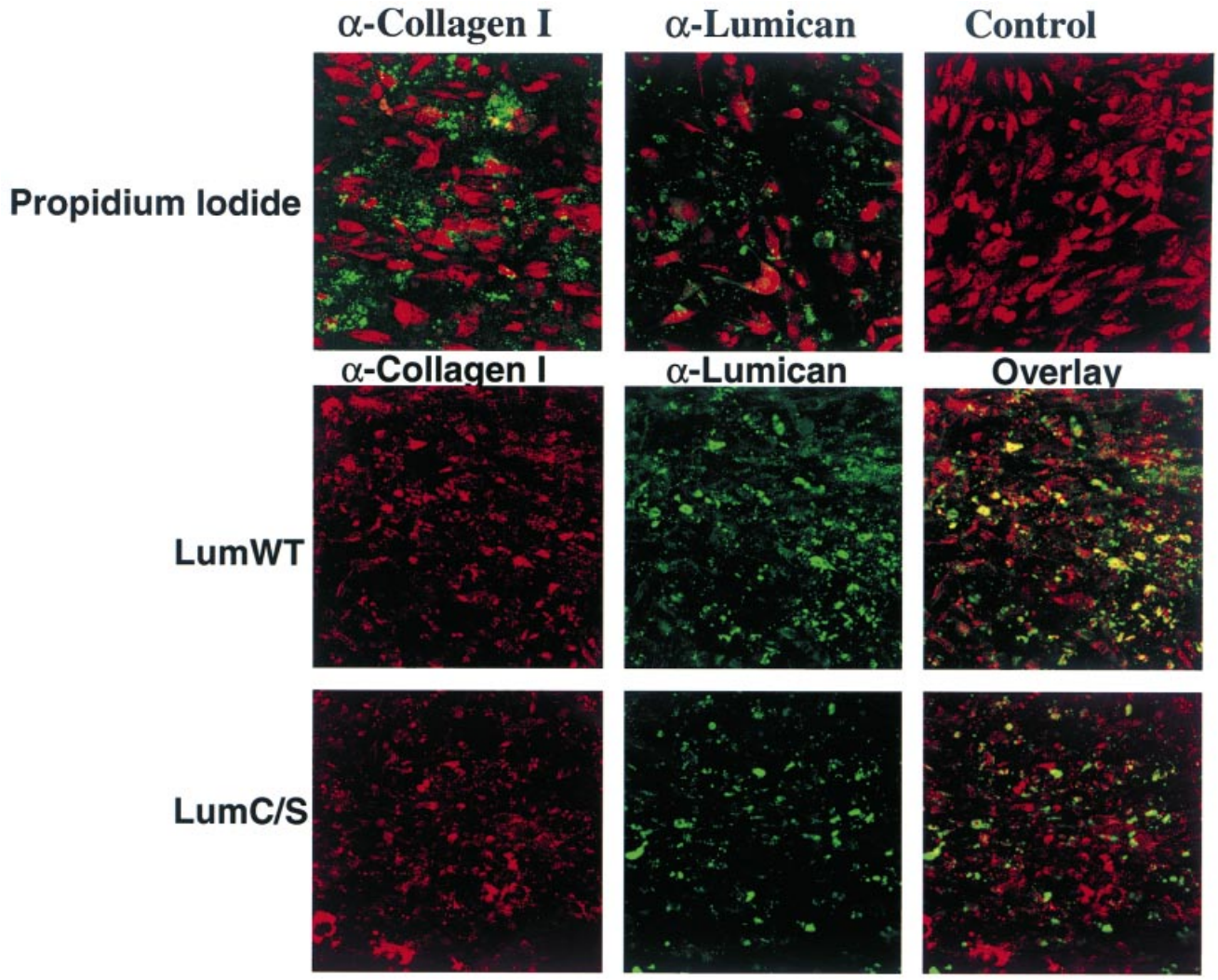

Figure 8 Immunofluorescent and propidium iodide staining analysed by confocal microscopy

Top panels: confocal images of three-dimensional cell cultures immunostained (green) for collagen I or lumican along with propidium iodide staining (red) show the secretion of both of these proteins into the ECM. The top right panel is the non-immune control with propidium iodide staining. Middle and bottom panels: confocal images of three-dimensional cell cultures immunostained for collagen I (red) and lumican (green). Both lumWT (middle panels) and lumC/S (bottom panels) cells show an ECM rich in collagen I, with recombinant lumican secreted into this matrix. An overlay (right panels) of lumWT immunostaining shows the strong presence of a lumican and collagen I co-localization (yellow). However, lumC/S cells show very little co-localization of lumican and collagen I as compared with lumWT cells. The middle and bottom right panels are confocal micrograph overlays of collagen I or lumican immunostaining with propidium iodide staining to show secretion of these two components into the ECM.

Cys^{37}-Ala-Pro-Glu-Cys-Asn-Cys-Pro-His-Ser-Tyr-Pro-Thr-Ala-Met-Tyr-Cys^{53} in the N-terminal domain of lumican core protein is essential for the proper folding of this N-terminal domain, which modulates the binding of lumican to collagen molecules. Our present results provide evidence that the presence of the lumC/S mutant core protein results in the formation of collagen fibrils with wider variation in diameter compared with the wild-type core. In addition, the mutant core is associated with poorly formed lamellae and a disorganized lamellar arrangement. These results suggest that the N-terminal domain of the lumican core protein has an important role in collagen fibrillogenesis. Furthermore, a population of fibrils with a larger diameter was present in lumC/S cultures at 6 weeks. This second population of larger fibrils may be due to the removal of fibrillogenesis constraints by functional proteoglycans (i.e. lumican). Data plotted in the histogram represent a multi-modal distribution with a broadened peak skewed towards larger fibril diameters, characteristic of fibril systems with no lateral growth constraints. Basically, the lumC/S culture matrix is populated initially by fibrils with a diameter similar to that in lumWT cultures. However, the lumC/S recombinant protein cannot regulate collagen fibrillogenesis, potentially due to its inability to bind collagen; therefore the collagen fibrils continue growing. This role is elucidated further by the immunofluorescent data, as viewed by confocal microscopy. Figure 8 confirms the secretion of both recombinant lumican and collagen I into the ECM, and shows clearly that the mutation of Cys^{41} to Ser inhibits the ability of lumican to co-localize with collagen I in the ECM of these cultures. It is not apparent if this amino acid is an essential residue in a binding site for collagen I, or is responsible for a structural component necessary for the interaction. Regardless of the mechanism, the matrix in these cultures is perturbed by the

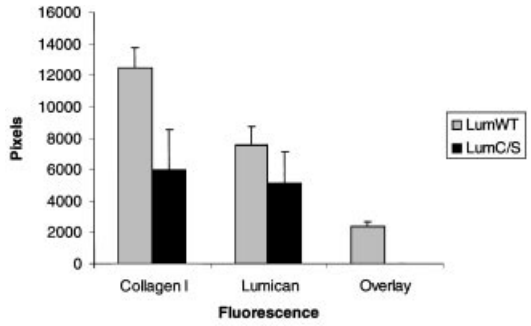

Figure 9 Histogram of laser scanning confocal microscopy data

LumWT and lumC/S confocal images were computer-analysed to determine the number of green (lumican), red (collagen I) or yellow (co-localization) pixels. LumWT clones secreted recombinant protein that co-localized with collagen I to a significantly greater extent than did lumC/S clones.

lumican mutant. Also noteworthy is the amount of collagen I in the lumWT matrix as compared with the lumC/S matrix. It is possible that the lumWT protein stabilizes collagen I in the ECM, preventing collagen degradation and/or metabolism. The inability of the lumC/S protein to bind collagen may provide a possible explanation for the significant decrease in the amount of collagen I seen in the matrix of these clones.

Perturbation of the collagen matrix in the corneal stroma leads to irreversible loss of vision. Organized orthogonal lamellae composed of fibrils of relatively small and uniform diameter are essential for the passage of light through the cornea. Disruption of this matrix leads to light refraction and reflection, which have a variety of effects on the perceived image. A matrix with these components not only inhibits the passage of light, but also is structurally unsound.

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