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Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon

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The small dermatan sulphate proteoglycan of bovine tendon demonstrated a unique ability to inhibit fibrillogenesis of both type I and type II collagen from bovine tendon and cartilage respectively in an assay performed *in vitro*. None of the other proteoglycan populations from cartilage, tendon or aorta, even those similar in size and chemical structure, had this effect. Alkali treatment of the small proteoglycan of tendon eliminated its ability to inhibit fibrillogenesis, whereas chondroitinase digestion did not. This indicates that its interaction with collagen depends on the core protein. Fibrillogenesis of pepsin-digested collagens was affected similarly, indicating that interaction with the collagen telopeptides is not involved. The results suggest that interactions between collagen and proteoglycans may be quite specific both for the type of proteoglycan and its tissue of origin.

The chondroitin sulphate and dermatan sulphate proteoglycans found in fibrous tissues (Anderson, 1975; Damle *et al.*, 1979, 1982; Cöster *et al.*, 1981; Pearson & Gibson, 1982) and produced by several types of fibroblasts in culture (Vogel & Peterson, 1981; Carlstedt *et al.*, 1981) appear to fall into two broad categories. One group of large molecules resemble the major proteoglycans of cartilage in hydrodynamic volume, being eluted from a Sepharose CL-2B column in 4M-guanidium chloride at $K_{av.}$ in the range 0.1–0.4. The numerous glycosaminoglycan chains are either chondroitin sulphate or dermatan sulphate containing a very low percentage of iduronic acid. By contrast, molecules of the group of small proteoglycans have a monodisperse characteristic core protein with an apparent weight-average M_r of approx. 45000 (for references see Heinegård & Paulsson, 1984). The number of glycosaminoglycan chains per molecule is small (one or two), and these may either be dermatan sulphate rich in iduronic acid or chondroitin sulphate. The relative proportion of the proteoglycans varies considerably in different tissues, from the extreme of cartilage, in which large proteoglycans can be more than 95% of the total, to the very fibrous portions of adult tendon, where the small proteoglycan represents about 90% of the total (K. G. Vogel & D. Heinegård, unpublished work). Whether or not corresponding proteoglycans from different tissues may be similar in terms of function, or if they differ according to their tissue of origin, has not yet been sufficiently investigated to allow generalizations.

In every connective tissue the proteoglycans exist in a milieu that is rich in collagen, and numerous studies in the last 25 years have explored the hypothesis that proteoglycans may influence the manner in which collagen fibrils are formed in the extracellular matrix. These studies have confirmed the ability of proteoglycans to induce the precipitation of collagen (Wood, 1960; Toole & Lowther, 1968), affect the rate of collagen fibrillogenesis (Oegema *et al.*, 1975; Snowden & Swann, 1980) and affect that rate differentially depending on the source of the collagen or the proteoglycans (Birk & Lande, 1981). Indeed, Scott & Orford (1981) have been able to show a localization of proteoglycans at the d-band of the collagen fibres of rat tail tendon by using electron-microscopic techniques. It was suggested that proteoglycans in this position could inhibit both radial fibril growth and matrix calcification.

Only glycosaminoglycans were used in the early studies *in vitro* of proteoglycan/collagen interactions, and in most of the more-recent studies mixed populations of proteoglycans have been added to collagen from a different tissue or species. This has made it difficult to draw conclusions about

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the specificity or physiological relevance of the interactions being investigated. In the present study the interaction *in vitro* between purified populations of large and small proteoglycans from various bovine tissue sources, and type I and type II collagen from these same tissues, was investigated.

Experimental

Collagen

Acid-extracted type I collagen. The feet of cows approx. 6 months old were obtained from the slaughterhouse, and only the fibrous proximal portion of the deep flexor tendon was taken. Soluble collagen was prepared essentially by the method described by Chandrakasan *et al.* (1976) for rat tail tendon. Collagen precipitated at NaCl concentrations between 3% and 4% was used. The yield was 34mg from 60g of tendon.

Pepsin-extracted type I collagen. Small pieces of bovine proximal flexor tendon were extracted twice for 24h at 4°C in 15 parts (w/v) of 0.5M-acetic acid containing 1/500 (w/w) of pepsin. After centrifugation collagen in the supernatant was precipitated by following the method of Chandrasakan *et al.* (1976). The yield was 812mg from 45g of tendon. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that this material contained greater amounts of cross-linked components not entering the gel than any of the other preparations. To remove these, samples of this collagen were resuspended in 0.5M-acetic acid and centrifuged at 90000*g* for 2h, dialysed against 0.1M-acetic acid, and freeze-dried.

Neutral-salt-extracted type II collagen. Cartilage was obtained from the cartilaginous portions of the long bones of a bovine foetus (6 months of gestational age) and ground in a Wiley mill while frozen in liquid N_2. The cartilage powder was extracted twice with 15vol. of 1M-NaCl/50mM-EDTA/50mM-6-aminohexanoic acid/5mM-benzamidine hydrochloride/50mM-sodium acetate buffer, pH6.0. Each extraction was done overnight at 4°C with continuous stirring. The insoluble residue was removed by centrifugation at 30000*g* for 30min, and the supernatants from the two extractions were pooled.

Collagen in the extract was precipitated in 20% (w/v) NaCl overnight and collected by centrifugation as above. The precipitate was then dissolved in 100ml of the extraction buffer and transferred to 0.2M-NaCl/50mM-Tris/HCl buffer, pH7.4, by extensive dialysis. Insoluble material was removed by centrifugation as above, and the supernatant was passed through a column (bed volume 40ml) of DEAE-cellulose (DE-52; Whatman, Maidstone, Kent, U.K.) equilibrated with the same buffer. The material that did not bind to the column was dialysed into 0.5M-acetic acid, and collagen was precipitated by addition of NaCl to a final concentration of 5% (w/v). After incubation at 4°C overnight, the precipitate was collected as described above and redissolved in 100ml of 0.5M-acetic acid. The material was again precipitated, this time by extensive dialysis against 20mM-sodium phosphate buffer, pH7.2, and the precipitate was collected as described above. The precipitate was dissolved in 0.5M-acetic acid, centrifuged at 30000*g* for 2h to remove particulate material, and dialysed into 0.1M-acetic acid before being freeze-dried. The yield of this sample was 14mg from 82g of cartilage.

By using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis it was found that this preparation still contained some type I collagen. Further purification was therefore attempted by using differential salt precipitation, as described by Trelstad *et al.* (1976). In short, the collagen was dissolved in 0.16M-sodium phosphate buffer, pH7.6, and type I collagen was precipitated by addition of NaCl to a final concentration of 1.5M. The supernatant containing type II collagen was dialysed into 0.1M-acetic acid and freeze-dried. The weight of this sample was 5.2mg.

Pepsin-extracted type II collagen. This collagen was purified after pepsin digestion of bovine nasal cartilage that had been pre-extracted with 4M-guanidium chloride, by using the procedure of Miller (1972). A DEAE-cellulose chromatography step, as described for the neutral-salt-soluble type II collagen, was included in the purification procedure to remove remaining proteoglycans.

Collagen analysis

Reduced samples containing 15μg of collagen were electrophoresed on polyacrylamide gels (5% acrylamide with 2.5% cross-link) in the buffer system of Neville (1971). Gels were stained with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.).

A 100μg portion of each collagen preparation was hydrolysed in 3ml of 6M-HCl for 24h at 110°C. A Durrum automatic amino acid analyser was utilized for determination of amino acids and hexosamines; a separate program was utilized to determine hydroxyproline. The mean hydroxyproline contents in residues/1000 residues were 104 and 129 for type I and type II preparations respectively. For glycine the contents were 368 and 322 residues/1000 residues for type I and type II respectively. Protein content calculated from amino acid analysis varied from a low of 68% for pepsin-digested type I collagen to 85% for pepsin-digested type II collagen.

Preparation of proteoglycans

Tendon. An outline of the procedure for the preparation and characterization of the proteoglycans from tendon is given below. Frozen tissue powder of adult bovine proximal flexor tendon was extracted with 25 vol. of 4M-guanidinium chloride containing 5mM-benzamidine hydrochloride/0.1M-6-aminohexanoic acid/10mM-EDTA/5mM-*N*-ethylmaleimide/50mM-sodium acetate buffer, pH5.8, for 24h at 4°C. After centrifugation, the supernatant was concentrated by ultrafiltration and subjected to CsCl-density-gradient centrifugation at a starting density of 1.33g/ml. The bottom quarter and top three-quarters of each tube were separately used for further purification of proteoglycans. Proteoglycans from the bottom quarter of each tube (density 1.39g/ml) contained 37% of the glycosaminoglycan hexosamine and were separated into two approximately equal populations by gel chromatography on Sepharose CL-2B eluted with 4M-guanidinium chloride. The peak for the smaller proteoglycan ($K_{av.}$ 0.63) was purified from remaining protein by density-gradient centrifugation in Cs_2SO_4 (starting density 1.44g/ml). The larger proteoglycans from Sepharose CL-2B chromatography were purified by another dissociative density-gradient centrifugation in CsCl at a starting density of 1.40g/ml; the large proteoglycans were found in the bottom fraction (density 1.60g/ml).

Proteoglycans from the top fractions of the initial CsCl-density-gradient centrifugation (63% of the glycosaminoglycan hexosamine) were isolated by ion-exchange chromatography in 7M-urea/10mM-Tris/HCl buffer, pH7, on DE-52 DEAE-cellulose eluted with an NaCl gradient. The proteoglycan recovered from this isolation procedure was identical with the smaller proteoglycan recovered from the initial D1 fraction when compared by agarose/polyacrylamide-gel electrophoresis, and both demonstrated a core protein that migrated on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with an apparent M_r of 48000 after chondroitinase ABC digestion of the proteoglycan. The glycosaminoglycan chains of the small proteoglycan were dermatan sulphate, rich in iduronic acid, having weight-average M_r 37000, and the chains of the larger proteoglycans were chondroitin sulphate and considerably shorter (weight-average M_r 17000). After extensive dialysis against water the proteoglycans were freeze-dried, and solutions (100μg/ml) in water were stored in the freezer. When combined with collagen in the fibrillogenesis assay, the small proteoglycan prepared by ion-exchange chromatography behaved identically with that prepared by gel chromatography.

Glycosaminoglycan chains of the small proteoglycan were prepared by alkaline cleavage (100μg of proteoglycan in 0.1M-NaOH for 4 days at 2°C). The mixture was then neutralized with acetic acid, freeze-dried to remove excess acetate, and resuspended in 1ml of water. Another portion of the proteoglycan was digested with chondroitinase ABC (0.05 unit/mg in 10mM-Tris/HCl buffer, pH8.0, for 5h at 37°C) to remove glycosaminoglycan chains from the protein core.

Other tissues. Purified proteoglycan fractions from other bovine tissues were prepared either by using an approach similar to that described above or by procedures already published for each. These included the high-M_r aggregating proteoglycans of nasal cartilage (Heinegård & Hascall, 1979), a non-aggregating fraction of these proteoglycans (Heinegård & Hascall, 1979), and low-M_r proteoglycans from cartilage (Heinegård *et al.*, 1981) and aorta (S. Gardell, unpublished work).

Fibrillogenesis assay

Collagen was redissolved in 10mM-acetic acid at either 0.4mg/ml (type I, acid-extracted) or 2mg/ml (others) and stored at 2°C for up to 6 weeks. All buffers used contained a final concentration of 0.14M-NaCl and 30mM-sodium phosphate, pH7.3 (saline/phosphate buffer). Tes buffer also contained 30mM-2-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}ethanesulphonic acid. All stock solutions were de-gassed before being combined. For a final volume of 1ml, 0.5ml of double-concentration buffer was combined with 0.25ml of proteoglycans and water in tubes on ice; 0.25ml of collagen was then added and the solution pulled up into a pipette five times to mix the components effectively. Within minutes this cold mixture was transferred to a cuvette and immediately placed in a Cary 210 spectrophotometer with a water-jacketed five-place cuvette chamber equilibrated to either 30°C or 37°C. Fibril formation was monitored by recording absorbance at 400nm at 5min intervals. Readings of absorbance are essentially measurements of turbidity.

Hydroxyproline assay

After termination of the fibrillogenesis assay, contents of cuvettes were centrifuged in a table-top centrifuge (Eppendorf) at 10000*g* for 2min, and 0.75ml of the supernatant was removed, hydrolysed in 6M-HCl at 100°C for 24h and the hydroxyproline content determined colorimetrically (Stegeman & Stalder, 1967). The amount incorporated into pelleted fibrils was calculated as the percentage of hydroxyproline lost from the supernatants by comparison with a control mixture of the same collagen that was not warmed to permit fibril formation. By this assay control

solutions contained 9–11 μg of hydroxyproline/100 μg of collagen.

Results

The electrophoretic patterns indicated that collagen from tendon was only type I whereas that from cartilage was type II (Fig. 1). Hexosamines were not detected in the type I preparations or in the neutral-salt-extracted type II preparation; it is estimated that these samples contained no more than one small proteoglycan molecule per 100 α-chains. In the pepsin-extracted type II collagen, galactosamine was detected at a level corresponding to a maximum of one proteoglycan molecule per 10 α-chains. (This estimate was achieved by noting the molar ratio between glycine and galactosamine in each collagen sample. From other structural information we then assumed that one-third of a collagen α-chain is glycine and that one-tenth of the dermatan sulphate proteoglycan of tendon or small cartilage proteoglycan is galactosamine, and calculated a molecular ratio of collagen to proteoglycan.)

Each preparation of collagen could be induced to undergo some type of precipitation, resulting in fibril formation, and this was monitored as changes in absorbance at 400nm over several hours. It was not possible to compare directly the kinetics of fibrillogenesis between these preparations, even in the same buffer, because the various collagens required different initial concentrations and temperatures for reproducible fibrillogenesis. However, each proteoglycan preparation at a similar ratio of proteoglycan to collagen (1:20) affected the fibrillogenesis process of all types of collagen in ways that were qualitatively similar.

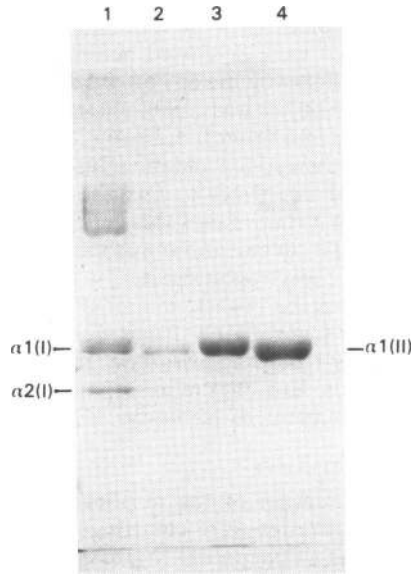

Fig. 1. *Electrophoresis of collagen prepared from bovine tendon and cartilage on sodium dodecyl sulphate/5%-polyacrylamide gels*

Each lane contains 15 μg of collagen that was reduced by heating at 37°C for 2h in sample buffer containing 5% (v/v) 2-mercaptoethanol. Lane 1, type I, acid-extracted; lane 2, type I, pepsin-extracted; lane 3, type II, neutral-salt-extracted; lane 4, type II, pepsin-extracted.

Type I: acid-extracted

Acid-extracted type I collagen at 100 μg/ml at 30°C formed a cohesive somewhat-opaque gel that remained in an inverted cuvette and was stable in the cold (absorbance declined by less than 10% after 4h at 4°C). In initial experiments the saline/phosphate buffer also containing Tes was used because it had been reported to produce fibrils *in vitro* having a microscopic morphology that was most like native fibrils (Williams *et al.*, 1978). Under these conditions the small dermatan sulphate proteoglycan of tendon produced a marked inhibition of the development of turbidity, whereas the large proteoglycans from tendon or cartilage and the small proteoglycans from cartilage and aorta had no effect (Fig. 2). Addition of 5 times as much of the small proteoglycan from aorta still inhibited the development of turbidity by much less than did the small proteoglycan of tendon (results not shown).

Collagen fibrillogenesis in the saline/phosphate buffer without Tes was affected by all proteoglycans, although the small dermatan sulphate proteoglycan of tendon still demonstrated a distinctly greater inhibition than any of the others (Fig. 3). This inhibition was similar when the small proteoglycan of tendon was added simultaneously with an equal amount of the large proteoglycan of tendon (results not shown). All of the proteoglycans produced some inhibition of the rate of fibrillogenesis and resulted in final absorbance values that were significantly higher than those of the control. Addition of 10 times as much of the large proteoglycans from cartilage increased the degree of inhibition only slightly. Interestingly, in this buffer, those samples containing the small proteoglycan of tendon eventually reached an absorbance value that was greater than that of the control. Fibrils formed in the presence of the small proteoglycan of tendon were just as stable to lowering the temperature as those formed without the proteoglycan.

The final absorbance values reached after completed fibrillogenesis with no added proteoglycan increased in an essentially linear fashion for both buffers over the range 25–200 μg of collagen/

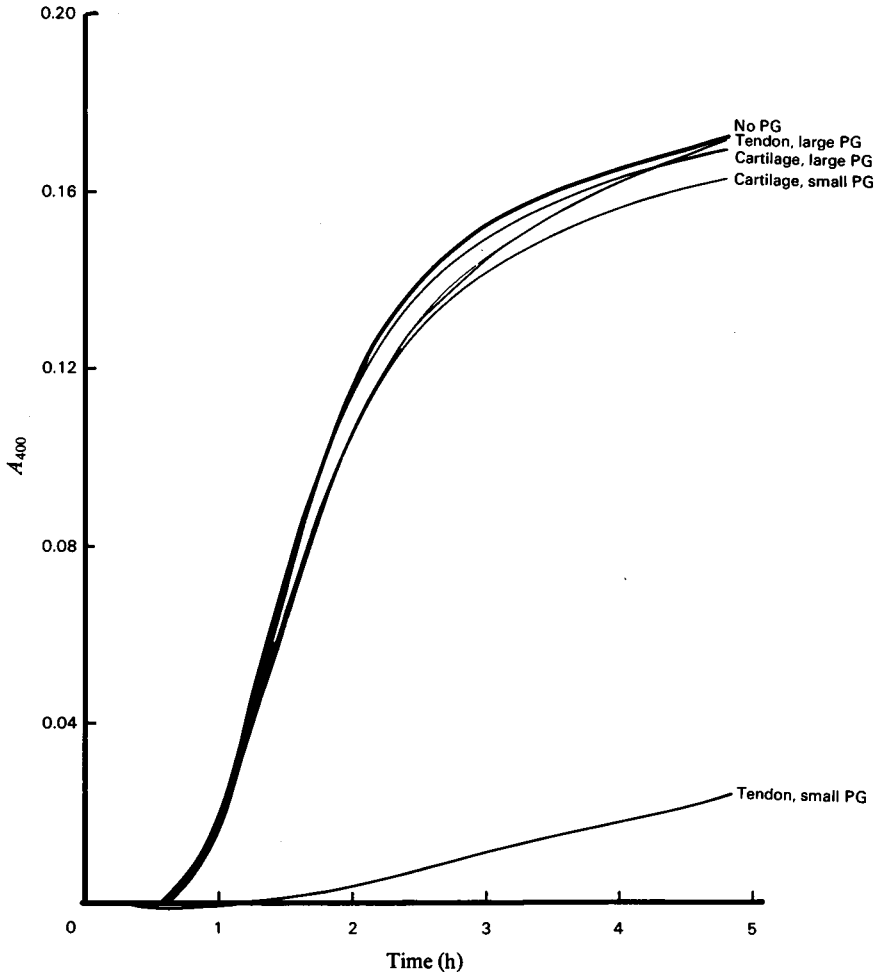

Fig. 2. *Effect of various proteoglycans on precipitation of acid-extracted type I collagen in phosphate/saline buffer containing 30 mM-Tes*

A 100μg portion of collagen and 5μg of each proteoglycan were combined in 1ml of saline/phosphate buffer containing Tes on ice. After mixing, samples were transferred to a cuvette and warmed to 30°C. Absorbance was monitored at 5min intervals. Abbreviation: PG, proteoglycan.

ml. No hydroxyproline (< 3%) could be detected in the supernatants after fibrillogenesis in saline/phosphate buffer. This was true for control as well as for all samples containing proteoglycans, once maximal absorbance was reached. It appears that the higher final absorbance that developed in the presence of proteoglycans was related to an alteration in fibrillar organization resulting in greater fibril turbidity rather than to enhanced efficiency in the incorporation of collagen into those fibrils. Somewhat more hydroxyproline was detected in the supernatants after fibrillogenesis in buffer containing Tes (range 6–15% of total). It is noteworthy that the amount of hydroxyproline remaining soluble after fibrillogenesis in saline/phosphate buffer containing Tes in the presence of the small proteoglycan of tendon was less than would be predicted by the linear relationship between final absorbance and collagen concentration of controls, i.e. absorbance was decreased by 85% whereas collagen incorporation was decreased by only 52%. Thus the small proteoglycan of tendon inhibited the increase in absorbance to a greater extent than it inhibited incorporation of collagen into insoluble fibrils, indicating that the fibrils that did form were structurally altered.

When the small proteoglycan of tendon was subjected to alkali treatment to release glycos-

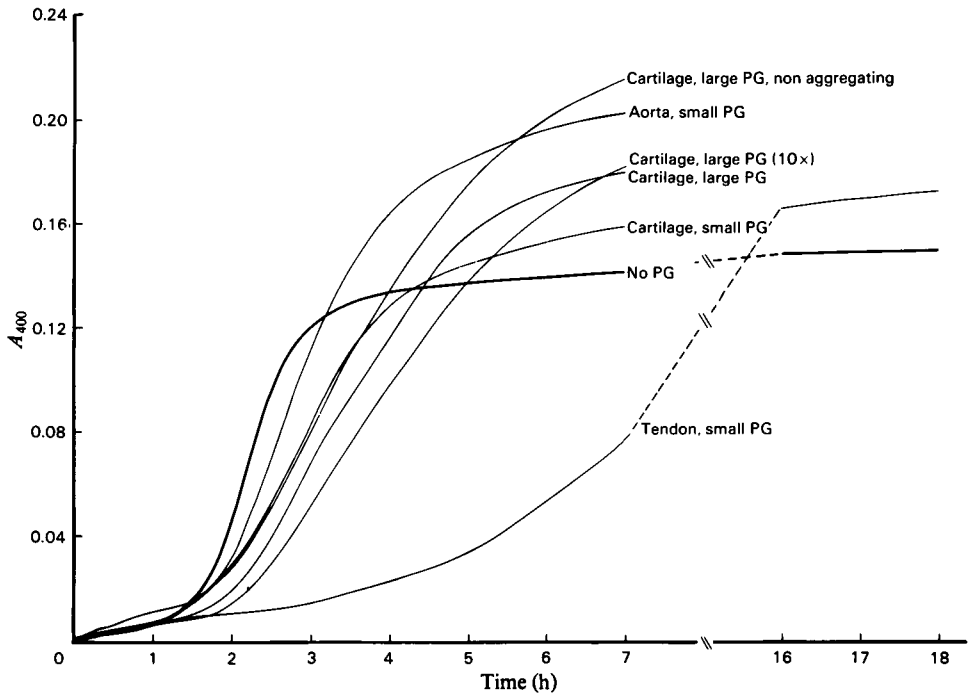

Fig. 3. *Effect of various proteoglycans on fibrillogenesis of acid-extracted type I collagen in phosphate/saline buffer*
A 100 μg portion of collagen and 5 μg of each proteoglycan were combined in 1 ml of saline/phosphate buffer on ice. After mixing, samples were transferred to a cuvette and warmed to 30°C. Absorbance was monitored at 5 min intervals. Abbreviation: PG, proteoglycan.

aminoglycan chains from the protein core and destroy the protein, its ability to inhibit fibrillogenesis was eliminated (Fig. 4). By contrast, proteoglycan that had been treated with chondroitinase ABC to degrade the glycosaminoglycan chains (but leave the core protein intact) still inhibited fibrillogenesis, although to a somewhat smaller extent when compared with the corresponding molar quantity of the intact proteoglycan.

Attempts were made to investigate the effect of adding proteoglycans during the lag phase of fibrillogenesis. It was found, however, that agitation of the collagen during lag phase resulted in an atypical increase in both the rate and magnitude of the absorbance that developed. This made it impossible to add proteoglycan during the lag phase and compare the kinetics with those obtained when collagen and proteoglycan were combined before raising the temperature. In other experiments bovine plasma fibronectin was added at concentrations up to 50 μg/100 μg of acid-extracted type I collagen. This protein either had no effect or slightly enhanced the rate of fibrillogenesis, and the final absorbance reached after completed fibrillogenesis was significantly enhanced (results not shown).

It was noticed that the kinetics for fibrillogenesis of the type I intact collagen preparation changed during the months when these experiments were being performed. Although stored as freeze-dried samples at −20°C, collagen resuspended in 10 mM-acetic acid after several months of storage generated fibrils with a shorter lag time and shorter half-time. This change was particularly noticeable in saline/phosphate buffer, and is responsible for the different kinetics demonstrated in Fig. 3 as compared with Fig. 4(*a*). The inhibitory effect of the small proteoglycan from tendon was more pronounced when the overall rate of fibrillogenesis was lower, although the specific inhibition was readily seen with all samples tested.

Type I: pepsin-extracted

Pepsin-extracted collagen from tendon generated reasonable fibrillogenesis curves in saline/phosphate buffer at 30°C, but it was necessary to use 5 times as much of this collagen in order to produce an absorbance value similar to that of

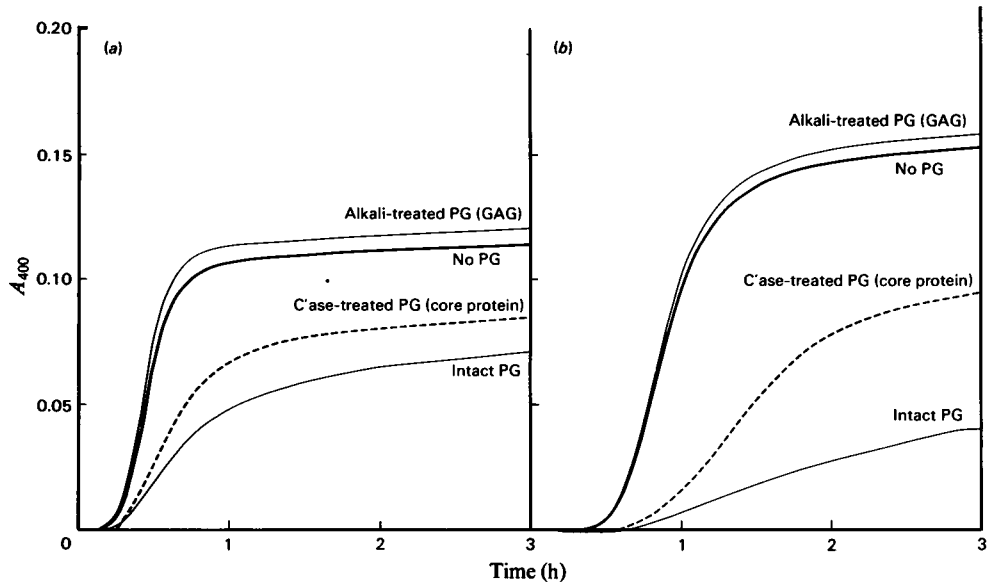

Fig. 4. *Effect of intact or altered small proteoglycans of tendon on fibrillogenesis of acid-extracted type I collagen*
A 100 μg portion of collagen and 5 μg of intact or treated small tendon proteoglycan were combined in 1 ml of (*a*) phosphate/saline buffer or (*b*) phosphate/saline buffer containing Tes. Absorbance was monitored at 5 min intervals. Abbreviations: PG, proteoglycan; GAG, glycosaminoglycans; C'ase, chondroitinase ABC.

acid-extracted collagen. The amount of proteoglycan added was also increased 5-fold in order to maintain a similar ratio of proteoglycan to collagen throughout the experiments. At a collagen concentration of 500 μg/ml a cohesive gel formed in the cuvette, and only 9% of the hydroxyproline remained in the supernatant after centrifugation of this gel.

The small proteoglycan of tendon produced a marked inhibition of the increase in absorbance during fibrillogenesis, whereas neither the large nor the small proteoglycans of cartilage had any measurable effect (Fig. 5). This inhibition was eliminated by alkali treatment of the proteoglycan, but it was not diminished by chondroitinase ABC digestion. From these results we conclude that the interaction between type I collagen and the small proteoglycan of tendon does not depend on the presence of collagen telopeptides. Although the small proteoglycan of tendon inhibited absorbance by 30–35%, analysis of the supernatants for hydroxyproline indicated that incorporation of collagen into the pelleted fibrils was inhibited by only 2–4%. Because of the very limited quantity of large tendon proteoglycan available, it was not tested with this collagen or any of the type II collagen preparations.

Type II: neutral-salt-extracted

The collagen extracted from foetal cartilage by neutral salt would not undergo detectable fibrillogenesis unless both concentration and temperature were increased in comparison with the tendon collagen. In saline/phosphate buffer at a concentration of 500 μg/ml and 37°C the absorbance that developed resembled the fibrillogenesis curves of type I collagen, although the final value was much higher than for any of the other collagen preparations. These fibrils did not form a cohesive gel. Instead, the cuvette contained a tangle of visible threads that could be poured easily from the cuvette.

The small proteoglycan of tendon dramatically decreased the absorbance developed during a 19 h assay (Fig. 6). The small proteoglycan of cartilage inhibited the rate of fibril formation more than the large proteoglycan of cartilage did, but in both cases that inhibition was minimal compared with that with the small proteoglycan of tendon. Analysis of hydroxyproline remaining in the supernatant of these assays indicated that 70–75% of this type II collagen was incorporated into pelleted fibrils formed in the absence of added proteoglycans or in the presence of either the large or the small cartilage proteoglycan populations. The final absorbance was increased by 26% and 14% in the presence of the large and the small proteoglycans of cartilage respectively. In the presence of small proteoglycan from tendon absorbance was inhibited by 73%, and only 50% of the collagen was incorporated into the pelleted fibrils. These fibrils were also seen as long tangled threads,

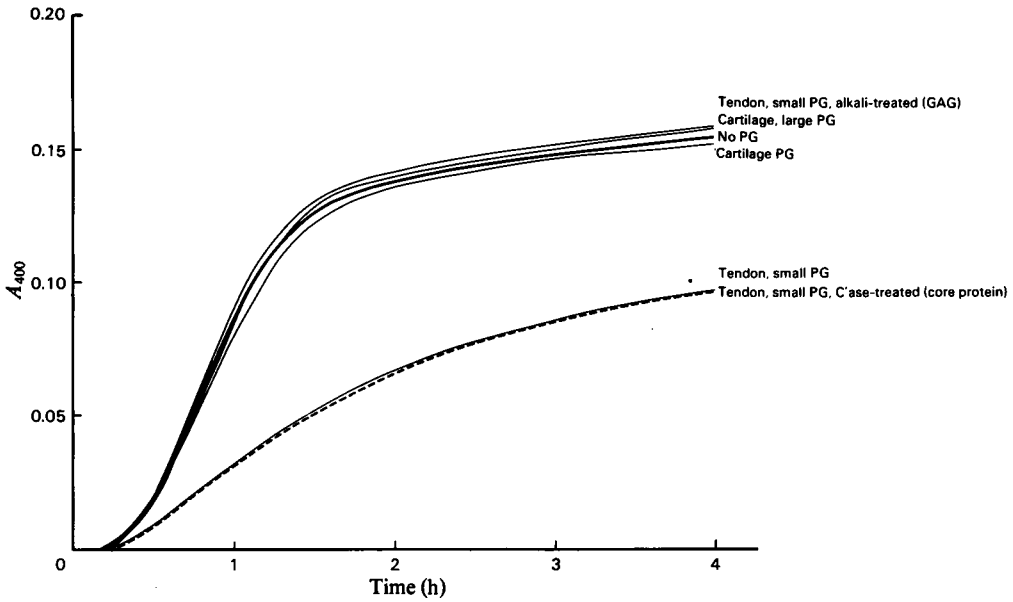

Fig. 5. *Effect of various intact proteoglycans and altered small proteoglycans of tendon on precipitation of pepsin-extracted type I collagen*

A 500 μg portion of collagen and 25 μg of each proteoglycan preparation were combined in 1 ml of phosphate/saline buffer on ice. After mixing, samples were transferred to a cuvette and warmed to 30°C. Absorbance was monitored at 5 min intervals. Abbreviations: PG, proteoglycan; GAG, glycosaminoglycans; C'ase, chondroitinase ABC.

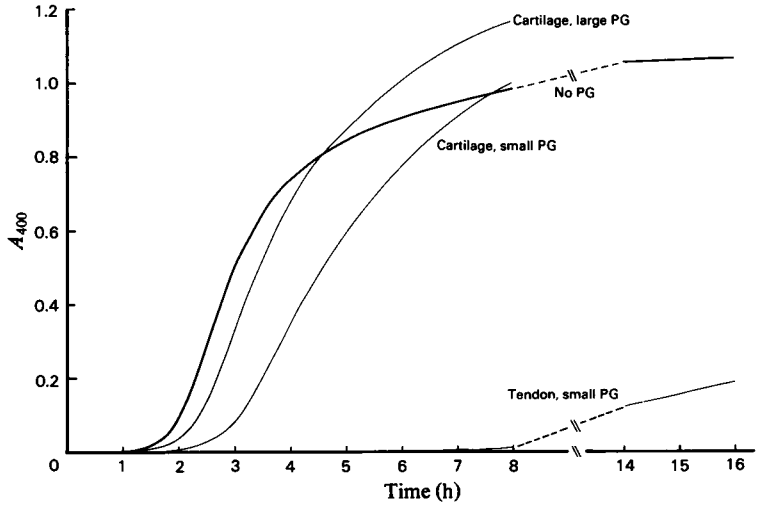

Fig. 6. *Effect of proteoglycans from cartilage and the small proteoglycans of tendon on precipitation of neutral-salt-extracted type II collagen*

A 500 μg portion of collagen was combined with 25 μg of proteoglycan in 1 ml of phosphate/saline buffer on ice. After mixing, samples were transferred to a cuvette and warmed to 37°C. Absorbance was monitored at 5 min intervals. Abbreviation: PG, proteoglycan.

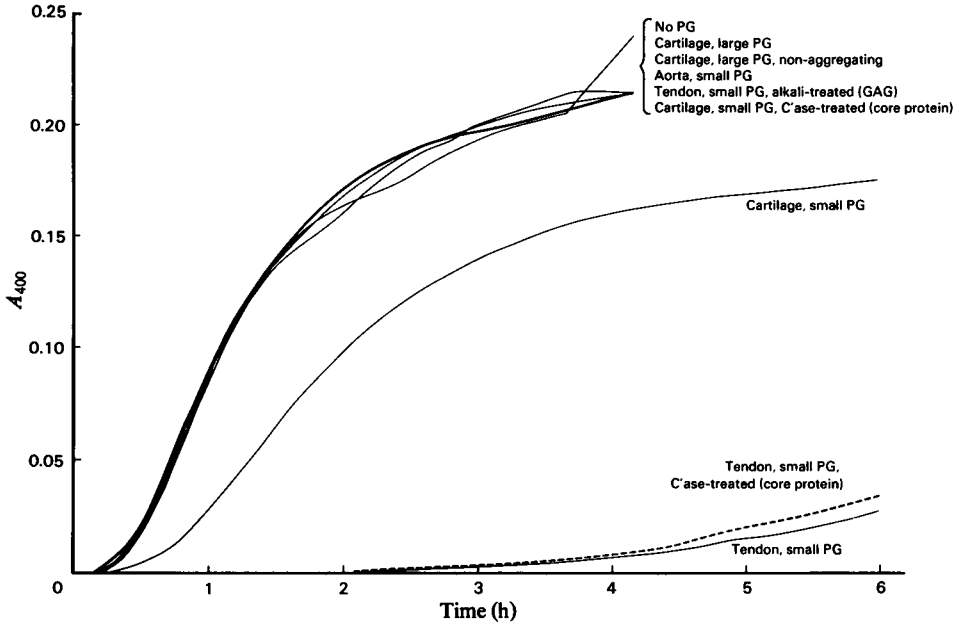

Fig. 7. *Effect of various intact and altered proteoglycans on precipitation of pepsin-extracted type II collagen*
A 500 μg portion of collagen with 25 μg of each proteoglycan preparation were combined in phosphate/saline buffer on ice. After mixing, samples were transferred to a cuvette and warmed to 37°C. Absorbance was monitored at 5 min intervals. Only a small percentage of the collagen was actually precipitated, and this precipitate had a tendency to float in the cuvette after 2–4 h, thereby interfering with measurements. Abbreviations: PG, proteoglycan; GAG, glycosaminoglycans; C'ase, chondroitinase ABC.

but there appeared to be less filamentous material formed in the cuvette. Additional characterization of the interaction in this system was not possible because of the small amount of neutral-salt-extracted type II collagen available.

Type II: pepsin-extracted

The pepsin-extracted type II collagen did not readily undergo fibrillogenesis. No increase in absorbance was observed at either 30°C or 37°C at any concentration up to 1 mg/ml when Tes was present in the saline/phosphate buffer. Even without the Tes there was no fibrillogenesis at 30°C unless the phosphate concentration was substantially lowered. In order to allow some comparisons between the various collagen preparations 30 mM-phosphate buffer was used for all experiments, but the temperature was increased to 37°C for the type II preparations. At 500 μg/ml a change in absorbance that resembled fibrillogenesis curves for the other preparations was also obtained with pepsin-extracted type II collagen, although the curves were not particularly smooth or stable. Rather than forming a firm gel, the precipitate tended to float to the top of the cuvette after 2–4 h. Furthermore, 90–95% of the collagen added to the assay was found in the clear supernatant after centrifugation of the formed fibrils, indicating that the kinetics observed involved only a small percentage of the original collagen. The absorbance of the precipitate declined by 85% when the temperature was lowered to 4°C.

The small proteoglycan of tendon greatly inhibited the increase in absorbance in this system (>97% at 4 h; Fig. 7). The small proteoglycan of cartilage also produced a distinct inhibition, although of smaller magnitude than that produced by the small proteoglycan of tendon. None of the other proteoglycans tested had an effect. Digestion of the small proteoglycans with chondroitinase ABC caused no change in the inhibition by tendon proteoglycan, but eliminated the inhibition of the small cartilage proteoglycan. Alkaline treatment of the small proteoglycan of tendon totally eliminated its inhibitory effect.

Discussion

Collagen molecules incubated at temperatures of 20°C to 37°C in buffers having a neutral pH will aggregate in a specific fashion and form fibrils;

these fibrils are insoluble in the buffer used and therefore can be observed visually as precipitates. The kinetics of this process can be monitored by turbidimetry, as was done in the present study. In the tissue, collagen fibril formation occurs in a matrix containing a number of molecules that can potentially interact with the collagen molecules or with various intermediate aggregates and therefore affect the properties of the completed fibril. A major component of many connective-tissue matrices is the proteoglycans. The present study was undertaken in order to determine if proteoglycans can interact with the collagen molecule and thereby affect fibril formation, as monitored in kinetic turbidimetric measurements of the forming precipitates. However, the absorbance recorded in such assays gives no information on the nature of the fibril. As a result, this technique alone does not distinguish between inhibition of fibril formation by way of preventing assembly or precipitation of the same quantity of collagen in fibrils with quite different optical properties. Determination of the proportion of the collagen that was precipitated permitted us to distinguish between these two alternatives, however, and we can conclude that both decreased precipitation of collagen and altered optical properties of the fibrils formed in the presence of proteoglycans were observed.

To determine whether or not the interaction was specific for certain proteoglycans, we studied the effects of the various subpopulations of proteoglycans isolated from a number of bovine tissues on fibril formation of two major types of collagen, i.e. type I collagen from tendon and type II collagen from cartilage. Of the various proteoglycans investigated, only the small dermatan sulphate proteoglycan from tendon demonstrated a consistent ability to significantly inhibit the rate of fibrillogenesis. None of the large proteoglycans inhibited this process, although they often produced an increased absorbance of the completed fibrils, indicating an effect on their assembly. In contrast, the absorbance of fibrils formed in the presence of the small proteoglycan of tendon was often decreased.

The preferential interaction of dermatan sulphate proteoglycans with collagen in fibrous tissues was suggested many years ago, when studies indicated that chondroitin sulphate and hyaluronic acid could be extracted from bovine skin with salt whereas hot 6M-urea or digestion of the collagen fibres was necessary to solubilize the dermatan sulphate (Toole & Lowther, 1966). Using morphological techniques, Scott *et al.* (1981) concluded that dermatan sulphate proteoglycan was found on the outside of the collagen fibrils in rat tendon. In cultures of human-embryo lung fibroblasts a small dermatan sulphate proteoglycan was the major proteoglycan constituent of medium when cells were grown on plastic, but when cells were grown on a collagen gel this proteoglycan was located in the gel (Vogel *et al.*, 1981). Similarly, human skin fibroblasts cultured on collagen gels produced an iduronic acid-rich dermatan sulphate that bound specifically to the collagen (Gallagher *et al.*, 1983).

The present study not only indicates a specific interaction between the small dermatan sulphate proteoglycan of tendon and collagen, but also shows that the interaction depends on the core protein rather than on the glycosaminoglycan chains. In fact, the glycosaminoglycan chains alone had no effect on the fibrillogenesis, contrary to the observation in previous studies that collagen under certain conditions can interact with dermatan sulphate (Öbrink, 1973). It has been demonstrated that the core proteins of large proteoglycans from both chick-embryo cartilage (Toole, 1976) and rat chrondrosarcoma (Oegema *et al.*, 1975) bind to type I collagen. However, core protein bound via this mechanism did not retard fibril formation, did not affect absorbance of the collagen fibrils, and appeared not to be released in the presence of high concentrations of intact proteoglycan (Oegema *et al.*, 1975). It was suggested that the proteoglycans and the protein core may bind to different sites on collagen. The alterations in fibrillogenesis rate and absorbance of the collagen fibrils produced in the presence of core protein of the small proteoglycan of tendon indicate that this interaction is different from that with the rat chondrosarcoma proteoglycan core. It is of note that the rat chondrosarcoma proteoglycan did not bind to fibrils once they had formed (Oegema *et al.*, 1975). Binding characteristics of the small proteoglycan of tendon to collagen are not yet known, although the results from our studies with collagen with and without the telopeptides indicate that a site on the protein core of the proteoglycan interacts with the triple-helical portion of collagen. The somewhat more-pronounced effects of intact proteoglycans with acid-extracted type I collagen, compared with the protein core, may indicate that the dermatan sulphate side chains provide additional sites of interaction, perhaps via the telopeptides.

We have no explanation for the observation that the small proteoglycan of tendon inhibited collagen fibrillogenesis much more effectively than did apparently similar small proteoglycans of cartilage and aorta. The result suggests that core proteins of proteoglycans from different tissues may carry unique structural regions for molecular interactions. The fact that the tendon proteoglycan inhibited type II collagen fibrillogenesis to a greater extent than did the small cartilage proteoglycan suggests that interactions between proteo-

glycans and collagen in a particular tissue could be determined by the amount and type of proteoglycan present rather than by the presence of unique proteoglycan-binding sites on the various types of collagen.

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