Resistance of Corneal RFUVA–Cross-Linked Collagens and Small Leucine-Rich Proteoglycans to Degradation by Matrix Metalloproteinases

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Purpose. Extracellular matrix metalloproteinases (MMPs) are thought to play a crucial role in corneal degradation associated with the pathological progression of keratoconus. Currently, corneal cross-linking by riboflavin and ultraviolet A (RFUVA) has received significant attention for treatment of keratoconus. However, the extent to which MMPs digest cross-linked collagen and small leucine-rich proteoglycans (SLRPs) remains unknown. In this study, the resistance of RFUVA–cross-linked collagens and SLRPs to MMPs has been investigated.

Methods. To investigate the ability of MMPs to digest cross-linked collagen and SLRPs, a model reaction system using purified collagen type I, type IV, and nonglycosylated, commercially available recombinant SLRPs, keratocan, lumican, mimecan, decorin, and biglycan in solution in vitro has been compared using reactions inside an intact bovine cornea, ex vivo.

Results. Our data demonstrate that corneal cross-linked collagen type I and type IV are resistant to cleavage by MMP-1, MMP-2, MMP-9, and MMP-13, whereas non–cross–linked collagen I, IV, and natively glycosylated SLRPs are susceptible to degradation by MMPs. In addition, both cross-linked SLRPs themselves and cross-linked polymers of SLRPs and collagen appear able to resist degradation. These results suggest that the interactions between SLRPs and collagen caused by RFUVA protect both SLRPs and collagen fibrils from cleavage by MMPs.

Conclusions. A novel approach for understanding the biochemical mechanism whereby RFUVA cross-linking stops keratoconus progression has been achieved. (*Invest Ophthalmol Vis Sci.* 2013;54:1014–1025) DOI:10.1167/iovs.12-11277

Keratoconus is a bilateral noninflammatory corneal ectasia, typically characterized by three histopathological signs: progressive corneal thinning, Bowman's layer breakage, and iron deposits in the basal layer of the corneal epithelium.[1,2] Keratoconus is detected when the normally spherical cornea begins to bulge outward acutely. This abnormal shape usually occurs as the central stromal region becomes thinner, preventing light from entering the eye and being focused correctly on the retina and causing distortion of vision.[3] Keratoconus may progress for 10 to 20 years and then slow down, and each eye may be affected differently. Keratoconus affects 1 in 2000 people[2] and was the leading indicator for penetrating keratoplasty in 2011 and 2010.[4]

The stroma comprises approximately 90% of the corneal thickness in humans.[5] Collagen gives the cornea its strength, elasticity, and form.[6] The unique molecular shape, paracrystalline arrangement, and very fine diameter of the evenly spaced collagen fibrils are essential in producing a transparent cornea.[7,8] Corneal stroma is composed primarily of orthogonal plies/lamellae of collagen fibrils, each of which consists of a core of type V collagen coated with type I collagen,[9,10] coated in turn by two classes of proteoglycans (PGs),[11] of which keratan sulfate PGs (KSPGs) are the predominant class. Through N-linked oligosaccharides, KS glycosaminoglycan (GAG) chains are attached covalently to three core proteins: lumican (LUM), keratocan (KER), and mimecan (MIM) to form KSPGs.[12–14] These three core proteins belong to a class of proteins known as small leucine-rich repeat proteins (SLRPs).[15–17] The other major class of PGs in corneal stroma is modified with chains of chondroitin/dermatan sulfate (CS/DS). Through O-linked oligosaccharide, CS/DS GAG chains are attached to the core SLRPs decorin (DCN)[18,19] and biglycan (BGN).[20,21] In the case of DCN, a single CS/DS linkage site is present near the amino terminus of the core protein, whereas BGN possesses two potential CS/DS linkage sites.[20,21] For LUM and KER, there are four or five potential KS attachment sites in their central leucine-rich repeat regions,[12,22,23] and MIM has two potential KS attachment sites.[24,25]

The most important clinical feature of keratoconus is thinning and ectasia of the cornea, suggesting that degradation of the stromal extracellular matrix may occur during the progression of keratoconus. In the stroma, a decrease in the number of lamellae and keratocytes,[26] changes in the gross organization of the lamellae, and uneven distribution of collagen fibrillar mass and inter- and intralamellae, particularly around the apex of the cone, have been observed.[27] Degradative extracellular enzymes, such as matrix metalloproteinases (MMPs), may play crucial roles in corneal degradation associated with keratoconus.[28–31] MMPs are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for tissue remodeling and degradation of the extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins, and PGs.[32,33] Under normal physiological conditions, MMPs are minimally expressed and homeostasis is maintained. The cornea is 70% collagen by weight, and the reduced collagen content of the keratoconic cornea suggests a degraded extracellular matrix.[27] Early studies detected increased MMP activities in keratoconus corneas, especially MMP-1, -2, -9, and -13.[34–38] MMPs are inhibited by tissue inhibitors of MMP

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(TIMPs) which comprise a family of four protease inhibitors, TIMP-1, -2, -3, and -4.[39] Thus, MMPs are widely assumed to have a central role in the pathogenesis of keratoconus.

Recently, a new technique for corneal cross-linking was devised that directly improves the biomechanical rigidity of the corneal stroma. This approach consists of irradiation of the cornea with ultraviolet A (UVA) in the presence of the photosensitizer riboflavin (RF), as a chromophore.[40,41] Cross-linking RFUVA treatment effectively stops the progression of the keratoconus syndrome, although the mechanism is not clear and remains under study. Our recent work demonstrated that RFUVA treatment causes cross-linking of collagen molecules among themselves and of PG core proteins among themselves, together with limited linkages between collagen and KER, LUM, MIM, and DCN.[42] However, the mechanism whereby RFUVA cross-linking stops the degradation processes associated with keratoconus has not been elucidated. In this study, a model reaction system using purified collagens and nonglycosylated, commercially available recombinant SLRPs in solution in vitro has been compared with reactions inside an intact cornea, ex vivo, revealing that RFUVA causes resistance of corneal cross-linked collagens and small leucine-rich PGs to degradation by MMPs.

Materials and Methods

Materials

Bovine eyeballs were freshly collected from Alta Vista Locker (Alta Vista, KS). Collagen type I from bovine skin was purchased from Inamed (Fremont, CA). All animals were used in accordance with the ARVO Statement for the Use of Animals for Ophthalmic and Vision Research. Collagen type IV from human placenta, 5′-monophosphate sodium salt RF and CS56 antibody were purchased from Sigma (St. Louis, MO). Anti-KS antibody J36 was kindly provided by Nirmala SundarRaj (University of Pittsburgh). Recombinant human MMPs, recombinant human LUM, recombinant mouse MIM, recombinant human DCN, recombinant human BGN proteins, anti-human LUM antibody, anti-mouse MIM antibody, anti-human DCN antibody, and anti-human BGN antibody were purchased from R&D Systems (Minneapolis, MN). Recombinant human KER and anti-KER polyclonal antibodies were purchased from Abnova Corp. (Taipei, Taiwan). Rabbit polyclonal antibody to collagen type I (code ab34710) was purchased from Abcam Inc. (Cambridge, MA). NuPAGE Novex tris-acetate precast gels and chromogenic Western blotting kits were purchased from Invitrogen Corp. (Carlsbad, CA).

Analysis of Amino Acids

Twenty-microliter volumes containing 1 μg/μL collagen and 0.1% RF (in phosphate-buffered saline [PBS]) in 0.5-mL plastic centrifugation tubes were irradiated with 370 nm of UVA light for 30 minutes at a distance of 5 cm from the light source at room temperature. The sample solution was irradiated with the UVA light shining directly onto the surface of the solutions, mixed twice by vortexing at 10-minute intervals while it was being irradiated. Prior to being hydrolyzed, the residual RF was not removed from the samples. Amino acids were analyzed by the AAA Service Laboratory (Damascus, OR). Briefly, 10 nM of norleucine as an internal standard was added to 20 μL of each sample. Samples were hydrolyzed at 110°C for 22 hours in 1 mL of 6*N*-HCl with 2% phenol under vacuum. After hydrolysis, samples were dried and then resuspended in 100 μL of sample buffer, of which 50 μL was injected into an amino acid analyzer (model L8900; Hitachi, Tokyo, Japan), and analysis was performed using post-column ninhydrin derivatization. Molar ratio (%) was represented by the molar concentration of each amino acid per total amino acids. Data are means ± standard deviations (SD) from three separate experiments.

MMPs Degradation System In Vitro

Soluble collagen type I was cross-linked by the photosensitizer RF and UVA under conditions that resembled those used for RFUVA clinical treatment of progressive keratoconus.[42] Briefly, 15 μL of the mixture of purified type I collagen (1 μg/μL in PBS) and RF (0.1% in PBS) was irradiated with UVA of 370 nm for 30 minutes at a distance of 5 cm from the light source (UV-X radiation system for corneal cross-linking; Iroc Medical, Zurich, Switzerland). The volume of the reaction solution was 20 μL in 0.5-mL plastic centrifugal tubes. After cross-linking treatment, the cross-linked collagen was used as a substrate for digestion by MMPs. A total of 25 μL of 100 mM Tris containing 30 mM NaCl and 20 mM $CaCl_2$ (pH 7.5) was added into the cross-linked solution (note that addition of 1, 10, and 100 μM $ZnCl_2$ did not increase the activity of MMP-1 [data not shown]). Aliquots of MMP (200 ng/μL) were added at a series of amounts of 40, 60, 80, and 100 ng, respectively. A 2-μL aliquot of 25 mM *p*-aminophenylmercuric acetate and water was added to produce a final reaction volume of 50 μL. The reaction solution was mixed by vortexing and then incubated for 24 hours at 37°C. Enzymatic digestions were terminated by heating for 10 min in boiling water, and then samples were lyophilized.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Briefly, 5 μL of NuPAGE LDS sample buffer (Invitrogen) and 2 μL of NuPAGE reducing agent (Invitrogen) were added to each sample solution, heated at 70°C for 10 minutes, and then loaded onto NuPAGE Novex Bis-Tris 4% to 12% gels (8 cm × 8 cm × 1.5 mm precast gel [Invitrogen]) and subjected to electrophoresis (100 mA/gel for 60 minutes). After electrophoresis, gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250.

Cross-Linking Treatment of Intact Whole Corneas Ex Vivo

The cross-linking procedure was performed as described previously.[42] Briefly, bovine corneal epithelium was mechanically removed using a blunt knife, and 0.1% (w/v) RF in PBS was applied to the exposed stromal surface by drips for 30 minutes before irradiation and performed every 5 minutes during irradiation. Corneas were irradiated with UVA of 370 nm for 30 minutes at a distance of 5 cm from the light source.

Guanidine-HCl Extraction of Corneal Tissue

De-epithelialized bovine corneas (0.6 g wet weight) treated or untreated with RFUVA were frozen by liquid nitrogen, pulverized, homogenized further in 4 M guanidine-HCl (GHCl) containing protease inhibitors,[43] and then incubated for 24 hours at 0°C to 4°C with gentle agitation. Tissue residue was removed by centrifugation at 10,000*g* for 30 minutes, and the supernatant was retained as the extract. The tissue residue pellet was re-extracted for a second 24 h with fresh 4 M GHCl solution. The two extracts were combined, neutralized by addition of 1 M NaOH, and then applied to a centrifugal filter (regenerated cellulose, 3000-molecular weight [MW] cutoff; Amicon Ultra; Millipore, Billerica, MA), centrifuged to desalt, and concentrated to 1/5 of the original volume. Retentates that did not pass through the filter were collected and evaluated for degree of collagen cross-linking and resistance to degradation without any further processing. Total protein concentrations of GHCl extraction samples were determined using a spectrophotometer (NanoDrop ND-2000c; Thermo Scientific).

Evaluation of Resistance of Cross-Linked Collagen to MMPs Ex Vivo

Antibodies to collagen types I and IV and to each of the PG core proteins were used to compare the resistance of non–cross-linked versus cross-linked collagen and PG core proteins to digestion with MMPs. The sample (15 μg of protein) was treated with MMP concentrations of 0.8, 1.2, 1.6, and 2.0 ng/μL for 24 hours at 37°C,

as described above. Enzymatic digestions were terminated by heating for 10 min in boiling water and then samples were lyophilized. The same proportions of each digested sample were subjected to 4% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then to Western blotting analysis. The antibodies used here are capable of detecting collagens I and IV and the SLRP core proteins.

Results

For collagen type I, nontreated versus RFUVA–cross-linked samples, as analyzed by SDS-PAGE and Coomassie Blue staining, RFUVA causes (a) α1, α2, and β chains to almost disappear; b) γ chains to increase slightly in intensity; c) protein staining at the base of the well to increase (Fig. 1A, compare lane 2 vs. 3; Figs. 1B–D, compare lane 1 vs. 2). These results support earlier studies that concluded that collagen treated with RFUVA cross-linking forms higher-molecular-weight polymers in response to irradiation with UVA in the presence of RF,[42,44] a process that simultaneously modifies several specific amino acids (Table 1). Anterior corneal stroma is the location of the major effects of RF plus UVA treatment,[40] and the stromal fibrils are composed primarily of collagen type I. This collagen type therefore was chosen for the following experiments.

Results of amino acid analyses are presented in Table 1. In the case of collagen type I, distinct decreases in molar percentages of methionine, tyrosine, histidine, hydroxylysine, and lysine in cross-linked collagen were observed compared to their respective percentages detected in untreated collagen type I, suggesting that these amino acids are significantly modified by RFUVA. Similar results were detected with collagen type IV (Table 1).

As can be seen in Figure 1A, using the current reaction conditions, incubation of samples of purified collagen type I with MMP-1 caused collagen type I to be degraded into peptides of lower molecular sizes (Fig. 1A, compare lane 2 [nondigested control] vs. 4, 6, 8, and 10), and the β and γ chain bands of collagen type I to virtually disappear (α1 and α2 chains diminish more slowly). In contrast, after exposure to RFUVA (Fig. 1A, compare lane 3 [nondigested control] vs. 5, 7, 9, and 11), the cross-linked collagen high-molecular-weight polymers that are present at the γ band position were not degraded by MMP-1. As expected, the collagen at the α and β band positions was reduced by incorporation into higher-molecular-size polymers after exposure to RFUVA, but the limited amounts of protein still remaining at those positions did not appear to undergo digestion with MMP-1. When collagen type I was exposed to three other MMPs (MMP-13, -2, and -9), the α, β, and γ chain bands of collagen type I were significantly altered into lower-molecular-weight forms (Fig. 1B, C, and D, lane 1 [nondigested control] versus lanes 3, 5, 7, and 9). In contrast, after exposure to RFUVA (Figs. 1B–D, lanes 2 [nondigested control] vs. 4, 6, 8, and 10), the cross-linked collagen high-molecular-weight polymers that were present at the γ band position were not degraded by MMP-13 (Fig. 1B), MMP-2 (Fig. 1C), or MMP-9 (Fig. 1D). Thus, collagen cross-linked by RFUVA, especially that in the γ band position, is resistant to degradation by MMPs-1, -2, -9, and -13.

Data presented in Figure 2 represent the results of scanning density analysis of SDS-PAGE bands with Image Quantity software TL (GE Health Care Biosciences Corp.). The intensity of γ bands in RFUVA-treated collagen relative to the intensity of γ bands in nontreated control collagen as shown in Figure 1 was chosen as an indication of the ability of type I collagen to resist degradation by MMP: when exposed to low concentrations of MMPs (0.8 ng/μL), approximately 40% to 50% of non-RFUVA–cross-linked control collagen remained resistant to MMP-1 and -13, whereas approximately 10% resisted digestion by MMP-2 and -9. When exposed to high concentrations of MMPs (2 ng/μL), barely 10% of non-RFUVA–cross-linked control collagen remained resistant after digestion by MMP-1, -2, -9, or -13. In sharp contrast, however, prior exposure of collagen to RFUVA caused most such cross-linked collagen to be resistant to even the high concentration (2 ng/μL) of MMP-1 (80% resistant), MMP-13 (85%), MMP-2 (90%), or MMP-9 (70%).

To study the ability of MMPs to digest SLRPs, recombinant SLRP core proteins, in the presence or absence of collagen type I and with or without prior treatment with RFUVA, were digested with MMP-1, MMP-2, MMP-9, and MMP-13, respectively. In the cases of KER, LUM, MIM, DCN, and BGN, RFUVA causes formation of polymers of a very wide range of MWs from 100 and/or 150 kDa up to the bottom of the gel sample well (Fig. 3A, lane 3 in each case). In the absence or presence of soluble collagen, RFUVA causes cross-linking of all SLRPs, thus forming polymers of a wide range of higher MWs, from 100 and/or 150 kDa up to the bottom of the gel sample well (Fig. 3A, lane 4 in each case). Importantly, after incubation with MMPs at a high concentration (2.0 ng/μL, as shown in Fig. 1), both the cross-linked SLRPs themselves (Fig. 3A, lane 3) and also the mixture of SLRPs and collagen (Fig. 1, lane 4) appear able to resist degradation. By quantitative analysis of the amount of each SLRP remaining undigested at its normal gel band position, as presented in Figure 3B, the results indicated that most commercially available recombinant, nonglycosylated SLRPs are not digested significantly by MMPs, as others also have noted.[45] This resistance to degradation by the four most common corneal MMPs, in contrast to their readily digestible behavior when treated with the same MMPs ex vivo (see Fig. 5), may reflect the fact that these SLRPs have been modified in three ways: (1) they have been modified greatly by biosynthesis in nonglycosylated form, (2) they have undergone modifications in amino acid sequence from wild type, and (3) they have been modified by addition of covalent tags to allow their solubility, even though they are very hydrophilic and nonglycosylated (Table 2).

Whole corneas were treated ex vivo as described in Materials and methods, either with saline (controls) or with RFUVA, followed by extraction of all proteins, treatment or not with MMPs, and analysis by Western blotting. Figure 4 shows Western blots of the electrophoretic migration pattern of the α, β, and γ component molecules of collagen types I and IV extracted from whole corneas ex vivo, nontreated controls versus cross-linked by RFUVA, after extraction with GHCl, and then digestion with MMPs-1, -13, -2, or -9. Digestion of extracted samples with MMP-1 (Fig. 4A) caused nontreated control collagen type I (Fig. 4A, lane 1) to generate a series of low-MW peptides and caused the disappearance of α chains, β chains, and γ chains (Fig. 4A, MMP-1 digestion: lanes 3, 5, 7, and 9). Conversely, corneas exposed ex vivo to RFUVA prior to extraction with GHCl yielded collagens that showed staining intensity of γ chains, and the cross-linked collagen polymers at the high-MW range remained unchanged by digestion with 0.4 to 1.6 ng/μL concentrations of MMP-1, with no new bands of low-MW peptides observed (Fig. 4A, nondigested controls, lane 2 versus MMP-digested, lanes 4, 6, 8, and 10). A similar result was obtained after digestion of extracted samples with MMP-13 and Western blotting with antibodies for type I collagen (Fig. 4B). In addition, samples extracted from control corneas and corneas exposed ex vivo to RFUVA alternatively were digested with MMP-2, separated by SDS-PAGE and subjected to Western blotting with antibodies to collagen type IV (Fig. 4C, nontreated controls [lane 1]) versus digestion with 0.4 to 1.6 ng/μL concentrations of MMP-2: lanes 3, 5, 7, and 9. Results indicated that type IV collagen in samples extracted from control corneas underwent degradation by MMP-2 to a range of

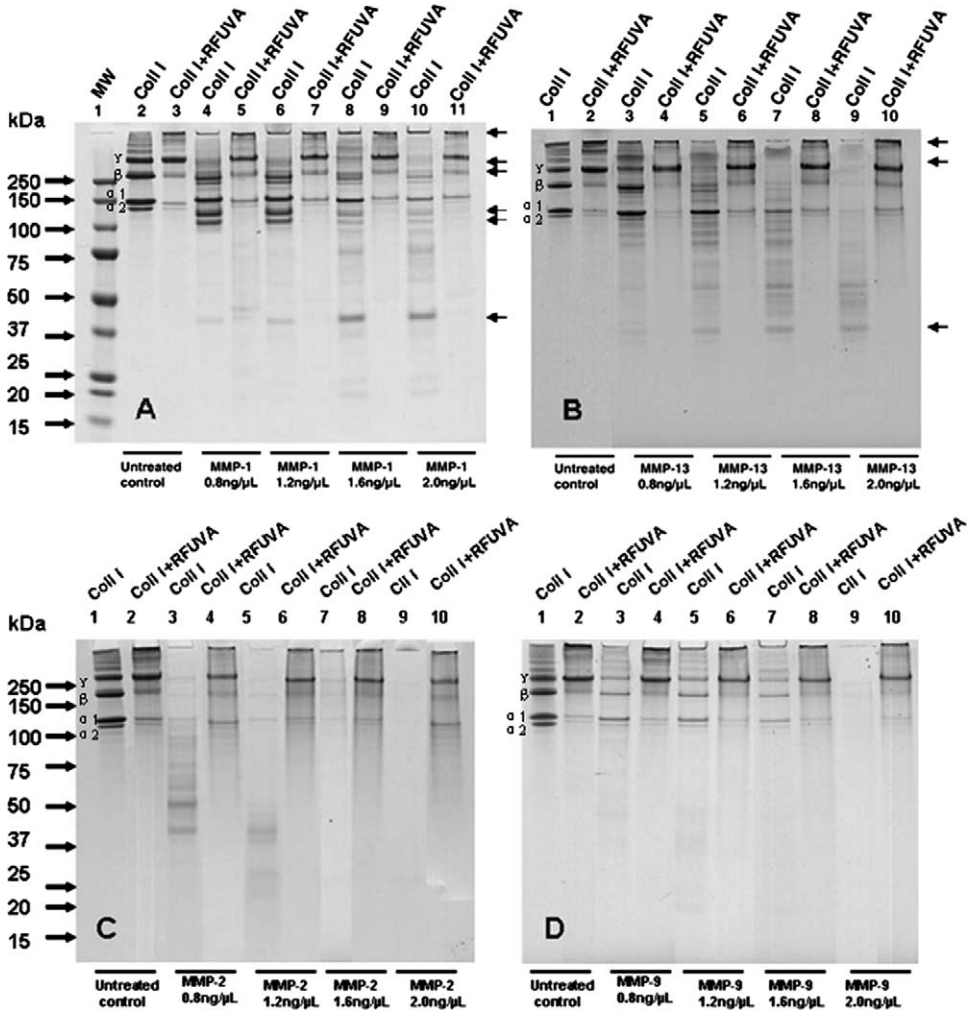

Figure 1. Type I collagen (from bovine skin, commercially obtained), control, and RFUVA–cross-linked, digested with MMPs. SDS polyacrylamide gels stained with Coomassie Blue. (**A**) (1) Compared to the nondigested control (lanes 2 and 3), cross-linked collagen in the gel well bottom was not digested by MMP-1 (lanes 5, 7, 9, and 11). (2) Similarly, cross-linked collagen in the γ band position was not digested by MMP-1 (nondigested control [lanes 2 and 3] versus lanes 5, 7, 9, and 11). (3). Native collagen (nondigested control lane 2), when exposed to a range of concentrations of MMP-1 generated a range of lower molecular size peptides (lanes 4, 6, 8, and 10). However, samples that had undergone RFUVA cross-linking generated no new lower-weight-molecular bands upon exposure to MMP-1 (nondigested, RFUVA-treated control: lane 3 versus lanes 5, 7, 9, and 11). Similar results were observed with MMP-13 (**B**), MMP-2 (**C**), and MMP-9 (**D**).

lower-MW bands whose density increased at higher enzyme concentrations. In sharp contrast, however, prior treatment of the cornea ex vivo with RFUVA generated patterns of cross-linked higher-MW polymers of type IV collagen that were not affected by digestion with MMP-2. A similar result also was observed when samples from control and RFUVA-treated ex vivo corneas were digested with MMP-9 and then analyzed by Western blotting with antibodies for type IV collagen (Fig. 4D). Thus, these results indicate that collagen types I and IV in nontreated control corneas ex vivo upon extraction and incubation with MMPs, are degraded to lower MW forms, generally to a "terminal digest" array of peptides whose concentrations are more prominent with use of higher MMP concentrations. In sharp contrast, samples extracted from corneas that had been exposed ex vivo to RFUVA upon incubation with MMPs show high-MW patterns of collagen types I and type IV cross-linked by RFUVA that resist cleavage by MMPs.

Relative susceptibility of the most common corneal SLRP proteins to digestion by MMPs before and after RFUVA cross-linking was described in Figure 3. However, those analyses were performed with commercially available and slightly modified (Table 2) purified recombinant nonglycosylated proteins in solution in vitro. In the natural environment of the cornea, those proteins would each carry at least one covalently bound chain of sulfated GAG, providing a degree of solubility sufficient to allow their exocytosis and diffusion within the extracellular matrix (whereas such normal core proteins, very hydrophilic and lacking such glycosylation, are insoluble). In addition, the core proteins of such native proteoglycan molecules are thought to normally wrap their hydrophobic domains around polymerized collagen fibrils, thus potentially presenting a conformation quite distinct from that of the nonglycosylated, artificially tagged (Table 2), purified recombinant proteins in solution used in Figure 3.

To determine the susceptibility of normal, glycosylated core SLRPs to MMPs before or after RFUVA treatment of whole corneas ex vivo, as described in Materials and Methods, RFUVA-treated and nontreated whole corneas were extracted with GHCl and the harvested macromolecules were incubated with or without MMP-1. Figures 5A, 5B, 5C, 5D, and 5E display the Western blot profiles of the main SLRPs extracted from such

Table 1. Amino Acid Analysis of Native and Treated Collagen with RFUVA

	Collagen Type I		Collagen Type IV	
Amino Acid	**Untreated**	**RFUVA Treated**	**Untreated**	**RFUVA Treated**
Hyp	9.43 ± 0.05	9.27 ± 0.05	11.04 ± 0.04	10.2 ± 0.06
Asp	4.7 ± 0.04	4.74 ± 0.01	5.16 ± 0.02	5.3 ± 0.05
Thr	1.54 ± 0.06	1.59 ± 0.01	2.38 ± 0.03	2.44 ± 0.05
Ser	3.16 ± 0.03	3.08 ± 0.05	3.96 ± 0.02	4.34 ± 0.17
Glu	7.07 ± 0.03	7.34 ± 0.06	8.55 ± 0.06	8.56 ± 0.03
Pro	12.75 ± 0.03	12.76 ± 0.05	8.13 ± 0.03	8.46 ± 0.25
Gly	33.92 ± 0.09	34.69 ± 0.11	31.56 ± 0.08	32.29 ± 0.46
Ala	10.32 ± 0.21	10.86 ± 0.05	3.55 ± 0.02	4.25 ± 0.02
Val	1.97 ± 0.03	1.91 ± 0.08	2.86 ± 0.01	2.84 ± 0.02
Met	0.58 ± 0.01	0.23 ± 0.07	1.22 ± 0.01	0.74 ± 0.05
Ile	1.21 ± 0.01	1.19 ± 0.01	3.24 ± 0.01	3.16 ± 0.03
Leu	2.3 ± 0.02	2.32 ± 0.04	5.24 ± 0.02	5.08 ± 0.02
Tyr	0.62 ± 0.01	0.32 ± 0.01	0.88 ± 0.03	0.53 ± 0.08
Phe	1.23 ± 0.02	1.3 ± 0.01	2.81 ± 0.04	2.52 ± 0.06
His	0.44 ± 0.01	0.16 ± 0.01	0.66 ± 0.02	0.21 ± 0.05
Hlys	0.67 ± 0.02	0.54 ± 0.02	4.66 ± 0.13	4.7 ± 0.03
Lys	2.74 ± 0.04	2.65 ± 0.24	0.83 ± 0.02	0.73 ± 0.03
Arg	5.23 ± 0.03	5.17 ± 0.03	3.28 ± 0.12	3.32 ± 0.02

Collagen (1 μg/μL in PBS) and RF (0.1% in PBS) was irradiated with UVA at 370 nm for 30 minutes at a distance of 5 cm from the light source at room temperature. Molar ratio (%) was represented by the molar concentration of each amino acid per total amino acids. Data are means ± SD from three separate experiments.

corneas incubated with or without MMP-1. Comparison of sample lanes 1 (nontreated control) in Figures 5A, 5B, 5C, 5D, and 5E versus lane 3 (MMP-digested). Figures 5A, 5B, 5C, 5D, and 5E indicates that each such control SLRP was cleaved by MMP-1, generating at least one peptide of lower-MW (note, the normal, glycosylated core proteins in Figs. 5A–E, lane 1, exist in a range of MWs, thus generating a diffuse smear of higher-MW antibody-positive staining, rather than the single band of core proteins in their nonglycosylated forms seen in Fig. 3). Staining patterns in lane 3 in Figures 5A, 5B, 5C, 5D, and 5E include at least one distinct band of lower-MW, probably a nonglycosylated peptide fragment, visibly indicating significant digestion of each of the four core SLRPs by MMP-1. In contrast, after exposure of whole corneas to RFUVA, followed by extraction of macromolecules with GHCl, RFUVA cross-linking between core proteins and other proteins generates a range of higher-MW polymers (Figs. 5A–E, lane 2). Importantly, when such samples from RFUVA-treated corneas are incubated with

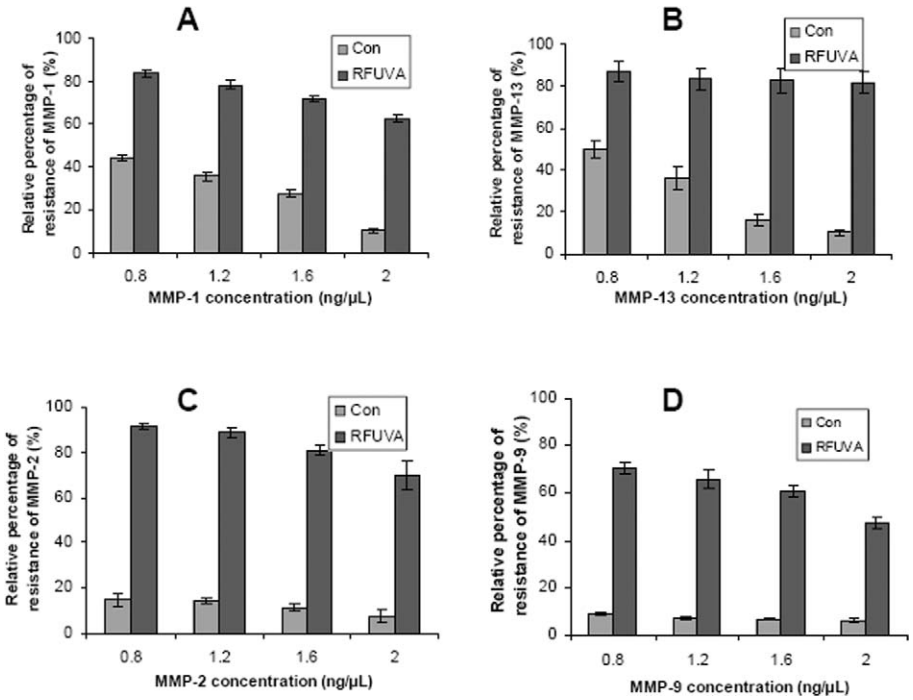

Figure 2. Quantitative analysis of the resistance of collagen type I to degradation by MMPs before and after RFUVA cross-linking. (**A**) MMP-1; (**B**) MMP-13; (**C**) MMP-2; (**D**) MMP-9. Data are percentages of the γ band remaining (resistant) in experimental samples after digestion with MMPs compared to the γ band from nontreated native control collagen.

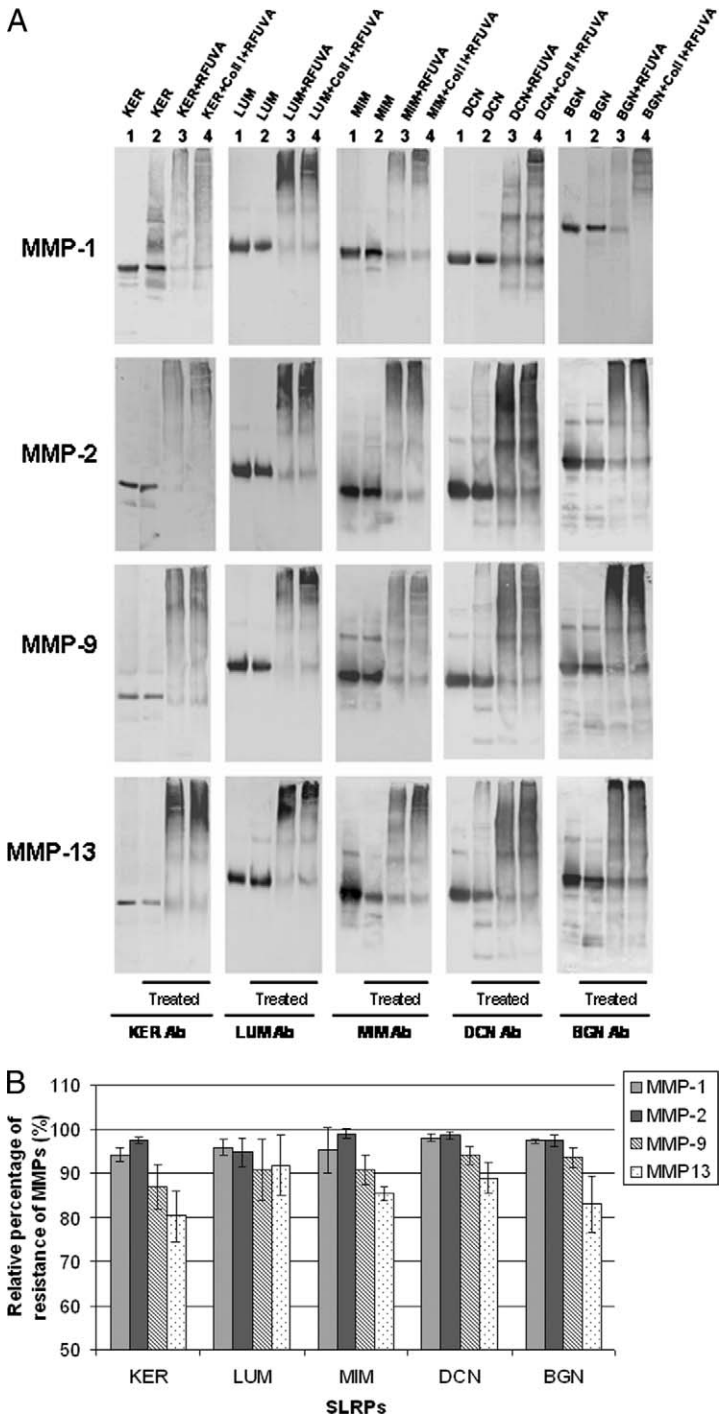

Figure 3. Effects of incubating SLRPs with MMPs. (**A**) Western blot analysis of the behaviors of core proteins in the absence or presence of collagen, then treated or not with RFUVA, and finally incubated with MMPs. (**B**) Quantitative analysis of the amount of each core protein remaining (resistant) after incubation with (lane[s] 2, *above*) or without (lane[s] 1, *above*) MMPs in the absence of collagen and in the absence of RFUVA treatment, as assessed by measurements of band intensities (performed in triplicate).

MMP-1, lower MW forms are not generated (Figs. 5A–E, lane 4), indicating that RFUVA treatment of corneas ex vivo cross-links SLRPs to one another and possibly to other proteins, too, and allows them to resist degradation by MMP-1.

Finally, as an independent approach to determining the response of native, glycosylated SLRPs in vivo before and after RFUVA treatment to digestion with MMPs, Figure 6 shows the patterns of KS chains and CS chains attached to SLRPs. Irradiation of whole corneas ex vivo with RFUVA causes SLRPs bearing KS chains or CS chains to migrate near the top of the gel (Figs. 6A, 6B, lane 2; in the same locations that antibodies to the SLRPs themselves were seen in Fig. 5, lane 2). Here, the locations of the KS and CS chains are used simply to reveal the locations of the SLRPs to which they are natively covalently bound, compared with that of the control samples in the absence of RFUVA (Figs. 6A, 6B, lane 1). It is noteworthy that previous work has indicated that RFUVA does not crosslink the GAG chains,[42] per se, but their presence on most of the SLRP

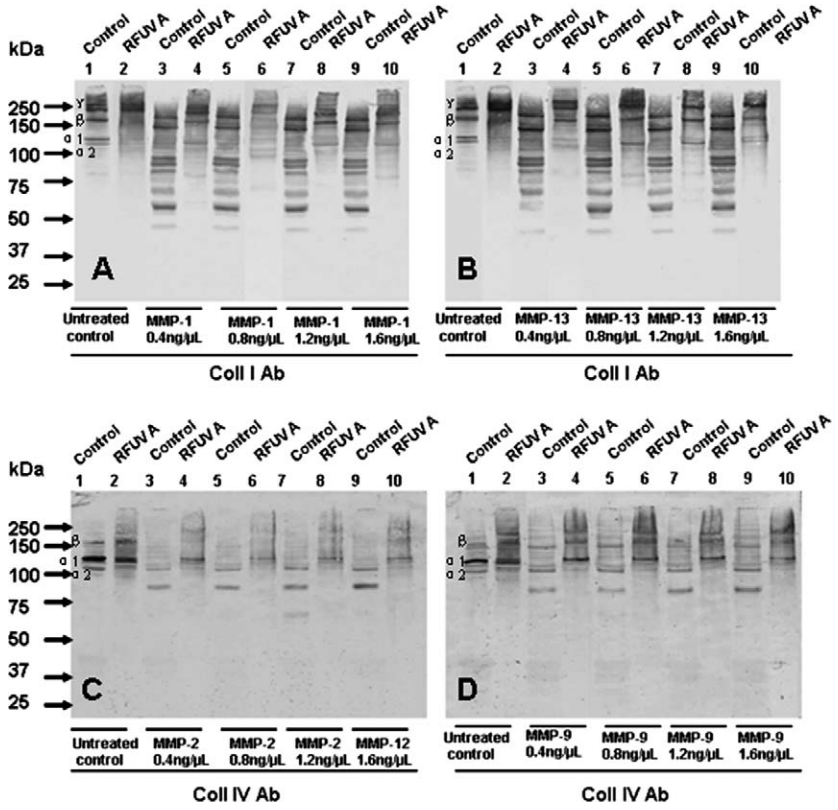

Figure 4. Western blot analysis of collagens I and IV in samples extracted from corneas treated ex vivo: control versus RFUVA–cross-linked, extracted with GHCl and then digested with MMPs. Extracted samples were treated with MMPs, then separated by gel electrophoresis, and subjected to Western blot analysis with antibodies specific to collagens I or IV (in each case, lanes 1 and 2 were not digested with MMPs, nondigested controls). (**A**) Treated with MMP-1, antibody to collagen type I. (**B**) Treated with MMP-13, antibody to collagen type I. (**C**) Treated with MMP-2, antibody to collagen type IV. (**D**) Treated with MMP-9, antibody to collagen type IV. (**A**–**D**) Compared to samples extracted from untreated control corneas (lane1), which displayed susceptibility to digestion with all MMPs tested here (lanes 3, 5, 7, and 9), samples extracted from RFUVA–cross-linked corneas (lane 2) exhibited resistance to MMP degradation (lanes 4, 6, 8, and 10).

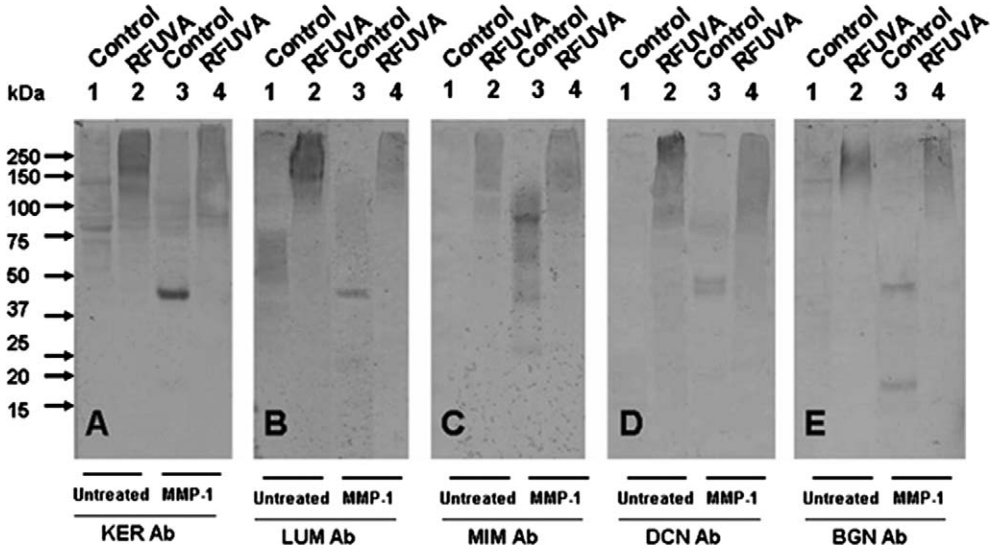

Figure 5. Migration of native PGs extracted from control and RFUVA–cross-linked ex vivo corneas, then subsequently digested with MMP-1. Detection of their SLRP core proteins via Western blotting with antibodies specific to each of the following SLRPs: (**A**) keratocan; (**B**) lumican; (**C**) mimecan; (**D**) decorin; and (**E**) biglycan. Compared to untreated samples (lane 1), all five PG core proteins as normally glycosylated corneal PGs, as a broad range of MWs, undergo digestion, generating at least one significant peptide of lower-MW upon incubation with MMP-1 (lane 3). In sharp contrast, corneal PGs extracted from corneas that had undergone RFUVA cross-linking migrated as a range of high-MW molecules, even after incubation with MMP-1, thus indicating that exposure of whole corneas to RFUVA generates cross-linked proteoglycan SLRP core proteins that can resist degradation by MMP-1.

Table 2. Molecular Modifications of Recombinant Human SLRPs from Wild-Type Sequences, To Foster Their Solubility as Non-Glycosylated Proteins

Recombinant Human SLRP	Molecular Mass, kDa	Location of Modification	Commercial Source
Keratocan	36.63	Partial 253AA–351AA, with GST tag at the N terminus	Abnova Corp.
Lumican	37.5	Mouse myeloma cell line NS0-derived Gln19–Asn338, with a C-terminal 6-His tag	R&D Systems
Mimecan	32.6	Mouse myeloma cell line NS0-derived Ala20–Phe298, with a C-terminal 6-His tag	R&D Systems
Decorin	38	*Spodoptera frugiperda* Sf21(baculovirus)-derived Gly17–Lys359, with a C-terminal 6-His tag	R&D Systems
Biglycan	37.5	Mouse myeloma cell line NS0-derived Asp38–Lys368, with a C-terminal 6-His tag	R&D Systems

core proteins, together with other potential sites of glycosylation, collectively generate PG molecules with a range of MWs (Figs. 6A, 6B, lane 1). In response to treatment with MMP-1, the intensity levels of KS and CS in control samples sharply decreased or disappeared (Figs. 6A, 6B, lane 3) because digestion of their attached core proteins released peptide fragments carrying the GAG chains, which thereafter were likely washed out of the gels following SDS-PAGE. However, in samples extracted from RFUVA-treated corneas, significant reactivity of KS and CS is detected in the same high MW region for RFUVA-treated samples treated with MMP-1 (Figs. 6A, 6B, lane 4) as for their respective nondigested controls (Figs. 6A, 6B, lane 1). This indicates that the core SLRP to which they are natively attached remain cross-linked to one another (as shown in Fig. 5) and to other proteins, and remain intact even after incubation with MMP-1, as revealed by the location of their GAG chain prosthetic groups. Thus, the presence of KS and CS chains occurs in the same high-MW regions of the same samples those probed for the presence of the core SLRP (Fig. 5, lanes 2 and 4). These results, significantly, indicate that RFUVA treatment of whole corneas not only effectively cross-links collagens and SLRP core proteins and renders them resistant to degradation by MMPs, but also immobilizes the posttranslational modifications attached to them, in this case, the KS and CS chains, which by themselves do not participate in RFUVA-induced cross-linking.

Discussion

In the cornea, collagen cross-linking occurs naturally with aging due to an oxidative deamination reaction that takes place within the end chains of the collagen.[46,47] Keratoconus is generally first diagnosed in young people at puberty or in their late teens, progresses for 10 to 20 years, and then slows or stabilizes by approximately the age of 40.[48] Irradiation of the cornea with UVA in the presence of RF[40,41] effectively stops the progression of keratoconus, although the mechanism by which RFUVA cross-linking stops it remains to be demonstrated.

In this study, we used both an in vitro and an ex vivo model reaction system to investigate the effects of RFUVA cross-linking of collagens and SLRPs on their ability to resist enzymatic digestion by MMPs, an interaction very likely to occur following clinical corneal cross-linking. Data presented in Table 1 indicate the changes in molar percentage of amino acids that are detected before and after RFUVA cross-linking of collagen types I and IV. Compared to native collagens, RFUVA causes distinct decreases in molar percentages of Met, Tyr, His, Hlys, and Lys in cross-linked collagens, suggesting that these amino acids are modified by RFUVA, just as they are as free amino acids in solution, with the latter being very susceptible to photodegradation in the presence of RFUVA.[49] During corneal RFUVA cross-linking treatment, RF is a key component as it is excited by UVA into its triplet state, generating singlet oxygen that can react further with various molecules,[41] inducing chemical bonds to form that covalently link amino groups of collagen fibrils[50] with those of other proteins in the corneal stroma extracellular matrix.[42] These reactions may involve tyrosine residues,[51] advanced glycation end products, [50,52] or changes in secondary or tertiary structure.[53,54] Tyrosine residues in the terminal, telopeptide domains of collagen alpha chains can form pi-pi complexes,[55] leading to dityrosine cross-links.[56–59] Histidine residues in collagen may produce cross-links in the presence of singlet oxygen.[60] Methionine residues in collagen fibrils may be involved in RFUVA cross-linking as indicated by changes in the patterns of CNBr cleavage products.[42] In addition, irradiation of RFUVA can induce lysine to form glycosyl-lysine cross-links (Maillard reaction) and so-called advanced glycation end products (AGEs),[40,52] suggesting that corneal cross-linking is carbonyl-dependent and involves the formation of AGE cross-links.[41,50] RFUVA thus causes apparent disappearance of tyrosine, histidine, methionine, and lysine/

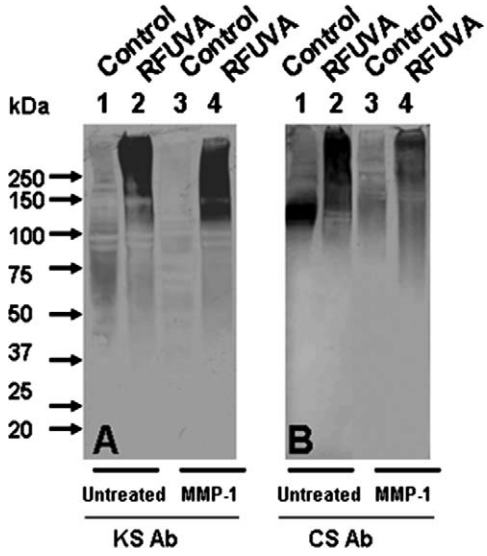

Figure 6. Migration of native PGs extracted from control and RFUVA–cross-linked ex vivo corneas, then subsequently digested with MMP-1. Locations of the PG core protein SLRPs are revealed by detection of their covalently attached GAGs. (**A**) Western blots using antibody for KS. (**B**) Western blots using antibody for CS. Compared to untreated samples (lane 1), the core SLRPs, which are natively covalently attached to KS or CS GAG chains in normal corneal PGs, undergo digestion to lower-MW forms upon incubation with MMP-1 (lane 3), carrying with them into consequent disappearance the attached GAG chains. In sharp contrast, corneal PGs extracted from corneas that had undergone RFUVA cross-linking, migrated as a range of high-MW molecules, even after incubation with MMP-1, thus indicating that exposure of whole corneas to RFUVA generates cross-linked PGs whose core proteins can resist degradation by MMP-1 and thus can retain their covalently attached GAG chains (lane 4), even though such GAG chains do not participate in RFUVA covalent cross-linking per se.

hydroxylysine while concomitantly converting their structures while still in peptide linkage to participate in covalent cross-links (which are not detected during normal amino acid analyses) (Table 1).

Preventing protein degradation by MMPs is an essential goal in the clinical efficacy of the cross-linking treatment for keratoconus because already demonstrated increased MMPs activity is one of the most important factors causing corneal thinning. The stroma comprises approximately 90% of the cornea thickness in humans.[5] Collagen gives the cornea its strength, elasticity, and form.[6] Type I collagen, the most abundant collagen in corneal stroma,[10] is composed of three polypeptide chains: two types of single chains, α1 and α 2, both of which are accessible to polymerization through intra- and intermolecular bonds.[61] Dimers of α chains are called β components (composed of α1-α2 or α1-α1 chains). Trimers of three α chains are called γ components. The strength of the collagen fibers depends on the formation of covalent cross-links between the N and C termini, nonhelical telopeptide domains, and adjacent helical domains of collagen molecules.[62] RF-sensitized photodynamic modification of collagen causes formation of cross-linked molecules in which an energy transfer between the excited RF and molecular oxygen produces singlet oxygen that then interacts with the collagen fibrils in an oxidation reaction to form physical, covalent cross-links.[52]

Previous studies demonstrated that RFUVA mainly causes α1, α2, β11, and β12 chains to cross-link into macromolecules at least as large and larger than γ components, including those trapped at the bottom of sample wells, and thus disappear from their normal α-, and β-chain positions on electrophoretic gels.[42,44] Intermicrofibrillar cross-links significantly affect the mechanical properties of corneal collagen tissue.[63] Earlier studies showed that RFUVA–cross-linked intact rabbit corneal tissues can resist digestion by bacterial collagenase.[64,65] However, effects of RFUVA on resistance of collagen fibrils and PG core proteins to digestion by corneal endogenous MMPs remained lacking. Our data presented here clearly demonstrate that the initial γ band of collagen type I in the absence of RFUVA (Fig. 1) is distinctly digested by MMPs, whereas the new γ-components formed by RFUVA are very resistant to digestion. For the stabilizing property of cross-linked collagen, one possible explanation may be the changes of the tertiary structure to prevent MMPs access to their specific cleavage sites by steric hindrance.[66,67] In addition, it is likely that some of the normal MMP cleavage sites within the collagen fibrils may be blocked during the RFUVA cross-linking, thus forming new high-molecular-weight polymers, as observed at the γ band position and the bottom of the sample wells (Fig. 1), suggesting that the MMPs cleavage sites may be modified by RFUVA cross-linking. In addition, data presented in Figure 2 show that non–cross-linked control type I collagen appears more susceptible to digestion by MMP-2 and MMP-9 than to that by MMP-1 or MMP-13, whereas RFUVA–cross-linked collagen appears totally resistant to MMP-13 and highly resistant to MMP-1, -2, and -9.

Data presented in Figure 3 reveal that the presence of soluble collagen during RFUVA does not increase the subsequent digestion of soluble, commercial, recombinant, nonglycosylated, chemically tagged (Table 2) SLRPs by MMPs. Thus, in both non-RFUVA–cross-linked and RFUVA–cross-linked form, such SLRPs are mostly resistant to digestion by the four most common SLRPs of the cornea, whereas in sharp contrast, they are quite digestible by those same MMPs when the SLRPs are available ex vivo in their normal glycosylated forms and bound to fibrillar collagen. In cornea and in other tissues, any of these core proteins may exist either in forms lacking glycosylation or with glycosylation consisting of GAGs chains of various lengths. Such PG core proteins, even when heavily glycosylated with GAG chains, are modeled as binding with their leucine-rich domains directly to the sides of collagen fibrils.[68,69] The narrow collagen fibrils of corneal stroma associate with the core SLRPs of the PGs, whose sulfated GAG chains appear as variable-length linear structures extending laterally from the collagen fibrils.[70] Considering these structural models, our data show that RFUVA causes PG core proteins to crosslink themselves, blocking the MMPs cleavage sites as effectively in the absence or presence of soluble collagen, leading core proteins to form high-MW copolymers (Fig. 3A, lane 4). In addition, the covalent cross-linking between PG core proteins and collagen induced by RFUVA may result in saturation of the MMPs cleavages sites on collagen fibrils.[42] The ability of cross-linked PG core proteins to protect collagen fibrils from catabolism by MMPs provides an additional potential effect of the interaction of SLRPs and collagen. Moreover, data presented in Figure 3B also suggested that nonglycosylated, commercially modified SLRPs, although mostly resistant to all 4 MMPs, appear to be digested by MMP-13 and MMP-9 somewhat more easily than by MMP-2 and MMP-1.

Soluble commercial, recombinant, nonglycosylated chemically tagged (Table 2) SLRPs generally were resistant to digestion with the four most common corneal MMPs (Figs. 3A, 3B), whereas those same core SLRPs were readily digested by those same MMPs when exposed to them after their extraction in their native glycosylated forms from whole corneas ex vivo. We propose three possible explanations for this contrast in sensitivity of these SLRPs to degradation by MMPs: (1) the commercial changes made in the recombinant versions of the SLRP proteins (to make them soluble) (Table 2) may render them mostly resistant to digestion by corneal MMPs; (2) glycosylation of SLRPs , generally with GAG chains of KS or CS, changes their conformation, allowing them to become digestible by MMPs; and/or (3) normal binding of the glycosylated SLRPs to their binding partners in vivo (e.g., collagen fibrils) allows them to become digestible by MMPs. Using native PGs extracted from corneas ex vivo, MMPs are able to digest SLRP core proteins. However, after treatment with RFUVA, cross-links form between those proteins in such a manner that they become much more resistant to digestion, even in the presence of soluble collagen, by the normal extracellular degradative enzymes of the corneal stroma, the MMPs, as demonstrated in this study.

The ECM of normal corneas becomes more cross-linked and insoluble during normal aging, and RFUVA treatment of the cornea (including the already-insoluble ECM mass that has slowly formed there) adds additional covalent cross-links within that mass between proteins already there and adds more proteins to it. In this study, 4 M GHCl was used to extract the soluble molecules of the corneal stroma from both normal control and RFUVA–cross-linked bovine corneas ex vivo. As a classical method for protein extraction, 4 M GHCl has been widely used to isolate PGs[43,71] and also extract collagens from corneal tissues.[72,73] In this type of extraction, noncovalent intra- and intermolecular bonds between macromolecules are broken, and the corneal aggregate structure is dissociated into its soluble components.[74] Our results demonstrated that GHCl extraction was an effective method for isolation of partially cross-linked collagens and PGs from normal control corneas, and from RFUVA–cross-linked bovine corneas, although the recovery of total PGs and collagen is incomplete. However, more details of molecular cross-linking could be found by studying the insoluble aggregated ECM of normal and RFUVA corneas and their responses to MMPs. Nonetheless, the cross-linking process can still be studied profitably in this representative, soluble portion of the stromal ECM.

Evidence presented in Figure 4 indicates that corneal collagen types I and IV, examined here after undergoing RFUVA cross-linking ex vivo followed by extraction with GHCl, both display resistance to degradation by MMPs. Collagen type IV is a primary component of corneal epithelial basement membranes, a structure important for corneal functions and cell adhesion.[75] Disrupted distribution of type IV collagen was noted in the basement membrane of the keratoconic corneas.[76,77] Basement membrane alterations may interfere with critical interactions of the corneal epithelium with the underlying basement membrane, as well as cell-matrix interactions and matrix organization in the stroma.[78] Interestingly, our data demonstrated that RFUVA–cross-linked collagen type IV can resist degradation by MMP-2 and MMP-9, suggesting that further damage to the corneal basement membrane of keratoconus may be halted by RFUVA treatment.

In the present study, we also examined the responses of ex vivo native and RFUVA–cross-linked PG core proteins to digestion with MMPs. Our recent work demonstrated that RFUVA causes linkages between collagens and PG core proteins both in vitro and ex vivo.[42] Here, we demonstrate that RFUVA–cross-linked PG core proteins display resistance to digestion with MMP-1 (Fig. 5). Moreover, data presented in Figure 6 reveal that the normal GAG chains, KS and CS, natively attached to PG core proteins, disappear along with the remains of their linkage region peptides upon digestion of non-RFUVA–cross-linked corneal stroma with MMPs. However, they remain intact, still attached to their nondigested core proteins after RFUVA cross-linking. Thus, complete, native PGs glycosylated with attached GAG chains in the corneal stroma are susceptible to degradation by MMPs, but become resistant to degradation in response to RFUVA treatment either in vitro solution or in native corneal stromal matrix ex vivo.

In summary, this work presents a novel approach for understanding the mechanism by which RFUVA cross-linking appears to stop the progression of keratoconus. Our results strikingly demonstrate that corneal RFUVA–cross-linked collagen and PG core proteins can resist cleavage by MMPs. Moreover, it is likely that the formation of covalent cross-links between collagen and SLRPs induced by RFUVA contributes added protection to collagen fibrils from degradation by MMPs. The ability of MMPs to digest several other corneal ECM proteins before and after RFUVA cross-linking in solution in vitro versus ex vivo (e.g., collagen types I with V and collagen type IV with laminin, fibronectin, and fibrillin) remain to be characterized.

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