Journal of Cellular Biochemistry 104:1684–1698 (2008)

Non-Enzymatic Glycation of Type I Collagen Diminishes Collagen–Proteoglycan Binding and Weakens Cell Adhesion

Kristin L. Reigle,[1] Gloria Di Lullo,[1] Kevin R. Turner,[1] Jerold A. Last,[2] Inna Chervoneva,[3] David E. Birk,[4] James L. Funderburgh,[5] Elizabeth Elrod,[6] Markus W. Germann,[6] Charles Surber,[7] Ralph D. Sanderson,[7] and James D. San Antonio[1,4]*

[1]Department of Medicine, Cardeza Foundation for Hematologic Research, Thomas Jefferson University, Philadelphia, Pennsylvania
[2]Pulmonary and Critical Care Medicine, University of California, Davis, California
[3]Department of Pharmacology and Experimental Therapeutics, Division of Biostatistics, Thomas Jefferson University, Philadelphia, Pennsylvania
[4]Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania
[5]Department of Ophthalmology, University of Pittsburgh, Pittsburgh, Pennsylvania
[6]Departments of Chemistry and Biology, Georgia State University, Atlanta, Georgia
[7]Department of Pathology, University of Alabama, Birmingham, Alabama

Abstract Non-enzymatic glycation of type I collagen occurs in aging and diabetes, and may affect collagen solubility, charge, polymerization, and intermolecular interactions. Proteoglycans[1] (PGs) bind type I collagen and are proposed to regulate fibril assembly, function, and cell–collagen interactions. Moreover, on the collagen fibril a keratan sulfate (KS) PG binding region overlaps with preferred collagen glycation sites. Thus, we examined the effect of collagen modified by simple glycation on PG–collagen interactions. By affinity coelectrophoresis (ACE), we found reduced affinities of heparin and KSPGs for glycated but not normal collagen, whereas the dermatan sulfate (DS)PGs decorin and biglycan bound similarly to both, and that the affinity of heparin for normal collagen decreased with increasing pH. Circular dichroism (CD) spectroscopy revealed normal and glycated collagens to assume triple helical conformations, but heparin addition caused precipitation and decreased triple helical content—effects that were more marked with glycated collagen. A spectrophotometric assay revealed slower polymerization of glycated collagen. However, ultrastructural analyses indicated that fibrils assembled from normal and glycated collagen exhibited normal periodicity, and had similar structures and comparable diameter distributions. B-cells expressing the cell surface heparan sulfate PG syndecan-1 adhered well to normal but not glycated collagen, and endothelial cell migration was delayed on glycated collagen. We speculate that glycation diminishes the electrostatic interactions between type I collagen and PGs, and may interfere with core protein-collagen associations for KSPGs but not DSPGs. Therefore in vivo, collagen glycation may weaken PG–collagen interactions, thereby disrupting matrix integrity and cell–collagen interactions, adhesion, and migration. J. Cell. Biochem. 104: 1684–1698, 2008. © 2008 Wiley-Liss, Inc.

Key words: glycation; type I collagen; proteoglycan; diabetes; aging; extracellular matrix; endothelial cells

Abbreviations used: PG, proteoglycans; KS, keratan sulfate; HS, heparan sulfate; DS, dermatan sulfate; CS, chondroitin sulfate; GAG, glycosaminoglycan; ACE, affinity coelectrophoresis; CD, circular dichroism; AGE, advanced glycation end products; CB, cyanogen bromide; MOPSO, 3-(*N*-morpholino)-2hydroxypropanesulfaonate.

Grant sponsor: NIH; Grant numbers: HL053590, AR049604, CA68494, EY05129.

*Correspondence to: James D. San Antonio, Department of Medicine, Cardeza Foundation for Hematologic Research, Thomas Jefferson University, 809 Curtis, 1015 Walnut St., Philadelphia, PA 19107-5099.
E-mail: james.sanantonio@jefferson.edu

Received 18 January 2008; Accepted 23 January 2008
DOI 10.1002/jcb.21735

© 2008 Wiley-Liss, Inc.

Type I collagen is ubiquitous in all vertebrates, and is among the largest and most complex of all macromolecules [see for review Linsenmayer, 1991; Prockop and Kivirikko, 1995]. It is synthesized as a soluble, procollagen form containing two α1, and one α2 chains, each of about 1,000 amino acids. Upon secretion from the cell the propeptides are cleaved, and the collagen monomer is assembled into the fibril, proposed to consist of aggregates of microfibrils, or 5-mer bundles of overlapping monomers. Type I collagen fibrils contribute to the integrity and function of many tissues via interactions with other collagens, PGs, and growth and differentiation factors [Di Lullo et al., 2002]. In aging, type I collagen becomes less flexible and more acid insoluble, which correlates with its content of cross-linked advanced glycation end products (AGE) [Brennan, 1989]. In diabetes, the collagen fibril also becomes abnormally stiff, insoluble, and cross-linked, correlating with high levels of circulating glucose [Andreassen et al., 1981; Schnider and Kohn, 1982]. It is proposed that alterations in type I collagen structure and function through glycation may underlie various pathologies associated with aging and diabetes [Paul and Bailey, 1996; Yan et al., 2003].

Glycation and AGE form in vivo and in vitro on collagen via non-enzymatic reactions that covalently add a sugar moiety onto the protein [see for review Paul and Bailey, 1996; Tsilibary, 2003]. The formation of a simple glycation product involves a reaction between the aldehyde, open chain glucose with the ε-amino group of a free lysine residue of collagen to generate a Schiff-base intermediate, followed by its rearrangement to the more stable Amadori product. Although the modified lysine side group has the potential to remain ionized, it is likely that the attached sugar moiety may sterically interfere with electrostatic interactions between it and its binding partners. Fructosyl-lysine residues may then, via a complex series of reactions, create intra- or intermolecular covalent cross-links with free amino groups of the protein, which are distinct from the enzyme-mediated cross-linking of collagen at its globular ends. The chemistry of mature AGE structures that occur in vivo are thought to include pentosidine and pyralline and *N*-epsilon-carboxymethyllysine intermolecular cross-links, among others. AGE formation takes several weeks and thus primarily affects proteins with long half-lives such as matrix constituents. The residues on type I collagen that serve as substrates for simple glycation have been identified on the α1(I) CB (cyanogen bromide) peptide 3 and α2(I) CB3-5 cleavage fragments which comprise approximately 25% of the molecule [Reiser et al., 1992]; in these regions, nearly twenty residues were found to be glycated, and of these, lysines α1(I) 434, and α2(I) 453, 479, and 924 were the most frequently modified.

PGs are matrix or cell surface molecules composed of core proteins to which one or more glycosaminoglycan (GAG) chains are covalently attached [Hascall et al., 1991]. PGs are proposed to play key roles in matrix assembly and function via their associations with collagens, fibronectin, and other matrix molecules, and on the cell surface as receptors for matrix components, growth factors, and cytokines [Ruoslahti, 1988; Bernfield et al., 1992; Klass et al., 2000; Sanderson et al., 2004]. It has been speculated that changes in type I collagen net charge due to glycation, which neutralizes basic charge on lysine residues, could significantly affect interactions with its binding partners including the anionic PGs [Paul and Bailey, 1996], that are proposed to bind to collagen electrostatically, at least in part [Ruoslahti, 1988; San Antonio et al., 1993]. Moreover, we have reported that several predominant glycation sites on the type I collagen fibril co-localize with regions proposed to bind KSPGs and HSPGs, but not DSPGs [Di Lullo et al., 2002]. Here we test the hypothesis that collagen–PG interactions are influenced by collagen glycation, by examining the binding of heparin, KSPGs and DSPGs to normal collagen and to collagen modified by simple glycation, as well as the consequences of simple glycation on collagen conformation, polymerization, and cell–collagen interactions.

MATERIALS AND METHODS

Collagen Preparation and Glycation

Type I collagen was isolated from rat tail tendon [San Antonio et al., 1992]. Collagen glycation was carried out as detailed [Reiser et al., 1992]. In brief, a collagen suspension of 8–25 mg/ml in a 1.0 ml reaction volume was incubated for 24 h in a 37°C water bath with D-glucose (500 mg/ml) in 20 mM Na Phosphate-0.9% NaCl, pH 7.4 (PBS), with 3.0 mM sodium azide added as a preservative in a sterile 12-ml

conical centrifuge tube, with occasional mixing. The reaction mixture became noticeably more viscous and clear as the collagen was glycated and solubilized. At the end of the incubation, the tube was placed on ice, and 1.0 ml of 95% ethanol slowly added with continuous mixing. The sample was centrifuged for 10 min at 4,000 rpm, and the ethanol layer decanted and discarded. Concentrations of acid soluble collagens were determined by amino acid analysis (AAA Service Laboratory, Boring, OR).

Fructosamine Assay

Levels of glycation of type I collagen were measured using the fructosamine kit (Catachem, Bridgeport, CT). Type I collagen in 0.5 M acetic acid was heat denatured for 1 h at 65°C and then neutralized with NaOH. Collagen samples and glycation standards supplied with the kit were mixed with the color reagent at a 1:19 ratio in wells of microtiter plates (Nalge Nunc Intl) for 1 min at room temperature, and then placed in a microplate spectrophotometer (Molecular Devices) at 37°C. OD_{530} measurements were taken at 1 min intervals from 5 to 10 min and plotted versus time. Only samples yielding curves with linear correlation coefficients (R) ≥ 0.8 were evaluated. Fructosamine concentrations were calculated from the ratio of the sample slope to that of the glycation standards, which gave concentrations readings 20–30% lower than those stated by the kit manufacturer.

Fibrillar Collagen Preparation

Type I collagen fibrils were prepared as described [San Antonio et al., 1994b]. In brief, lyophilized normal and glycated collagens were dissolved in 0.5 N acetic acid at 1.2 mg/ml and rocked gently at 4°C overnight. The collagens were dialyzed against 0.02 M Na_2HPO_4 for 48 h at 4°C. Following dialysis, the collagen formed a white precipitate. The collagens were sonicated on ice with 5–8 pulses, each pulse lasting ~5 s, with ~15 s between pulses to avoid heating and melting the collagen. Afterwards, a homogenous white solution was formed. The collagens were serially diluted with the same buffer, mixed with an equal volume of 2× ACE running buffer, and prepared for ACE as described below.

Radiolabeled Heparin and PG Preparation

Whole heparin from pig intestinal mucosa (Sigma; Grade I-A) was tyramine end-labeled as described [San Antonio et al., 1993]. Recombinant native-type human decorin and biglycan preparations were the generous gift of Dr. David McQuillan and LifeCell, Inc. For KSPG isolation, total PGs were extracted from minced bovine corneas at 4°C twice in 20 volumes of 0.2 M NaCl, 0.1% CHAPS detergent, 0.1 M 6-aminohexanoic acid, 0.01 M Na_2 EDTA, 0.05 M sodium acetate pH 5.8 with freshly added benzamidine (0.8 mg/ml) and phenylmethylsulfonyl fluoride (0.174 mg/ml). The extract was clarified by filtration and passed over a DEAE-Sephacel fast flow column which was subsequently washed with 0.5 M NaCl, 0.1% CHAPS in 0.02 M Tris–HCl pH 7.4. PGs were eluted by 2.0 M NaCl in the same buffer. The PG fraction was concentrated by ultrafiltration and dialyzed to 0.1 M Tris–acetate pH 7.5. Chondroitin-dermatan sulfate was digested at 37° for 4 h with 0.1 unit/mg protein of affinity-purified chondroitinase ABC (Seikagaku). The PGs were chromatographed a second time on DEAE-Sephacel and eluted in 20 mM Tris–HCl pH 7.4, 0.1% CHAPS using a NaCl gradient from 0.1 to 1.0 M. Fractions were screened by immunoblotting using anti-keratan sulfate antibody and the identified keratan sulfate PGs were pooled, concentrated using ultrafiltration, and dialyzed to 20 mM Na phosphate pH 7.0. Aliquots were stored at −80°C until use.

Heparin and PGs were radiolabeled with ^{125}I Na (MP Biomedicals, Costa Mesa, CA) to specific activities of $\cong 1 \times 10^7$ cpm/μg. For some experiments the radiolabeled heparin was Sephadex G-100 fractionated and the final ≅12% to elute was retained as the low M_r material of ≤6 kDa [Jordan et al., 1979].

Electrophoretic Analysis of Binding of Heparin and PGs to Type I Collagens

Binding of radioiodinated heparin and various PGs to type I collagen was studied by ACE [Lee and Lander, 1991; San Antonio et al., 1993; San Antonio and Lander, 2001]. Collagens were dissolved in 0.5 N acetic acid at 2.4 mg/ml, and serially diluted in 0.5 N acetic acid. Samples were neutralized with 0.5 N NaOH, mixed with warm 2× concentrated ACE running buffer (1× buffer was 50 mM sodium MOPSO/125 mM sodium acetate [pH 7.0]), mixed with 2% agarose and poured into agarose wells. Alternatively, native-type fibrils were formed from collagen samples as described previously and serially diluted in 2× running buffer then mixed

1:1 with 2% agarose and loaded into wells. In experiments where ACE was conducted at pHs other than 7.0, the following running buffers were instead used: 240 mM sodium acetate (pH 5.0) and 300 mM Tris–HCl (pH 8.0). Electrophoresis of radioiodinated heparin or PGs through the collagen-containing wells was then conducted. Gels were dried and PG or heparin mobility measured using a phosphorimager (Molecular Dynamics) by scanning each protein lane and determining relative radioactivity content per 88 μm pixel along the length of the lane. Calculation of retardation coefficients, curve fitting of binding isotherms, and determination of apparent K_ds were performed as detailed [Lee and Lander, 1991; San Antonio et al., 1993; San Antonio and Lander, 2001].

CD Spectroscopy

CD recordings of collagens were made using a Jasco 710 Spectropolarimeter. Spectra were measured in 0.5% acetic acid buffer and path lengths of the cuvettes used were 1.0, 2.0, and 5.0 mm. Collagen concentrations of 0.1 mg/ml were analyzed and four scans were averaged for each spectrum. To determine the effects of heparin on normal and glycated collagens, heparin was titrated at various concentrations into the collagen samples. All measurements were taken at 190–300 nm at room temperature.

Collagen Fibrillogenesis Assay

Type I collagen fibrillogenesis was studied as detailed [Sweeney et al., 1998], with slight modifications. To wells of 96-well Costar microplate dishes were added 170 μl of 2× buffer (0.28 M NaCl, 60 mM $NaPO_4$, pH 7.3), and 170 μl of 0.4 mg/ml normal or glycated type I collagen. Controls were run as buffer alone and water. The solutions were mixed by pipetting, plates were covered with lids coated with fog-X (Unelko Corp.) to reduce condensation, and absorbance at $\lambda = 405$ nm was monitored for 3–18 h at 1.5 min intervals at 37°C in a microplate spectrophotometer (Molecular Devices). In some experiments the effect of heparin on fibrillogenesis was studied as described above but with the addition of 20 μl of heparin solution (1.0 μg/μl water) to the wells.

Transmission Electron Microscopy and Fibril Diameter Analyses

Negatively stained samples were prepared as previously described [Birk et al., 1990]. Briefly, fibrils were absorbed onto formvar-coated copper grids and negatively stained with 2.0% aqueous uranyl acetate. For each of the different fibril preparations, that is, collagen and glycated collagen with and without heparin, three grids were prepared in quadruplicate sets. The negatively stained fibrils were examined and photographed using a Tecnai 12 transmission electron microscope operating at 80 kV equipped with a Gatan Ultrascan US1000 2K digital camera. Collagen fibril diameters were measured within a defined area of 1.83 μm^2 in 33 images per group using the following criteria: one diameter measurement per fibril, with only fibrils displaying distinct striations and edges being measured. Fibril diameter measurements were analyzed in a linear mixed effects model [Vonesh and Chinchilli, 1997] incorporating sample-to-sample variability. The assumption of a normal distribution was appropriate for fibrils not treated with heparin. The diameter measurements for fibrils treated with heparin were log transformed to satisfy the normality assumptions.

Cell–Collagen Attachment

Preparation of ARH-77 cells expressing syndecan-1 was as previously described [Liebersbach and Sanderson, 1994]. Briefly, ARH-77, a cell line established from a human plasma cell leukemia, were transfected with the cDNA for syndecan-1 and clones stably expressing syndecan-1 on the cell surface were isolated. Cell binding to collagen was assayed as described previously [Sanderson et al., 1992; Langford and Sanderson, 2002]. Briefly, 8.0×10^4 cells were incubated for 15 min at room temperature in 96-well plates (polyvinyl U-bottomed plates; Dynatech, Chantilly, VA) that had been coated with either type I collagen (1 mg/ml) or BSA (1 mg/ml). The plate was centrifuged at 120*g* for 20 min. Following centrifugation, cells were fixed and stained with 4% trypan blue in phosphate-buffered saline. In this assay, unbound cells form a densely stained pellet while bound cells remain as a uniform coating on the well surface.

Endothelial Cell Migration Assay

Human umbilical vein endothelial cells were isolated and cultured as described [Sweeney et al., 2003]. The migration assay was modified from a published protocol [Majack and Clowes, 1984]. Cells were plated at $1 \times 10^5/cm^2$ cells/well

onto 12-well plates coated with 100 μg/ml normal or glycated type I collagen in 10 mM acetic acid at 25 $\mu g/cm^2$ and supplemented with endothelial cell growth media and placed in the tissue culture incubator. On the third day, a "scrape wound" was fashioned across the center of the well by dragging a 200 μl plastic pipette tip through the cell monolayer. The cells were rinsed with Dulbecco's Phosphate Buffered Saline (DPBS+, Mediatech), and re-supplemented with culture media and at various times micrographs were taken of the wound area by phase contrast microscopy. Wound widths at their narrowest points were measured from the micrographs and data from cells cultured on normal vs. glycated collagen were compared using the Student's *t*-test.

RESULTS

We first used a fructosamine assay to estimate the level of glycation of normal and glycated collagen used in these studies. In four experiments, normal collagen exhibited undetectable levels of glycation, whereas glycated collagen contained 46 ± 15 ($n = 11$) mol glucose/mol triple helical collagen, confirming that our glycation protocol produced highly modified collagen.

To determine whether PGs exhibit altered interactions with normal versus glycated type I collagen, we first studied their heparin binding characteristics. Heparin is a structural analog of heparan sulfate (HS) GAG chains, which are integral components of HSPGs, ubiquitously expressed on cell surfaces and in the matrix [Hascall et al., 1991]. We used ACE to examine the binding of heparin to normal rat tail tendon type I collagen, or to the same collagen that had been glycated. ACE is a gel shift binding assay in which trace concentrations of radiolabeled GAGs or PGs are electrophoresed through a protein present at various concentrations in lanes of an agarose gel. Afterwards, based on the retardation of the mobility of the GAG or PG through binding at the various protein concentrations, as visualized by autoradiography or phosphorimaging, the dissociation constant (K_d) of GAG/PG–protein binding can be calculated as detailed in Materials and Methods Section. ACE provides accurate estimates of affinity of GAG and PG interactions with proteins including the collagens, fibronectin, laminin, and basic fibroblast growth factor [Lee and Lander, 1991; San Antonio et al., 1993, 1994a; San Antonio and Lander, 2001], as well as revealing binding heterogeneity when present, for example, for heparin–antithrombin III interactions [Lee and Lander, 1991]. Although studying the binding interactions of acid soluble collagens with purified GAGs and PGs may not ideally mimic PG–collagen fibril interactions in vivo, the approach does avoid complications of dealing with insoluble, heterotypic collagen fibrils, and is deemed to be an appropriate first step towards understanding the effects of collagen glycation on PG–collagen interactions.

For initial experiments, native-type fibrils were prepared from normal or glycated collagens; both produced fibrous precipitates, although that formed by the glycated collagen appeared sparser. ACE revealed appreciably stronger heparin-binding by normal ($K_d \cong 100$ nM) as compared with glycated collagen ($K_d \cong 250$ nM) (Fig. 1); the heparin-binding affinity of the normal sample is within the range of affinities we and others have reported for heparin–collagen interactions [McPherson et al., 1988; San Antonio et al., 1993, 1994b]. To determine whether the difference could be attributed to a defect in fibrillogenesis of the glycated sample, we next examined the heparin-binding characteristics of normal and glycated collagens neutralized from the acid soluble state and cast into agarose gels before they could undergo fibrillogenesis. Again, we found that in comparison with normal collagen the glycated sample bound heparin two- to three-fold more weakly (data not shown). These data show that glycated type I collagen displays defective heparin-binding, and that the effect is independent of the supramolecular state of the collagen.

To examine whether the reduced affinity of glycated collagen for heparin was specific for this GAG, we analyzed collagen binding of a mixture of native corneal KSPGs including lumican, keratocan, and mimecan, as well as human native recombinant forms of decorin and biglycan DSPGs. For these experiments, to conserve samples modified ACE gels were used where binding was examined at 100 or 500 nM protein concentrations, and the migration distances, or retardation coefficients (R) of the PGs through protein samples were measured. PG migration through normal versus glycated collagen was expressed as: R, glycated

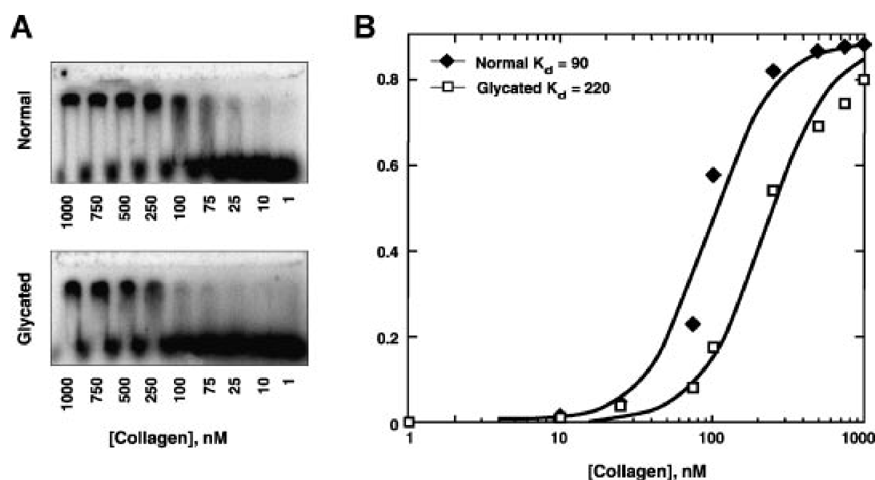


Fig. 1. Glycated collagen exhibits weaker affinity for heparin compared with normal collagen. Retardation coefficients (R) for the migration of whole ^{125}I-tyramine-heparin through fibrillar normal and glycated collagens were determined from ACE gel electrophoretograms (**panel A**) and are plotted against protein concentration (**panel B**). Smooth curves represent nonlinear least squares fits to the equation $R = R_\infty/(1 + (K_d/[\text{collagen}])^2)$. In three experiments glycated collagen showed reduced heparin binding affinity ($K_d = 250 \pm 40$ nM) compared to normal collagen ($K_d = 98 \pm 9$ nM).

collagen/R, normal collagen; ratios of approximately 1.0 indicate little or no difference in binding whereas values significantly less than 1.0 indicate weaker binding to glycated collagen. Significant binding of heparin and each of the PGs to normal collagen was seen, as evidenced by a retardation of heparin and PG migration at protein concentrations of 100 and 500 nM. Again, heparin exhibited weaker affinities for glycated collagen. This effect was also seen with KSPGs binding to glycated collagen at a concentration of 500 nM, whereas, in contrast, decorin and biglycan exhibited similar affinities for both collagens at 100 and 500 nM concentrations (Table I). That weaker affinities of KSPGs for collagen were observed only at the high collagen concentration may be due to the possibility that the affinity of KSPG–collagen binding is moderate in strength, falling in the 500 nM range. The contribution of collagen charge density to heparin binding affinity was also examined by performing ACE of heparin–collagen interactions at pH 5.0, 7.0, and 8.0. Affinity of heparin for collagen exhibited a strong pH dependency, ranging from $K_d \cong 10$ nM at pH 5.0 to nearly 100-fold weaker at pH 8.0 (Fig. 2).

The potential structural basis for the reduced binding affinity of glycated collagen for heparin and KSPGs was next examined. By CD spectroscopy, acid soluble samples of normal and glycated collagen showed positive elipticity at 223 nm and a large negative minimum at 198 nm, which are spectral characteristics of a

TABLE I. Reduced Binding Affinities of Glycated Collagen for KSPGs and Heparin

PG or GAG	$R_{glycated}/R_{normal} \pm SD$	n	*P* value
Biglycan			
100 nM	1.01 ± 0.15	6	≥ 0.25
500 nM	0.94 ± 0.09	6	≥ 0.05
Decorin			
100 nM	1.09 ± 0.16	6	≥ 0.10
500 nM	0.94 ± 0.15	6	≥ 0.15
Heparin			
100 nM	0.66 ± 0.22	5	≤ 0.02
KSPGs			
100 nM	1.24 ± 0.56	5	≥ 0.15
500 nM	0.68 ± 0.19	5	≤ 0.01

The relative binding affinities of PGs/GAGs for normal and glycated collagens were analyzed by ACE at collagen concentrations of 100 and 500 nM. R values were calculated as described in Materials and Methods Section, and values of $R_{glycated}/R_{normal}$ were used to compare the relative PG/GAG binding affinities for collagen samples; values were compared with1.0 (no difference in binding) by *t*-test to establish significance.

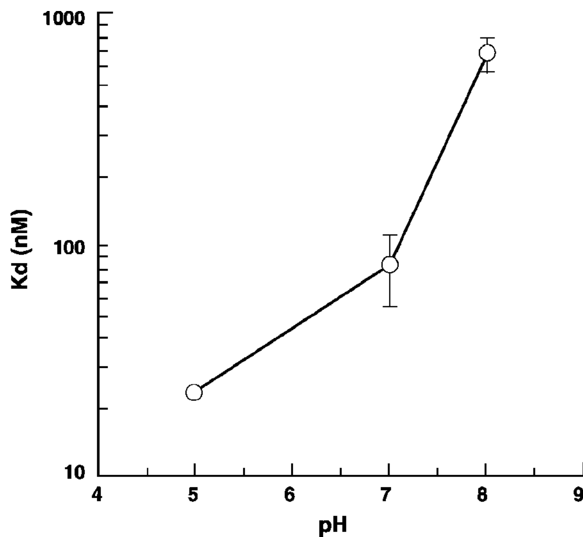
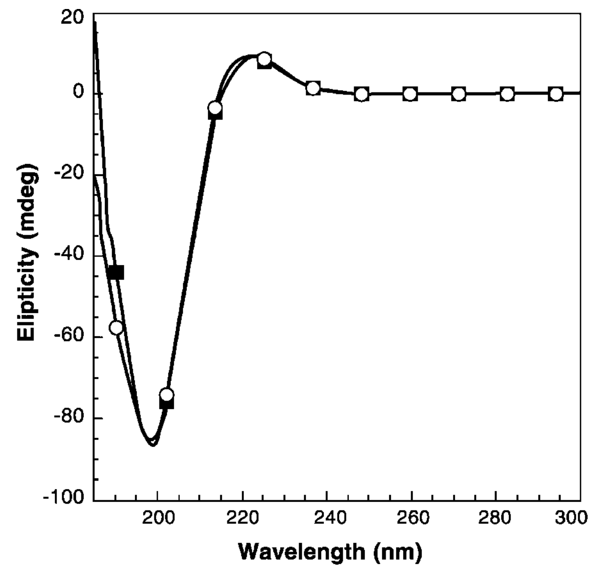


Fig. 2. Affinity of heparin for type I collagen increases with decreasing pH. ACE analysis of the binding of low M_r ^{125}I-T-heparin to acid soluble rat type I collagen was conducted in 240 mM sodium acetate (pH 5.0), MOPSO/Sodium acetate (pH 7.0), and 300 mM Tris–HCl (pH 8.0), and K_d values of heparin–collagen binding determined as described in the legend to Figure 1. Values represent data from three experiments.



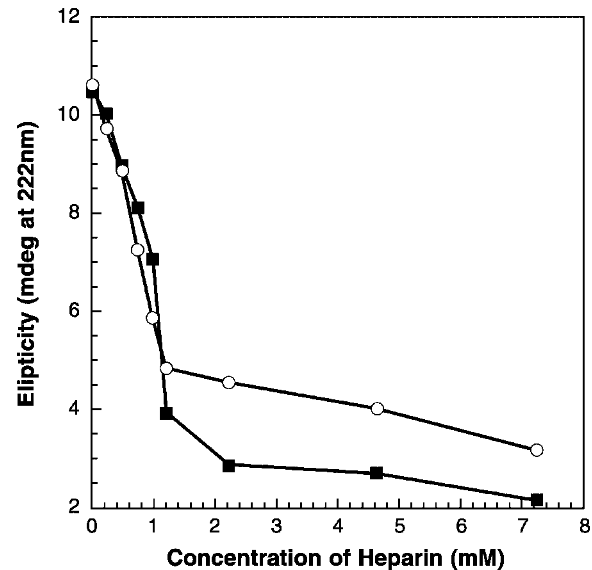


Fig. 3. Normal and glycated collagen assume a triple helical conformation but in the presence of heparin glycated collagen loses more triple helical content and precipitates more readily than normal collagen. **A**: CD Spectra of normal (open circles) and glycated (filled squares) collagen. Data were collected in a 1.0 mm cuvette for 0.2 mg/ml collagen in 0.5% HAC. **B**: Titration of normal (open circles) and glycated (filled squares) collagen with heparin. Spectra were collected in a 2.0 mm cuvette, for 0.1 mg/ml collagen in 0.5% HAC. Experiments were repeated three times.

collagen triple helix (Fig. 3A). The addition of heparin caused an apparent decrease in helical content of the collagen, which was more pronounced for the glycated sample (Fig. 3B). This is a reflection of the formation of aggregates and ultimately precipitation, which was observed at ratios higher than 3:1 heparin:glycated collagen (data not shown). Further CD experiments corroborated that in the presence of low heparin concentrations, both collagen samples eventually precipitated completely, although this happened sooner for the glycated sample. In each case, at the 6 h end-point, removing the precipitate by centrifugation left no CD signal in the soluble fraction (data not shown). Thus, by CD, the conformations of the soluble forms of the normal and glycated collagens appear similar, and heparin is more efficient at precipitating glycated collagen.

The kinetics of fibrillogenesis of the collagen preparations was examined by neutralizing the proteins from an acid soluble state and following sample turbidity spectrophotometrically. For normal collagen, polymerization initiated within min, proceeded exponentially within the first hr and reached a plateau within 1–3 h (Fig. 4). In contrast, the glycated collagen showed a significant delay in the onset of fibril formation and a slower rate of polymerization, yet achieved a plateau within 12–24 h (not shown). In some wells 20 μg of whole heparin was supplemented to sample wells at the time of collagen neutralization and the polymerization of normal collagen was delayed, but that of the glycated samples appeared unchanged (Fig. 4). Transmission electron microscopy was next used to examine the fine structure of the normal and glycated collagen fibrils formed in the

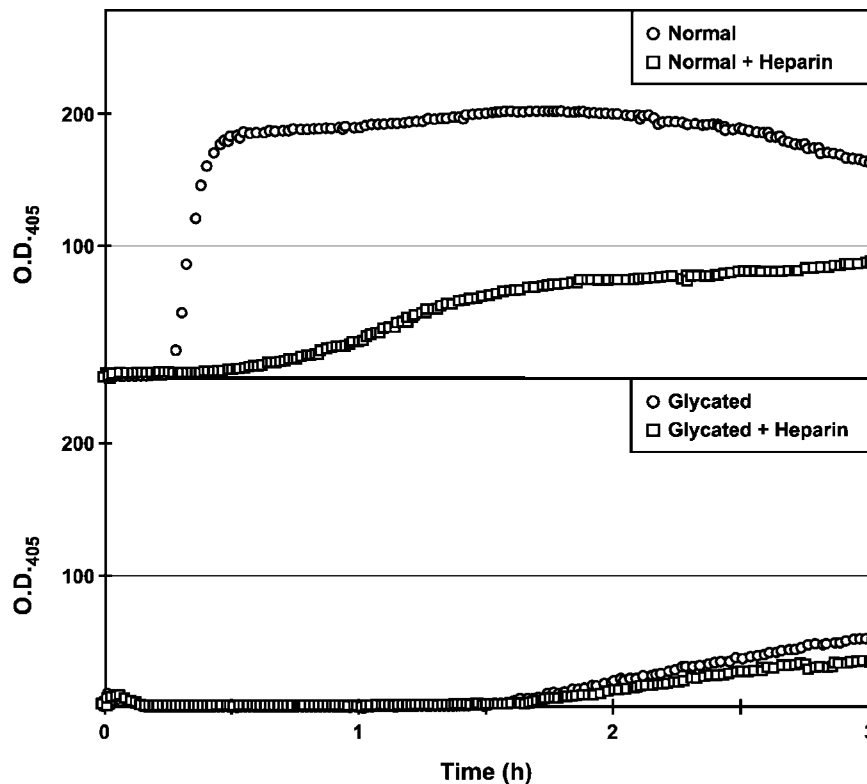


Fig. 4. Glycated collagen exhibits delayed fibrillogenesis compared with normal collagen. Fibrillogenesis was examined by neutralizing normal and glycated type I collagens from the acid soluble state ± heparin and following OD_{405} in a microplate spectrophotometer. Fibrillogenesis of glycated collagens was delayed but sample turbidity reached a plateau within 6–12 h (data not shown); heparin delayed fibrillogenesis only for normal collagen. Experiments were repeated three times.

presence or absence of heparin after 17 h in the polymerization assays. All fibril preparations displayed the typical 67 nm banding pattern characteristic of collagen fibrils (Fig. 5A). Fibril diameters were measured and their sizes compared between samples (Fig. 5B). For fibrils not treated with heparin, the mean difference between glycated and non-glycated fibril diameters was not significant ($P = 0.328$). On average, glycated fibrils were smaller by 1.2 nm. For fibrils treated with heparin, the mean difference between glycated and non-glycated fibril diameters was also not significant ($P = 0.719$). On average, glycated fibrils had a 2.4% decrease in diameter. The distribution of diameters of fibrils not treated with heparin was adequately represented by the normal distribution. Meanwhile, for fibrils treated with heparin, a log-normal right-skewed distribution was appropriate. Thus, for the normal distribution, the center location is the mean and the median, which are the same. For a right-skewed distribution, the center location is represented by the median, which is smaller than the mean. For the log-normal distribution, this median is computed as the back transformed mean of the log-transformed original measurements. Thus, there is a statistically significant increase in the proportion of smaller diameter fibrils in normal and glycated collagens polymerized in the presence of heparin, as compared with the same collagens not exposed to heparin.

Further experiments examined whether cell surface PGs exhibit altered interactions with glycated collagen, and how this may impact on cell–collagen interactions. For this purpose, the ARH-77 B-cell line was used as it lacks cell surface PGs but can be transfected to express the HSPG syndecan-1, a known ligand for type I collagen. Thus, the attachment of syndecan-1 expressing and control transfected cells to normal or glycated type I collagen or BSA-treated plastic substrata were examined in a microplate assay. It was found that syndecan-1 expressing cells attached strongly to normal collagen and weakly to glycated collagen, and not at all to BSA-treated plastic (Fig. 6). Finally, the effect of glycation of collagen to its ability to

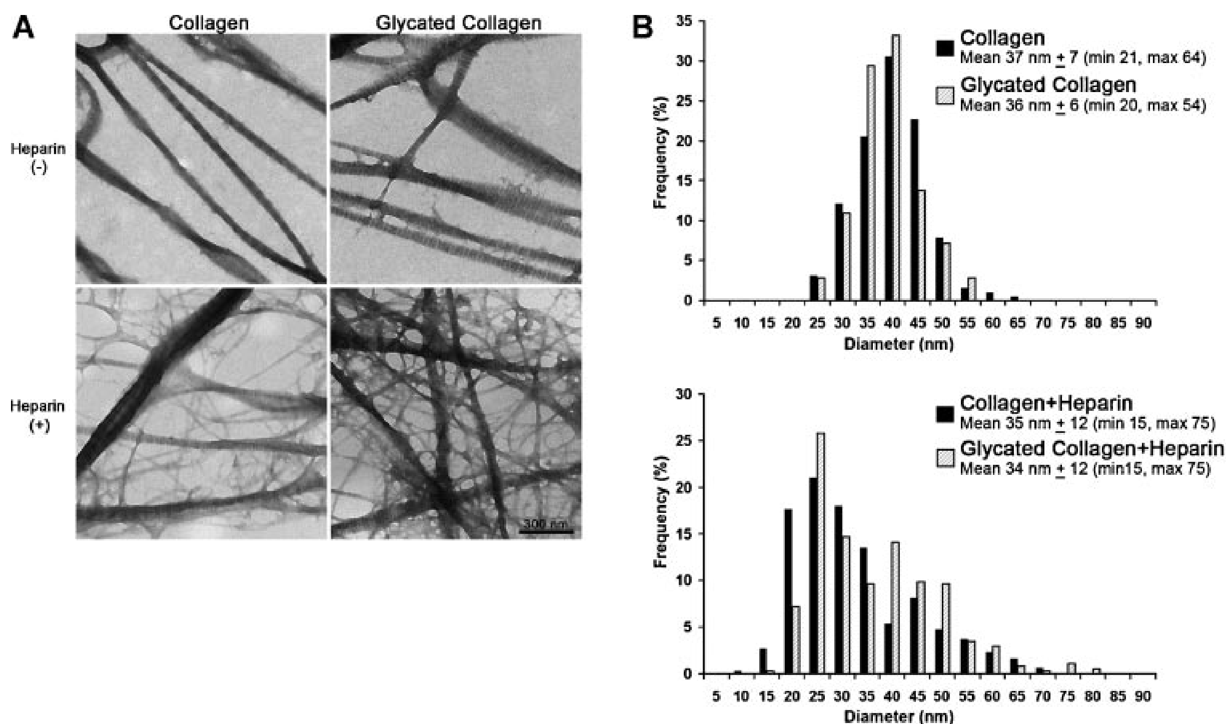


Fig. 5. Fibrils from normal and glycated collagens display normal periodicities and are of similar diameters. **A**: Collagen fibrils formed in fibrillogenesis assays (e.g., Fig. 4) were examined by TEM for morphology. Micrographs are representative samples from two experiments. **B**: Fibril diameter measurements were carried out as described in Materials and Methods Section using fibril preparations from at least two fibrillogenesis experiments.

support endothelial cell migration was examined in an in vitro endothelial cell "scrape wound" assay (Fig. 7). Thus, endothelial cells were plated onto normal or glycated collagen films and allowed to reach confluence. A scrape wound was then created in the cell monolayer, and its recovery was examined over time. It was observed that endothelial cell migration on glycated collagen was significantly delayed in comparison with the normal collagen, although all cultures repopulated the scrape wound area within 24 h.

DISCUSSION

Defective interactions between endothelial cells [Kuzuya et al., 1998; Chen et al., 2001], keratinocytes [Morita et al., 2005], osteosarcoma cells [Paul and Bailey, 1999], and fibroblasts [Kawano et al., 1990] with glycated type I collagen may be attributed to altered interactions between cell surface integrins and PGs with collagen fibrils. Moreover, glycation affects basement membrane protein interactions [Tsilibary et al., 1988], and may also influence the matrix binding properties of PGs. Finally, on the collagen fibril, one of the two major binding regions for KSPGs overlaps with several preferred glycation sites [Reiser et al., 1992; Hadley et al., 1998; Di Lullo et al., 2002]. The aim of this study was thus to determine whether type I collagen modified by simple glycation impacts collagen–PG interactions.

Heparin–collagen interactions are thought to rely upon both non-specific electrostatic binding and the fine structural features of both molecules. Thus, these interactions are disrupted by high salt concentrations [Koda et al., 1985; Keller et al., 1986]; however, affinity of heparin for collagen does not correlate solely with heparin charge density [San Antonio et al., 1993]; rather, the types and distributions of sulfated disaccharides, the presence of highly sulfated iduronic acid-rich domains, or even discrete collagen-binding sequences may also play roles. Although collagen binding sequences in heparin have not been identified, for B-cells, affinity for type I collagen correlates with high N-sulfation and 2-O-sulfation, and low 6-O-sulfation of the HS chains of cell surface syndecan-1 [Sanderson et al., 1994].

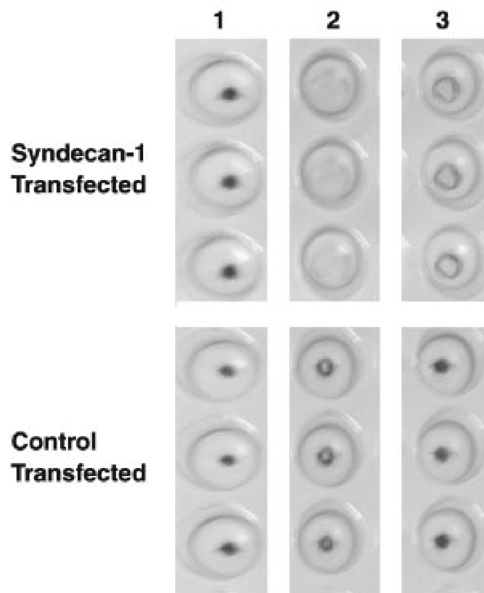


Fig. 6. Heparan sulfate-mediated binding of cells to glycated type I collagen is diminished as compared to normal type I collagen. Syndecan-1-transfected or control transfected ARH-77 cell binding to type I collagen coated microtiter wells was assessed. In this assay, following centrifugation, cells binding to collagen form a uniform coat over the well surface, cells not binding form a pellet. Cells expressing syndecan-1 bind tightly to normal type I collagen (lane 2), but not to control wells coated with BSA (lane 1), and weakly to wells coated with glycated type I collagen (lane 3). Control-transfected cells that lack syndecan-1 expression fail to bind BSA and collagens. Triplicate wells for each condition are shown. Wells shown are from a single plate from a representative experiment.

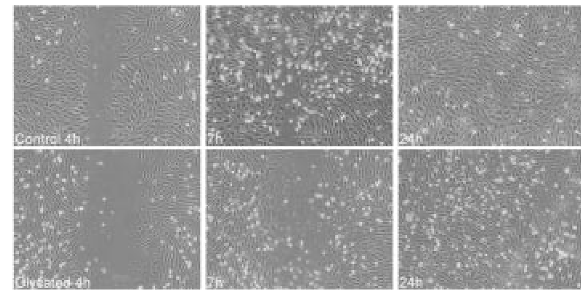


Fig. 7. EC migration is delayed on glycated collagen. EC plated on normal or glycated collagen films and allowed to reach confluence were ''scrape wounded,'' rinsed with PBS+, then re-supplemented with culture media. Scrape wound widths were greater in cultures on glycated collagen at 4 and 6 h (not shown) ($P < 0.01$; n = 7 cultures/time point) as compared with controls, and complete wound recovery took longer for cells cultured on glycated collagen. Experiments were repeated twice.

Several features of type I collagen are proposed to confer affinity for heparin. First, the basic charge (pI $\cong$ 8.0) of the polymer may favor electrostatic interactions with the anionic heparin chains. Second, the triple helical conformation is necessary for heparin binding, as melting of collagen greatly reduces its affinity for heparin [Keller et al., 1986; San Antonio et al., 1992]. Third, a discrete heparin-binding site at amino acids 87–93 of type I collagen may play a role [San Antonio et al., 1994b; Sweeney et al., 1998]. Therefore, the reduced affinity of heparin for glycated collagen seen here could result from the effects of glycation on any of collagen's electrostatic binding potential, triple helical conformation, or heparin-binding site function. Of these, our data are consistent with a role for glycation in altering the electrostatic binding potential of collagen. Thus, it was found that affinity of heparin for type I collagen is highly sensitive to its ionization state as the K_d of heparin–collagen binding varied from $\cong$10 nM at pH 5.0 to 1 μM at pH 8.0. These data imply that the affinity of heparin for collagen strengthens with an increase in the ionization state of the basic residues of collagen. By extension, for glycated collagen, reducing the availability of lysine residues to interact electrostatically, perhaps via steric hindrance of collagen–PG interactions by the glucose adducts, may reduce heparin-binding affinity. CD revealed that although the normal and glycated collagens each assume triple helical conformations, in the presence of heparin, the glycated collagen precipitated more readily, perhaps owing to a reduction in its number of available lysines. This study cannot evaluate whether the function of the heparin-binding sequence of type I collagen is affected by glycation, since the N-terminal region of the protein including the binding site has not been examined in this regard; however, potential glycation acceptor sites are present within and near the heparin-binding sequence. Moreover, the binding of heparin to the heparin-binding sequence could also be influenced by the glycation of residues elsewhere in the collagen fibril, since heparin can span the distance between the heparin-binding site on one monomer and some of the sites of preferential glycation on other monomers, for example, lysines 453 and 479 [Reiser et al., 1992].

In this study the effects of collagen glycation to PG binding were also examined. In many

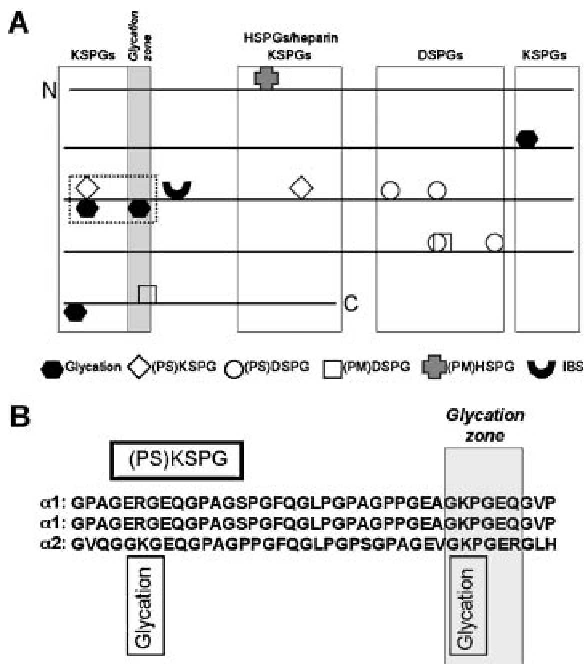


Fig. 8. Glycation and KSPG-binding regions co-localize on type I collagen. **A**: Schematic of the type I collagen D-period, or repeating structural subunit, showing cross-fibril zones for KSPG, KSPG/HSPG/heparin, and DSPG binding, predominant residues modified by glycation, the major glycation region of the fibril (shaded rectangle), proposed sequences (PS) for DSPG and KSPG binding, physically mapped (PM) sites for DSPG and HSPG binding, and the GFPGER integrin binding site (IBS). Collagen triple helical monomers are shown as black lines with N- and C-termini indicated; fibril structure is according to Chapman [1974]. For the sake of clarity monomers are separated from each other by considerable distances; however, in the native fibril they are tightly associated. Note that preferred glycation sites overlap with KSPG binding regions and two sites localize to the same triple helical regions as one of the proposed sequences for KSPG binding (dashed-lined rectangle). The electron dense banding pattern seen in EM preparations of positively stained fibrils is not shown; however, the KSPG binding region on the left includes the C1–C3 bands; the HSPG/heparin/KSPG region includes the A1–A4 bands; and the DSPG binding region the D and E1–E2 bands. **B**: Sequence of the triple helical region enclosed by the dashed-lined rectangle in panel A of this figure. Note that the GERGEQGPAGS sequence of the α1 chain proposed as a consensus site for KSPG binding [Scott and Glanville, 1993] co-localizes with a preferred glycation site on the α2 chain, and is only 18 residues away from a second glycation site and the major glycation region of the fibril (shaded rectangle), also in panel A of this figure.

tissues PGs are full-time binding partners of type I collagen; for example, fibrils may associate with decorin in tendon and KSPGs in the cornea [Ayad et al., 1998]. Thus, DSPGs including decorin were found to localize to the D and E1–E2 band region, and KSPGs including lumican, keratocan, and others mapped to the C1–C3 and A1–A4 band regions [Scott, 1991] (Fig. 8A). These PGs are thought to interact via their core proteins with discrete triple helical domains limited to one monomer within the D-period of the fibril; PG GAG chains are also proposed to bind to the collagen fibril and to project outwards and regulate inter-fibril interactions [Weber et al., 1996; Vesentini et al., 2005]. Binding sites for the DSPG/decorin core protein(s) were also mapped at high resolution to two sequences (Fig. 8A,B) [Scott et al., 1997].

The PGs tested here are members of the small leucine-rich PG family, comprised of core proteins each containing approximately 10 leucine-rich repeats, to which one or more GAG chains are attached [Ayad et al., 1998]. The DSPGs include recombinant forms of decorin and biglycan isolated under non-denaturing conditions and shown to exhibit native-type conformations and matrix interactive behaviors [Hocking et al., 1996; Ramamurthy et al., 1996]. The KSPG mixture was isolated from bovine cornea under similar conditions and contains lumican, keratocan and mimecan. In spite of the homology between the DSPGs and KSPGs, only the latter exhibited weaker affinity for glycated collagen. One interpretation is that KSPG binding to collagen, like that of heparin, may have a significant electrostatic component that is impacted by glycation, whereas the other PGs do not. This seems unlikely since the net charge densities of the various PGs used here are similar (data not shown). Another possibility is that the binding sites on the collagen fibril for KSPGs, but not DSPGs are directly impacted by glycation. This notion is consistent with the report that several preferred sites of glycation on the collagen fibril fall within one of the cross-fibril regions proposed to bind KSPGs including the C1–C3 bands [Di Lullo et al., 2002] (Fig. 8A). In that KSPG binding region, a candidate consensus sequence for core protein binding was identified [Scott and Glanville, 1993] (Fig. 8B) that contains one of the preferred glycation sites (lysine 453), and the other three sites are its near neighbors on the collagen fibril [Reiser et al., 1992], falling within less than 5% of the area of the entire D-period (Fig. 8A). On the fibril the C1 band is the most heavily impacted by glycation [Hadley et al., 1998]; interestingly, this region falls within the cross-fibril KSPG-binding zone including the C1-C3 bands. It is

also only 18 residues, or approximately 5.5 nM away from the candidate consensus KSPG binding sequence (Fig. 8A,B), but is distant from the DSPG binding region [Hadley et al., 1998; Di Lullo et al., 2002].

Glycation has been reported to affect various physical properties of type I collagen, resulting in the appearance of fluorescent cross-links, decreased acid solubility, pepsin resistance at low temperatures [Bai et al., 1992], altered packing density of fibrils and intermolecular cross-linking [Bai et al., 1992]. Collagens from rat tail tendon and diabetic human skin contain up to one glycated residue or cross-link per two moles of type I collagen [Brennan, 1989; Bailey et al., 1995]. We propose that such values be corrected to establish an estimate of the extent of glycation of the monomers on the fibril periphery, since most glycation is assumed to occur after the fibril is formed [Paul and Bailey, 1996]. Considering the ratio of inaccessible/exposed monomers in collagen fibrils may provide the "glycation correction factor" to convert moles glucose/monomer to moles glucose/exposed monomer. Thus, the fibril and component monomers may be modeled in cross-section as a circle containing tightly packed circles of equal area. Monomers on the periphery are assumed to be glycation substrates; all others are the non-glycated or inaccessible monomers. Using a database including the best known packings of equal circles in unit circles (http://hydra.nat.unimagdeburg.de/packing/cci/cci. html), and assuming the collagen monomer to be 1.5 nm in diameter, examples can be found representative of small fibrils of diameters 16 nm [Piez and Reddi, 1984], with 25 exposed monomers and 61 inaccessible monomers, and 36 nm, with 65 exposed monomers and 425 inaccessible monomers. The "glycation correction factor" for the 16 and 36 nm fibrils would be 2.4 and 6.5, respectively. Although models for larger fibrils are unavailable, our extrapolations for fibrils of 250 or 500 nm diameters typical in tendon [Bai et al., 1992] reveal glycation correction factors of at least 42 and 86, respectively (data not shown). Thus, for a homogenate generated from tendon comprised of 500 nM fibrils, if glucose/type I collagen monomer is measured to be 0.5:1.0 (mol/mol), the glycation density on the exposed monomers in that preparation may be as high as 42 mol glucose/mol collagen, which is in the range of the sample we used for our studies.

Here, that normal and glycated collagen exhibit similar triple helical conformations and fibrillar structures again suggests that glycation influences PG–collagen interactions by altering fine structural features of collagen such as the availability of its basic residues for intermolecular interactions. Glycated collagen was reported to delay fibrillogenesis, which was attributed to the disruption of charge–charge interactions between monomers needed for polymerization [Guitton et al., 1981]. In the present study, that fibrillogenesis of normal but not glycated collagen was delayed by heparin may also be a consequence of collagens reduced potential for electrostatic interactions. Indeed, it has been shown that KSPGs, DSPGs, and heparin reduce the rate of type I collagen polymerization [Vogel et al., 1984; Gavriel and Kagan, 1988; Rada et al., 1993], and therefore may regulate collagen fibril diameters in vivo. Our finding that normal and glycated collagen fibrils are similar in diameter and exhibit normal periodicity in the presence or absence of heparin implies that the reduced heparin-binding affinity of glycated collagen is likely not due to a difference in the availability or number of heparin-binding sites on the fibril.

KSPGs are present in many tissues as full-time binding partners of type I collagen and are most abundant in the cornea where they may regulate fibril organization and tissue transparency [Funderburgh et al., 1991; Ayad et al., 1998]. Lumican null mice exhibit corneal opacity, fragile skin and defects in collagen fibril size and organization [Chakravarti et al., 1998, 2000], and this PG may play roles in cell migration, adhesion, and growth in other tissues [Saika et al., 2000; Vij et al., 2004]. Based on our results, it is proposed that glycation may weaken KSPG–collagen interactions in vivo, by disrupting core protein- and GAG-fibril binding, and contributing to altered fibril organization, which are observed in corneal pathologies in aging and diabetes [Paul and Bailey, 1996]. Cell surface HSPGs including the syndecans are considered to be part-time binding partners of type I collagen, and to function with integrins to promote cell adhesion to type I collagen and other matrix components [Koda et al., 1985; Woods and Couchman, 1998]. In B-cells, the lack of cell surface syndecan-1 correlates with the propensity to invade a type I collagen gel, consistent with the malignant phenotype [Liebersbach and Sanderson, 1994].

Syndecans are also proposed to play roles in matrix assembly, turnover, and wound healing [Bernfield et al., 1992; Klass et al., 2000; Echtermeyer et al., 2001]. Consistent with these findings, we observed a disruption of syndecan-mediated B-cell adhesion to glycated collagen. Moreover, we also found that the migration of human primary endothelial cells was delayed on glycated versus normal collagen. Potentially, glycation of collagen may disrupt endothelial cell–collagen interactions in at least two ways. First, endothelial cell surfaces are rich in PGs [Vargas et al., 1990], which may bind to collagenous substrates electrostatically and such interactions may be weakened due to the reduced charge density of the glycated collagen. Second, the predominant type I collagen receptors on endothelial cells are the $\alpha1\beta1/\alpha2\beta1$ integrins, which bind the $GFPGER_{502-507}$ sequence of the collagen, localized only about 10 nM from the glycation substrate K479 and the major glycation zone of the fibril (Fig. 8A) [Knight et al., 2000; Di Lullo et al., 2002]. Therefore, integrin-collagen ligation could also be sterically disrupted by collagen glycation.

In summary, our results imply that in vivo, collagen glycation may inhibit PG-mediated cell adhesion or migration in the collagenous stroma, and PG–collagen interactions required for maintaining ECM structure and function. Collagen glycation may thus be predicted to contribute to pathologies associated with diabetes and aging including delayed wound healing and tumor metastasis.

ACKNOWLEDGMENTS

We gratefully acknowledge Diana Menezes and Biao Zuo for expert assistance with electron microscopy, Kari Habursky for performing endothelial cell experiments, and the gift of recombinant human decorin and biglycan from Dr. David McQuillan and LifeCell Inc, N.J. This work was supported by NIH HL053590 and AR049604 to JSA, CA68494 to RDS, and EY05129 to DEB.

REFERENCES

Andreassen TT, Seyer-Hansen K, Bailey AJ. 1981. Thermal stability, mechanical properties and reducible cross-links of rat tail tendon in experimental diabetes. Biochim Biophys Acta 677:313–317.

Ayad S, Boot-handford RP, Humpries MJ, Kadler KE, Shuttleworth CA. 1998. The extracellular matrix facts book. San Diego: Academic Press.

Bai P, Phua K, Hardt T, Cernadas M, Brodsky B. 1992. Glycation alters collagen fibril organization. Connect Tissue Res 28:1–12.

Bailey AJ, Sims TJ, Avery NC, Halligan EP. 1995. Non-enzymic glycation of fibrous collagen: Reaction products of glucose and ribose. Biochem J 305(Pt 2):385–390.

Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EJ. 1992. Biology of the syndecans: A family of transmembrane heparan sulfate proteoglycans. Annu Rev Cell Biol 8:365–393.

Birk DE, Fitch JM, Babiarz JP, Doane KJ, Linsenmayer TF. 1990. Collagen fibrillogenesis in vitro: Interaction of types I and V collagen regulates fibril diameter. J Cell Sci 95(Pt 4):649–657.

Brennan M. 1989. Changes in solubility, non-enzymatic glycation, and fluorescence of collagen in tail tendons from diabetic rats. J Biol Chem 264:20947–20952.

Chakravarti S, Magnuson T, Lass JH, Jepsen KJ, LaMantia C, Carroll H. 1998. Lumican regulates collagen fibril assembly: Skin fragility and corneal opacity in the absence of lumican. J Cell Biol 141:1277–1286.

Chakravarti S, Petroll WM, Hassell JR, Jester JV, Lass JH, Paul J, Birk DE. 2000. Corneal opacity in lumican-null mice: Defects in collagen fibril structure and packing in the posterior stroma. Invest Opthalmol Visual Sci 41:3365–3373.

Chapman JA. 1974. The staining pattern of collagen fibrils. I. An analysis of electron micrographs. Connect Tiss Res 2:137–150.

Chen J, Brodsky S, Li H, Hampel DJ, Miyata T, Weinstein T, Gafter U, Norman JT, Fine LG, Goligorsky MS. 2001. Delayed branching of endothelial capillary-like cords in glycated collagen I is mediated by early induction of PAI-1. Am J Physiol Renal Physiol 281:F71–F80.

Di Lullo GA, Sweeney SM, Korkko J, Ala-Kokko L, San Antonio JD. 2002. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem 277:4223–4231.

Echtermeyer F, Streit M, Wilcox-Adelman S, Saoncella S, Denhez F, Detmar M, Goetinck P. 2001. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. J Clin Invest 107:R9–R14.

Funderburgh JL, Funderburgh ML, Mann MM, Conrad GW. 1991. Physical and biological properties of keratan sulphate proteoglycan. Biochem Soc Trans 19:871–876.

Gavriel P, Kagan HM. 1988. Inhibition by heparin of the oxidation of lysine in collagen by lysyl oxidase. Biochemistry 27:2811–2815.

Guitton JD, Le Pape A, Sizaret PY, Muh JP. 1981. Effects of in vitro N-glucosylation on type-I collagen fibrillogenesis. Biosci Rep 1:945–954.

Hadley JC, Meek KM, Malik NS. 1998. Glycation changes the charge distribution of type I collagen fibrils. Glycoconj J 15:835–840.

Hascall VC, Heinegard DK, Wight TN. 1991. Proteoglycans: Metabolism and pathology. In: Hay EB, editor. Cell biology of extracellular matrix. New York: Plenum Press. pp 149–172.

Hocking AM, Strugnell RA, Ramamurthy P, McQuillan DJ. 1996. Eukaryotic expression of recombinant biglycan. Post-translational processing and the importance of secondary structure for biological activity. J Biol Chem 271:19571–19577.

Jordan R, Beeler D, Rosenberg R. 1979. Fractionation of low molecular weight heparin species and their interactions with antithrombin. J Biol Chem 254:2902–2913.

Kawano E, Takahashi S, Sakano Y, Fujimoto D. 1990. Nonenzymatic glycation alters properties of collagen as a substratum for cells. Matrix 10:300–305.

Keller KM, Keller JM, Kuhn K. 1986. The C-terminus of type I collagen is a major binding site for heparin. Biochem Biophys Acta 882:1–5.

Klass CM, Couchman JR, Woods A. 2000. Control of extracellular matrix assembly by syndecan-2 proteoglycan. J Cell Sci 113(Pt 3):493–506.

Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. 2000. The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)-beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. J Biol Chem 275:35–40.

Koda JE, Rapraeger A, Bernfield M. 1985. Heparan sulfate proteoglycans from mouse mammary epithelial cells. Cell surface proteoglycan as a receptor for interstitial collagens. J Biol Chem 260:8157–8162.

Kuzuya M, Satake S, Ai S, Asai T, Kanda S, Ramos MA, Miura H, Ueda M, Iguchi A. 1998. Inhibition of angiogenesis on glycated collagen lattices. Diabetologia 41:491–499.

Langford JK, Sanderson RD. 2002. Measurements of glycosaminoglycan-based cell interactions. Methods Cell Biol 69:297–308.

Lee MK, Lander AD. 1991. Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: Development of a sensitive electrophoretic approach. Proc Nat Acad Sci USA 88:2768–2772.

Liebersbach BF, Sanderson RD. 1994. Expression of syndecan-1 inhibits cell invasion into type I collagen. J Biol Chem 269:20013–20019.

Linsenmayer TF. 1991. Collagen. In: Hay ED, editor. Cell biology of extracellular matrix. New York: Plenum Press. pp 7–40.

Majack RA, Clowes AW. 1984. Inhibition of vascular smooth muscle cell migration by heparin-like glycosaminoglycans. J Cell Physiol 118:253–256.

McPherson JM, Sawamura SJ, Condell RA, Rhee W, Wallace DG. 1988. The effects of heparin on the physiochemical properties of reconstituted collagen. Collagen Rel Res 1:65–82.

Morita K, Urabe K, Moroi Y, Koga T, Nagai R, Horiuchi S, Furue M. 2005. Migration of keratinocytes is impaired on glycated collagen I. Wound Repair Regen 13:93–101.

Paul RG, Bailey AJ. 1996. Glycation of collagen: The basis of its central role in the late complications of ageing and diabetes. Int J Biochem Cell Biol 28:1297–1310.

Paul RG, Bailey AJ. 1999. The effect of advanced glycation end-product formation upon cell–matrix interactions. Int J Biochem Cell Biol 31:653–660.

Piez KA, Reddi AH. 1984. Extracellular matrix biochemistry. New York: Elsevier.

Prockop DJ, Kivirikko KI. 1995. Collagens: Molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 64:403–434.

Rada JA, Cornuet PK, Hassell JR. 1993. Regulation of corneal collagen fibrillogenesis in vitro by corneal proteoglycan (lumican and decorin) core proteins. Exp Eye Res 56:635–648.

Ramamurthy P, Hocking AM, McQuillan DJ. 1996. Recombinant decorin glycoforms. Purification and structure. J Biol Chem 271:19578–19584.

Reiser KM, Amigable MA, Last JA. 1992. Nonenzymatic glycation of type I collagen. The effects of aging on preferential glycation sites. J Biol Chem 267:24207–24216.

Ruoslahti E. 1988. Structure and biology of proteoglycans. Annu Rev Cell Biol 4:229–255.

Saika S, Shiraishi A, Liu CY, Funderburgh JL, Kao CW, Converse RL, Kao WW. 2000. Role of lumican in the corneal epithelium during wound healing. J Biol Chem 275:2607–2612.

San Antonio JD, Lander AD. 2001. Affinity coelectrophoresis of proteoglycan-protein complexes. In: Iozzo RV, editor. Proteoglycan protocols. Totowa NJ: Human Press. pp 401–414.

San Antonio JD, Lander AD, Wright TC, Karnovsky MJ. 1992. Heparin inhibits the attachment and growth of Balb c/3T3 fibroblasts on collagen substrata. J. Cell. Physiol 150:8–16.

San Antonio JD, Slover J, Lawler J, Karnovsky MJ, Lander AD, 1993. Specificity in the interactions of extracellular matrix proteins with subpopulations of the glycosaminoglycan heparin. Biochemistry 32:4746–4755.

San Antonio JD, Karnovsky MJ, Gay S, Sanderson RD, Lander AD. 1994a. Interactions of syndecan-1 and heparin with human collagens. Glycobiology 4:327–332.

San Antonio JD, Lander AD, Karnovsky MJ, Slayter HS. 1994b. Mapping the heparin-binding sites on type I collagen monomers and fibrils. J Cell Biol 125:1179–1188.

Sanderson RD, Sneed TB, Young LA, Sullivan GL, Lander AD. 1992. Adhesion of B lymphoid (MPC-11) cells to type I collagen is mediated by integral membrane proteoglycan, syndecan. J Immunol 148:3902–3911.

Sanderson RD, Turnbull JE, Gallagher JT, Lander AD. 1994. Fine structure of heparan sulfate regulates syndecan-1 function and cell behavior. J Biol Chem 269:13100–13106.

Sanderson RD, Yang Y, Suva LJ, Kelly T. 2004. Heparan sulfate proteoglycans and heparanase–partners in osteolytic tumor growth and metastasis. Matrix Biol 23:341–352.

Schnider SL, Kohn RR. 1982. Effects of age and diabetes mellitus on the solubility of collagen from human skin, tracheal cartilage and dura mater. Exp Gerontol 17:185–194.

Scott JE. 1991. Proteoglycan:collagen interactions in connective tissues. Ultrastructural, biochemical, functional and evolutionary aspects. Int J Biol Macromol 13:157–161.

Scott JE, Glanville RW. 1993. Homologous sequences in fibrillar collagens may be proteoglycan binding sites. Biochem Soc Trans 21:123S.

Scott JE, Ritchie M, Glanville RW, Cronshaw AD. 1997. Peptide sequences in glutaraldehyde-linked proteodermatan sulphate: Collagen fragments from rat tail tendon locate the proteoglycan binding sites. Biochem Soc Trans 25:S663.

Sweeney SM, Guy CA, Fields GB, San Antonio JD. 1998. Defining the domains of type I collagen involved in heparin- binding and endothelial tube formation. Proc Natl Acad Sci USA 95:7275–7280.

Sweeney SM, DiLullo G, Slater SJ, Martinez J, Iozzo RV, Lauer-Fields JL, Fields GB, San Antonio JD. 2003. Angiogenesis in collagen I requires alpha2beta1 ligation of a GFP*GER sequence and possibly p38 MAPK activation and focal adhesion disassembly. J Biol Chem 278:30516–30524.

Tsilibary EC. 2003. Microvascular basement membranes in diabetes mellitus. J Pathol 200:537–546.

Tsilibary EC, Charonis AS, Reger LA, Wohlhueter RM, Furcht LT. 1988. The effect of nonenzymatic glucosylation on the binding of the main noncollagenous NC1 domain to type IV collagen. J Biol Chem 263:4302–4308.

Vargas FF, Osorio HM, Basilio C, De Jesus M, Ryan US. 1990. Enzymatic lysis of sulfated glycosaminoglycans reduces the electrophoretic mobility of vascular endothelial cells. Membr Biochem 9:83.

Vesentini S, Redaelli A, Montevecchi FM. 2005. Estimation of the binding force of the collagen molecule-decorin core protein complex in collagen fibril. J Biomech 38: 433–443.

Vij N, Roberts L, Joyce S, Chakravarti S. 2004. Lumican suppresses cell proliferation and aids Fas-Fas ligand mediated apoptosis: Implications in the cornea. Exp Eye Res 78:957–971.

Vogel KG, Paulsson M, Heinegard D. 1984. Specific inhibition of type I and II collagen fibrillogenesis by the small proteoglycan of tendon. Biochem J 223:587–597.

Vonesh EF, Chinchilli VM. 1997. Linear and nonlinear models for the analysis of repeated measurements. New York: M. Dekker.

Weber IT, Harrison RW, Iozzo RV. 1996. Model structure of decorin and implications for collagen fibrillogenesis. J Biol Chem 271:31767–31770.

Woods A, Couchman JR. 1998. Syndecans: Synergistic activators of cell adhesion. Trends Cell Biol 8:189–192.

Yan SF, Ramasamy R, Naka Y, Schmidt AM. 2003. Glycation, inflammation, and RAGE: A scaffold for the macrovascular complications of diabetes and beyond. Circ Res 93:1159–1169.