Structural Aspects of the Extracellular Matrix of the Tendon: An Atomic Force and Scanning Electron Microscopy Study

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Summary. The mutual interactions of small proteoglycans with collagen fibrils in the extracellular matrix remain to be completely understood. The present research investigated the extracellular matrix of the rat tail tendon by atomic force microscopy (AFM) as well as by scanning electron microscopy (SEM). Observations showed simply dehydrated specimens made of large heterogeneous fibrils, tightly packed in mutual contact with no visible interfibrillar spaces. Proteoglycans usually extended onto neighboring fibrils, forming an intricate interfibrillar weaving highly sensitive to chondroitinase digestion. Pre-treatment with cupromeronic blue only affected the proteoglycans side chains, which appeared better preserved but somewhat thickened. Observation of hydrated specimens by AFM confirmed the close packing of collagen fibrils and the abundance of collagen-bound proteoglycans. Interfibrillar bridges were only occasionally observed in this tissue, whose fibrils are instead tightly bound together by proteoglycans in a structure quite consistent with its functional requirements. The molecular machinery responsible for these interactions is the subject of ongoing research.

From a structural standpoint, all connective tissues are fiber-reinforced composites made of tough fibers immersed in a softer interfibrillar matrix, mostly made of hydrated proteoglycans bound to the fibril surface. Lesser (and tissue-specific) amounts of water, minerals, electrolytes, glycoproteins, adhesion molecules, and growth factors give rise to a wide variety of different tissues, each tailored to fulfill a particular functional role.

Tendons and ligaments represent an extreme simplification of this common blueprint. Although internal differences do exist (AMIEL et al., 1984), they are all made of almost parallel collagen fibrils aligned with the tension lines. For these reasons, tendons

have become a *de facto* standard for biomechanical studies and been the subject of myriad mechanical and functional investigations. Despite this, it seems that certain key points have been overlooked or inadequately addressed.

1) Almost all studies make the implicit assumption that mechanical stress is borne by collagen fibrils alone. This would be reasonable only if fibrous tissues were made of continuous fibers, while the length of the individual native collagen fibrils of tendons has been estimated to be just within the millimeter range (CRAIG et al., 1989; BIRK et al., 1997; GRAHAM et al., 2000). In other terms, fibrils are shorter than most — if not all — of the tendons they form, and some mechanism must transmit tensile forces from fibril to fibril along the interfibrillar matrix.

2) The functional contribution of the interfibrillar matrix has never been accounted for in biomechanical studies because of its extremely low shear stress (estimated as $1 \cdot 10^{-5}$ N/m² by PINS et al. (1997), as opposed to approximately $1 \cdot 10^9$ N/m² for the tensile strength of collagenous components). However, their extreme length and thinness give collagen fibrils a relatively large surface: even if the interfibrillar matrix is weakly bound to them, the total traction it exerts on the fibrils adds up incrementally with length until, at a certain critical point, it becomes great enough to break the fibrils themselves. In other words, the interfibrillar matrix can match the strength of collagen fibrils when their length is greater than a critical value: this *critical length* has been estimated by CRAIG et al. (1989) as just 30–100 μ m.

3) Virtually all studies of the collagen/proteoglycan interaction (see HOCKING et al., 1998) deal with the interaction of collagen with the core protein.

Fewer studies address the interaction of side chains with growth factors or other small molecules, while almost nothing is known of the interactions of their polysaccharide side chains with other chains radiating from neighboring fibrils, or with the fibrils themselves.

4) It is known that tensile stresses exerted on tendons cause axial gliding and the reciprocal rearrangement of collagen molecules within collagen fibrils (FOLKHARD et al., 1987; SASAKI and ODAJIMA, 1996). However, the strain within collagen fibrils is always smaller than in the whole tendon (FRATZL et al., 1997). As much as 60% of the strain can be borne by non-fibrillar components, so that some other coupling mechanism must take place at a super-fibrillar level.

It therefore seems obvious that proteoglycans must be heavily involved in the structural integrity of the tendon. Few studies, however, have focused on the structural (rather than merely hydrodynamic) role of proteoglycans, and only one structural model of the interactions of their side chains exists (SCOTT, 1995; CRIBB and SCOTT, 1995; SCOTT and THOMLINSON, 1998). These studies, however, were all carried out by transmission electron microscopy (TEM) on dehydrated, embedded, and sectioned specimens. This is a complex technique with a vast potential for artifacts, and it is not uncommon for this approach to find wildly altered values even for the well-known 67 nm D-period of collagen (see YOUNG, 1985).

Some authors have demonstrated the structure of collagen fibrils in three dimensions using atomic force microscopy (USHIKI et al., 1996; MELLER et al., 1997; YAMAMOTO et al., 1997, 2000), and our group was first to demonstrate collagen-bound proteoglycans using this method (RASPANTI et al., 1997). In the present research, these macromolecules and their interactions were investigated on a classic, well known connective tissue — the rat tail tendon — by tapping mode atomic force microscopy in fluid and by

scanning electron microscopy. Both techniques are capable of a very high resolution and are compatible with a minimal pretreatment of the specimens, in order to produce as few artifacts as possible.

MATERIALS AND METHODS

Four albino rats of either sex, 2–6 months old, were sacrificed by an excess of ethyl ether anesthesia, and the proximal portion of the tail tendon was dissected immediately after death. Some of the specimens were then either immediately dehydrated in graded ethanol and hexamethyldisilazane (NATION, 1983), while others were either treated overnight in 0.05% cupromeronic blue (Seikagaku Corp., Tokyo, Japan) in a 25 mM Na-acetate buffer containing 0.1 M $MgCl_2$ and 2.5% glutaraldehyde, pH 5.8, 20°C, followed by dehydration as above, or treated in chondroitinase ABC (Sigma, St. Louis, USA), 0.5 U/ml in a 0.2 M Tris-HCl buffer, pH 8, overnight at 37°C and dehydrated as above. Specimens intended for atomic force microscope (AFM) observation in fluid were briefly fixed in a mixture of 0.5% glutaraldehyde and 0.5% paraformaldehyde in a 0.1 M Na-cacodylate buffer, pH 7.2 and 4°C, and stored in a cold Na-cacodylate buffer. Tiny fragments were removed from all specimens with fine tweezers and immobilized on appropriate stubs with conductive bi-adhesive tape.

Hydrated specimens immersed in the Na-cacodylate buffer were observed on a Multi-Mode Nanoscope IIIa scanning probe microscope (Digital Instruments, Santa Barbara, California, USA) with Olympus OTR-8 oxide-sharpened silicon nitride probes (force constant approximately 0.68 N/m; resonance frequency approx. 11–13 kHz) and operated in tapping-mode at a scan rate of 1 Hz. Unfixed, dehydrated specimens were also observed in tapping-mode but with amorphous carbon super-tips grown atop Nanosensors TESP silicon probes (force constant 30–60 N/m,

Fig. 1. Extracellular matrix of the rat tail tendon imaged by tapping mode AFM. **A** and **B**. Unfixed, dehydrated tissue. In **A** collagen fibrils, running from top left to bottom right, are covered with a webbing of small, disordered filaments (*arrows*). In **B** individual glycosaminoglycan chains (*arrows*) can be seen running orthogonal to the fibril axis, passing from fibril to fibril. **C** and **D**. Pre-treatment with cupromeronic blue. In **C** this technique thickens the glycosaminoglycan chains (*arrows*), makes them more visible and keeps them more ordered. In **D** the chains maintain a visible, precise relationship with the collagen D-period of 67 nm while still interconnecting adjoining fibrils (*arrows*). **E** and **F**. Fibrils imaged in their fully hydrated state while immersed in fluid. These datasets have been filtered with a 3x3 low-pass filter to reduce the random noise, which is noticeably higher in images taken in fluid. With a high tapping force (**E**) we can image the surface of the collagen fibrils right through their softer proteoglycan coverage; a slender fibril (*arrow*) can be seen to lie directly on a thicker one, their gap- and overlap zones perfectly matching. With a minimal tapping force (**F**) the proteoglycan layer can be imaged and the fibril surface appears covered by a fine granular or filamentous webbing. The close packing is maintained and some interfibrillar connections are still evident (*arrows*). Bar (in all figures) = 100 nm

Fig. 1. Legend on the opposite page.

resonance frequency approximately 300 kHz) at a scan rate of 2 Hz. All pictures were deliberately taken so as to maintain the collagen fibrils at an angle of approximately 45 degrees with the scan axes in order to better differentiate scanning artifacts from actual surface features of the specimen.

All tapping-mode AFM data files were then rendered in three dimensions by proprietary software and a photo-realistic ray-tracing rendering package (POV-Ray v3.0, 1993 POV-Team; <http://www.povray.org>) running on a standard, Pentium III-based personal computer.

Specimens used for scanning electron microscopy (SEM) study were identical to those used for AFM study in air, and underwent no other treatment than dehydration with graded ethanol and hexamethyldisilazane. Fragments were mounted on appropriate stubs with conductive bi-adhesive tape, coated with 2 nm of platinum-carbon evaporated in a Balzers Union BAF-301 freeze-etching apparatus fitted with two EK 552 Electron Beam Guns and a QSG 201-D Quartz Crystal Thickness Monitor, and observed in secondary electron detection with a Philips XL-30 FEG-SEM operated at 7 kV.

RESULTS

Unfixed and dehydrated specimens observed by tapping mode AFM appeared composed of tight bundles of straight, rod-like fibrils, which were closely packed and ran in a parallel or slightly winding course within each bundle. The fibril diameter varied widely. Individual fibrils were well resolved and their distinct 67 nm D-banding was always readily visible. This technique often revealed slender filaments covering the fibril surface (Fig. 1A), encircling neighboring fibrils as orthogonal "belts" (Fig. 1B), or running occasionally along the fibril as interconnecting belts. Our previous research using the same approach (RASPANTI et al., 1997) demonstrated these filaments to be sensitive both to cupromeronic blue staining and to chondroitinase ABC digestion, allowing their identification with the glycosaminoglycan side chain of proteoglycans. Pre-treatment with cupromeronic

blue in a critical electrolyte concentration did not alter the general layout of the tissue nor the relationship of the glycosaminoglycan filaments with the collagen fibrils, and merely thickened the filaments themselves (Fig. 1C). The glycosaminoglycans were always clearly visible and again gathered adjoining fibrils; their relationship with the D-period of collagen was now better preserved (Fig. 1D).

Fully hydrated specimens observed by tapping mode AFM in fluid were very soft and very sensitive to the force exerted by the oscillating probing tip. When imaged with a high tapping force, they showed a clear 67 nm D-banding with sharp gap/overlap transitions, and usually appeared in small bundles, their gap- and overlap zones often perfectly aligned (Fig. 1E). By contrast, when the tapping force was reduced to the bare minimum required to follow the specimen surface, the D-period became less visible and the fibrils appeared covered by small, irregular bump-like protrusions. Some interfibrillar connections were occasionally recognizable. The close packing of the fibrils was always maintained (Fig. 1F).

SEM observation was not performed on unfixed tissue, since the glycosaminoglycans are easily altered by the preparation techniques and the observation itself (RASPANTI et al., 1997). SEM pictures of cupromeronic blue-treated fibrils were consistent with tapping mode AFM and typically showed collagen fibrils cross-tied by an intricate lacing of glycosaminoglycans (Fig. 2A), whose sheer amount was at times high enough to mask the D-banding and the collagen fibrils themselves. Due to the lower spatial resolution of SEM with respect to tapping mode AFM, the relationship of glycosaminoglycans with collagen fibrils and their intra-period location were not as readily apparent as above, but they could easily be seen winding around collagen fibrils or lying on their surface for a tract, rather than simply radiating from the fibrils themselves. The vast majority of the chains extended from one collagen fibril to another, spanning two or more fibrils and forming a characteristic basket-like weaving. Treatment with chondroitinase ABC removed the fibril-covering web almost completely, leaving only sparse debris and occasional interfibrillar bridges (Fig. 2B); the tissue now showed

Fig. 2. Extracellular matrix of the rat tail tendon imaged by high-resolution SEM. **A.** Tissue pre-treated with cupromeronic blue. Collagen fibrils, running from top left to bottom right, are interwoven with glycosaminoglycan filaments (arrows) in a tight, tough structure. Glycosaminoglycans can easily be seen to wind around collagen fibrils, rather than radiate from them. Bar = 1 μ m. **B.** Tissue digested with chondroitinase ABC. Collagen fibrils, running as in the previous picture, have been freed of most of their glycosaminoglycan covering and now clearly show their 67 nm D-period. Only occasional interfibrillar bridges, possibly corresponding to keratan sulfate-containing proteoglycans, have survived the treatment (arrows). Bar = 1 μ m

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more clearly the different diameters of its collagen fibrils and their periodic D-banding.

DISCUSSION

All the techniques used in the present study consistently revealed substantial amounts of glycosaminoglycan chains tying together neighboring fibrils. Although some filaments lay parallel to the fibril axis and interacted with just one fibril, the vast majority ran at an angle to the fibril axis and contacted one or more other fibrils.

In specimens observed in their fully hydrated state, the proteoglycans did not collapse onto the specimen surface but covered the collagen fibrils with an irregular, filamentous sheath, too soft to clearly reveal its structure but somewhat reminiscent of the "fractal perifrillar hydrated layer" described in the cornea by FRATZ and DAXER (1993). This layer was, in fact, so soft that even in tapping mode AFM it was frequently displaced along the X-axis by the probe, and a slight increase in the tapping force was enough to reveal the underlying collagen fibrils, with excellent resolution, throughout the proteoglycan layer. The collagen fibrils maintained the distinctive tight packing observed in our previous research (RASPANTI et al., 1990), with their gap- and overlap zones aligned in phase, a feature clearly implying some interfibrillar coupling. Our data could not confirm the hypothesis that each proteoglycan type binds only to a specific intra-period site (SCOTT, 1988), a concept already challenged by other recent studies (SVENSSON et al., 2000; KEENE et al., 2000), nor did they confirm an inter-chain interaction model proposed by the same author (SCOTT, 1995) where the proteoglycan side chains form interfibrillar "spacers". On the contrary, the three-dimensional interfibrillar web evident in our pictures, and especially in our SEM micrographs, was more consistent with the findings of YOUNG (1985), who also observed side chains encircling collagen fibrils and tying them together.

It seems that also from a quantitative standpoint a merely inter-chain interaction cannot account for the biomechanical properties of the fibrous tissues. Collagen-based fibrous structures are indeed credited with an ultimate strength in the range of $1 \cdot 10^9$ N/m², and with this value a fibril with a diameter of 200 nm (a not uncommon value in tendons) should break slightly above 30 μ N. If we consider that the mutual interaction of two glycosaminoglycan chains has been estimated at 40 ± 15 pN (DAMMER et al., 1995) and that an average of 3.5 chains are reportedly bound to the fibril surface per D-period (YOUNG,

1985), then glycosaminoglycans should exert on a collagen fibril a total shearing force of 140 ± 52.5 pN per D-period. With this value we would need 150,000–350,000 D-periods, or 10–25 cm, to reach the critical length, while the latter has been estimated at just 30–100 μ m, in the 1,000 D-periods range (CRAIG et al., 1989). In other words, to reach 30 μ N in just 1,000 D-periods we should postulate 150–350 interacting chains per single D-period, which seems quite an unlikely assumption. Since it is implausible that measurement uncertainties alone can account for a discrepancy in excess of two orders of magnitude, some mechanism other than a mutual chain interaction must be at work in the extracellular matrix. A direct interaction of glycosaminoglycan chains with the collagen fibrils surface is an obvious candidate.

It has been observed that the addition of chondroitin-6-sulphate to a collagen gel *in vitro* causes so dramatic an increase in tensile strength that any subsequent cross-linking treatment has a negligible effect (OSBORNE et al., 1998). It is remarkable that such a vast improvement in mechanical properties is brought about by the glycosaminoglycan alone, and this indicates quite obviously that the side chains are capable of multiple interactions with the collagen fibrils even in absence of their core protein.

In this respect, the three-dimensional interweaving of proteoglycans with collagen fibrils revealed by our pictures appears highly significant. It confirms a direct interaction of glycosaminoglycans side chains with the fibril surface, and suggests that glycosaminoglycans are directly responsible for the functional interconnection of adjoining collagen fibrils in the extracellular matrix.

REFERENCES

- AMIEL, D., C. FRANK, F. HARWOOD, J. FRONEK and W. AKESON: Tendons and ligaments: a morphological and biochemical comparison. *J. Orthopaed. Res.* 1: 257–265 (1984).
- BIRK, D. E., E. I. ZYCBAND, S. WOODRUFF, D. A. WINKELMANN and R. L. TRELSTAD: Collagen fibrillogenesis in situ: fibril segments become long fibrils as the developing tendons matures. *Devel. Dynam.* 208: 291–298 (1997).
- CRAIG, A. S., M. J. BIRTLES, J. F. CONWAY and D. A. D. PARRY: An estimate of the mean length of collagen fibrils in rat tail-tendon as a function of age. *Connect. Tiss. Res.* 19: 51–62 (1989).
- CRIBB, A. M. and J. E. SCOTT: Tendon response to tensile stress: an ultrastructural investigation of collagen-proteoglycan interaction in stressed tendon. *J. Anat.* 187: 423–428 (1995).

- DAMMER, U., O. POPESCU, P. WAGNER, D. ANSELMETTI, H. J. GÜNTHERODT and G. N. MISEVIC: Binding strength between cell adhesion proteoglycans measured by atomic force microscopy. *Science* 267: 1173-1175 (1995).
- FOLKHARD, W., E. MOSLER, W. GEERCKEN, E. KNÖRZER, H. NEMETSCHKE-GANSLER, T. NEMETSCHKE and M. J. KOCK: Quantitative analysis of the molecular sliding mechanism in native tendon collagen—Time-resolved dynamic studies using synchrotron radiation. *Int. J. Biol. Macromol.* 9: 169-175 (1987).
- FRATZL, P. and A. DAXER: Structural transformation of collagen fibrils in corneal stroma during drying. An X-ray scattering study. *Biophys. J.* 64: 1210-1214 (1993).
- FRATZL, P., K. MISOF, I. ZIZAK, G. RAPP, H. AMENITSCH and S. BERNSTORFF: Fibrillar structure and mechanical properties of collagen. *J. Struct. Biol.* 122: 119-122 (1997).
- GRAHAM, H. K., D. F. HOLMES, R. B. WATSON and K. E. KADLER: Identification of collagen fibril fusion during vertebrate tendon morphogenesis. The process relies on unipolar fibrils and is regulated by collagen-proteoglycan interaction. *J. Mol. Biol.* 295: 891-902 (2000).
- HOCKING, A. M., T. SHINOMURA and D. J. MCQUILLAN: Leucine-rich repeat glycoproteins of the extracellular matrix. *Matrix Biol.* 17: 1-19 (1998).
- KEENE, D. R., J. D. SAN ANTONIO, R. MAYNE, D. J. MCQUILLAN, G. SARRIS, S. A. SANTORO and R. V. IOZZO: Decorin binds near the C-terminus of type I collagen. *J. Biol. Chem.* 275: 21801-21804 (2000).
- MELLER, D., K. PETERS and K. MELLER: Human cornea and sclera studied by atomic force microscopy. *Cell Tiss. Res.* 288: 111-118 (1997).
- NATION, J. L.: A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Stain Technol.* 58: 347-351 (1983).
- OSBORNE, C. S., J. C. BARBENEL, D. SMITH, M. SAVAKIS and M. H. GRANT: Investigation into the tensile properties of collagen chondroitin-6-sulfate gels—the effect of cross-linking agents and diamines. *Med. Biol. Eng. Comput.* 36: 129-134 (1998).
- PINS, G. D., D. L. CHISTIANSEN, R. PATEL and F. H. SILVER: Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties. *Biophys. J.* 73: 2164-2172 (1997).
- RASPANTI, M., V. OTTANI and A. RUGGERI: Subfibrillar architecture and functional properties of collagen: a comparative study in rat tendons. *J. Anat.* 172: 157-164 (1990).
- RASPANTI, M., A. ALESSANDRINI, V. OTTANI and A. RUGGERI: Direct visualization of collagen-bound proteoglycans by Atomic Force Microscopy. *J. Struct. Biol.* 119: 118-122 (1997).
- SASAKI, N. and S. ODAJIMA: Elongation mechanism of collagen fibrils and force-strain relations of tendon at each level of structural hierarchy. *J. Biomech.* 29: 1131-1136 (1996).
- SCOTT, J. E.: Proteoglycan-fibrillar collagen interactions. *Biochem. J.* 252: 313-323 (1988).
- : Extracellular matrix, supramolecular organization and shape. *J. Anat.* 187: 259-269 (1995).
- SCOTT, J. E. and A. M. THOMLINSON: The structure of interfibrillar proteoglycan bridges ('shape modules') in extracellular matrix of fibrous connective tissues and their stability in various chemical environments. *J. Anat.* 192: 391-405 (1998).
- SVENSSON, L., I. NÄRLID and Å. OLDBERG: Fibromodulin and lumican bind to the same region on collagen type I fibrils. *FEBS Lett.* 470: 178-182 (2000).
- USHIKI, T., J. HITOMI, S. OGURA, T. UMEMOTO and M. SHIGENO: Atomic force microscopy in histology and cytology. *Arch. Histol. Cytol.* 59: 421-431 (1996).
- YAMAMOTO, S., J. HITOMI, M. SHIGENO, S. SAWAGUCHI, H. ABE and T. USHIKI: Atomic force microscopic studies of isolated collagen fibrils of the bovine cornea and sclera. *Arch. Histol. Cytol.* 60: 371-378 (1997).
- YAMAMOTO, S., H. HASHIZUME, J. HITOMI, M. SHIGENO, S. SAWAGUCHI, H. ABE and T. USHIKI: The subfibrillar arrangement of corneal and scleral collagen fibrils as revealed by scanning electron and atomic force microscopy. *Arch. Histol. Cytol.* 63: 127-135 (2000).
- YOUNG, R. D.: The ultrastructural organization of proteoglycans and collagen in human and rabbit scleral matrix. *J. Cell Sci.* 74: 95-104 (1985).

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