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Different patterns of collagen-proteoglycan interaction: a scanning electron microscopyand atomic force microscopy study

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SUMMARY

The extracellular matrix of unfixed, unstained rat corneal stroma, visualized with high-resolution scanning electron microscopy and atomic force microscopy after minimal preliminary treatment, appears composed of straight, parallel, uniform collagen fibrils regularly spaced by a three-dimensional, irregular network of thin, delicate proteoglycan filaments. Rat tail tendon, observed under identical conditions, appears instead made of heterogeneous, closely packed fibrils interwoven with orthogonal proteoglycan filaments. Pre-treatment with cupromeronic blue just thickens the filaments without affecting their spatial layout. Digestion with chondroitinase ABC rids the tendon matrix of all its interconnecting filaments while the corneal stroma architecture remains virtually unaffected, its fibrils always being separated by an evident interfibrillar spacing which is never observed in tendon. Our observations indicate that matrix proteoglycans are responsible for both the highly regular interfibrillar spacing which is distinctive of corneal stroma, and the strong interfibrillar binding observed in tendon. These opposite interaction patterns appear to be distinctive of different proteoglycan species. The molecular details of proteoglycan interactions are still incompletely understood and are the subject of ongoing research.

INTRODUCTION

Recent years have seen a renewed interest in the biology of glycoconjugates. The older classification of proteoglycans, based on their side-chain, has been superseded by a new definition based on their core protein. Small proteoglycans are now better characterized, in particular the so-called small leucine-rich proteoglycans (SLRPs) (Hocking *et al.*, 1998; Iozzo, 1999), consisting of a minute horseshoe-shaped core protein (Weber *et al.*, 1996) covalently bound to one or more glycosaminoglycan side-chains. Because of their very high affinity for their respective binding sites, most of these proteoglycans are usually entirely bound to specific locations on the fibril surface (Scott, 1984; Brown & Vogel, 1989). Several studies have indicated that each core protein binds non-covalently to a specific intraperiod site on the surface of collagen fibrils (Scott & Haigh, 1988; Pringle & Dodd, 1990; Hedbom & Heinegård, 1993), possibly involving two

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or more collagen molecules (Scott, 1995). As a consequence, the core proteins are highly effective in regulating the fibrils diameter by blocking their lateral accretion (Rada *et al.*, 1993; Fosang & Hardingham, 1996; Weber *et al.*, 1996; Hocking *et al.*, 1998; Iozzo, 1999); in fact, this may be the main fibril size-limiting element in any tissue.

The interactions of the flexible, highly polyanionic glycosaminoglycan side-chains which radiate from the fibrils surface like the bristles of a bottlebrush are, however, far less understood. X-ray diffraction studies and ultrastructural investigations substantially agree in reporting, at least in some tissues, a highly hydrated, fractal perifibrillar layer (Meek *et al.*, 1986; Fratzl and Daxer, 1993; Studer *et al.*, 1996) which supposedly acts as an interfibrillar spacer.

This elegant concept seems, however, inadequate to explain the structural and functional peculiarities of different proteoglycans, and many details of the crucial interactions among proteoglycans, collagen and water are incompletely understood. A major obstacle is that all these interactions depend heavily on hydration, pH and ionic strength and are easily destroyed by the very investigation techniques necessary to their study. In transmission electron microscopy (TEM), tissues are known to be substantially altered by the fixation, dehydration and embedding processes (Yamabayashi *et al.*, 1991; Fullwood & Meek, 1993; Fratzl & Daxer, 1993). Proteoglycans then need to be stained with electron-dense cationic dyes in order to be visible on thin sections, whose thickness is, in turn, usually unable to contain a whole proteoglycan molecule.

These drawbacks can only be overcome by a different technical approach, or by a comparison and cross-correlation of results obtained by different techniques. Atomic force microscopy (AFM) recently emerged as an aggressive competitor of electron microscopy techniques (Ushiki *et al.*, 1996; Diaspro & Rolandi, 1997) in the extracellular matrix field (Chernoff & Chernoff, 1992; Baselt *et al.*, 1993; Revenko *et al.*, 1994; Aragno *at al.*, 1995; Odetti *et al.*, 1995; Fullwood *et al.*, 1995; Meller *et al.*, 1997; Yamamoto *et al.*, 1997; Paige *et al.*, 1998). In previous studies, this technique compared favorably with freeze-etching (FE) and with scanning electron microscopy (SEM; Raspanti *et al.*, 1996), and made possible to display finely-detailed collagen-proteoglycan interactions in unfixed, unstained specimens (Raspanti *et al.*, 1997). SEM, on the other hand, is a more mature technique and offers some advantages in ease of observation and specimen handling, a wider magnification range and a comparable resolution on biological specimens.

In the present study, AFM and SEM were used together to analyze the ultrastructure of the extracellular matrix of tendon and corneal stroma.

MATERIAL AND METHODS

Four albino rats of either sex, 2-months to 6-months old, were sacrificed by an excess of ethyl ether anesthesia. Several specimens of both corneas and of the proximal portion of tail tendon were carefully dissected immediately after death, and were then either

a) immediately dehydrated in graded ethanol and hexamethyldisilazane, or
b) immersed in 0.05% cupromeronic blue (Seikagaku Corp., Tokyo) in 25mM Na-acetate buffer containing 0.1M $MgCl_2$ and 2.5% glutaraldehyde, pH 5.8, overnight at 20°C, and dehydrated as in (a), or
c) treated in chondroitinase ABC, 0.5 U/ml in 0.2M Tris-HCl buffer, pH 8, overnight at 37°C, treated with cupromeronic blue as in (b) and dehydrated as in (a).

Tiny fragments were removed from all specimens with fine tweezers and glued on appropriate stubs with conductive bi-adhesive tape.

Half of the specimens were then observed in tapping-mode atomic force microscopy (TMAFM), on a Digital Instruments Multi-Mode scanning probe microscope fitted with carbon super-tips grown on top of Nanosensors silicon cantilevers (force constant 30-60 N/m, resonance frequency 300-350 kHz). All observations were made in atmosphere, at a scan rate of approx. 2 Hz. TMAFM data files were then rendered in 3D 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-based PC.

Specimens intended for SEM study were coated with 2nm of platinum-carbon in a Balzers Union BAF-301 freeze-etching apparatus fitted with two EK552 Electron Beam Guns and a QSG 201-D quartz crystal thickness monitor, while continuously

rotating under a vacuum better than $1 \cdot 10^{-6}$ Torr, and were then observed in secondary electron detection with either a JEOL JSM-890 "in lens" FEG-SEM or a Philips XL-30 FEG-SEM, both operated at 7 kV.

RESULTS

Unfixed specimens of corneal stroma observed by AFM (Fig. 1/A) appeared made of thin, paral-

Fig. 1 - Unfixed, unstained specimens. In AFM pictures of corneal stroma (A, scan size=600nm) the collagen fibrils (here running diagonally from top left to bottom right), interfibrillar spaces and the interconnecting proteoglycan filaments (arrows) are all clearly visible. Unfixed tendon (B, scan size=2μm), by contrast, appears made of bundles of tightly packed collagen fibrils interwoven with proteoglycan filaments (arrows). In SEM micrographs of both cornea (C, field width=2μm) and tendon (D, field width=2μm) the tissue appears more altered, but proteoglycans (arrows) are still detectable.

lel collagen fibrils, approx. 32-33 nm in diameter and running approx. 40-50 nm centre-to-centre. All fibrils were separated by an evident empty space and interconnected by a number of roughly orthogonal filaments, approximately 4-6 nm thick, forming a complex three-dimensional meshwork from fibril to fibril. In most cases these filaments ran directly from one fibril to another, occasionally bracing three neighboring fibrils with a single bridge, or running in pairs. Frequently they ran obliquely from one fibril to another, and generally they did not show any obvious periodicity. Only occasionally did their spacing correspond to the known distance between binding sites (approx. 24nm and 16nm).

Tendon specimens, on the other hand (Fig. 1/B), appeared made of tightly packed bundles of large inhomogeneous collagen fibrils, with no visible interfibrillar spaces. Proteoglycans were usually regularly spaced at one binding site per period and, because of the reciprocal contact of collagen fibrils, they extended on the surface of neighboring fibrils forming a sort of periodically spaced, orthogonal ties.

SEM micrographs of similar, untreated specimens of corneal stroma and of tendon (Fig. 1/C-D) usually showed a sort of thin, amorphous coat covering the collagen fibrils, and individual proteoglycans were only occasionally demonstrated. This is consistent with previous studies (Raspanti *et al.*, 1996) indicating that unfixed proteoglycans are easily altered by the thermal load exerted on the specimens during the coating process and the observation itself.

Specimens of both tissues treated with cupromeronic blue and visualized by AFM exhibited just slight differences from untreated ones, but AFM pictures and the corresponding SEM micrographs were now almost superimposable. In corneal stroma, in particular (Fig. 2/A and 2/C), both techniques clearly confirmed the organization of collagen fibrils in parallel layers and the meshwork of proteoglycan particles which spans the interfibrillar spaces, but on AFM pictures (Fig. 2/A), the interfibrillar bridges appeared now noticeably thicker, with a diameter of approximately 12-15nm.

Similarly, tendon micrographs (Fig. 2/D) clearly exhibited the regular fabric of glycosaminoglycan side chains lying on the surface of the collagen fibrils and binding them together into tight bundles, and again on the corresponding AFM pictures (Fig. 2/B) the proteoglycans appeared similar but somewhat thickened.

Pre-treatment with chondroitinase ABC (which digests chondroitin- and dermatan-sulfate side chains but leaves keratan-sulfates unaffected) had opposite effects on the two tissues examined. Although corneal stroma was somewhat depleted of proteoglycans, its spatial architecture remained undisturbed and again it showed the usual array of collagen fibrils, clearly spaced apart and interconnected by proteoglycan bridges, not unlike the untreated tissue (Fig. 3/A and 3/C). Tendon specimens, on the contrary, even at low magnification appeared soft and "woolly", their fibrils now freed from any interconnection. At higher magnification, their axial 67 nm D-banding, no longer masked by surface-bound structures, was much more readily appreciable, while proteoglycan filaments were always undetectable (Fig. 3/B and 3/D).

In both tissues, AFM pictures and the corresponding SEM micrographs were again absolutely consistent.

DISCUSSION

Interfibrillar filaments positive to phthalocyanins or to cationic dyes have long been observed with TEM in thin sections of embedded specimens, and they usually appear anywhere collagen fibrils are present. They are, however, much more evident, even without phthalocyanin staining, in the three-dimensional visualization afforded by SEM and AFM.

From a technical standpoint, AFM provides far better pictures than SEM when imaging unfixed, untreated specimens. Once the proteoglycans have been stabilized by cupromeronic blue, both techniques provide high quality pictures with almost identical results. SEM has a wider magnification range and a far superior ease of observation; AFM, which allows a direct comparison with untreated specimens, reveals more clearly the artifactual thickening caused by the phthalocyanin covering of the polysaccharidic chains. SEM seems preferable for the study of fixed and stabilized specimens, while AFM has an evident edge in the observation of native, untreated tissues. Altogether, our observations provide a highly

Fig. 2 - Specimens treated with cupromeronic blue appear not unlike their unfixed counterparts. In AFM pictures (A, corneal stroma, scan size=1.2μm; B, tendon, scan size=2μm), proteoglycan filaments are now visibly thickened by their phthalocyanin coating. SEM micrographs (C, corneal stroma, field width=1μm; D, tendon, field width=2μm), on the contrary, are now directly comparable to the corresponding AFM pictures and their proteoglycan texture is immediately evident.

detailed visualization of the extracellular matrix and disclose wide differences in the collagen-proteoglycan interaction patterns.

Corneal stroma consists of a delicate meshwork of straight, parallel, regularly-spaced collagen fibrils, extensively interconnected by a complex three-dimensional web with no evident pattern or periodicity. The diameter of collagen fibrils corresponds quite well to the values obtained by X-ray diffraction on intact, hydrated tissue (Meek &

Fig. 3 - Specimens digested with chondroitinase ABC and treated with cupromeronic blue exhibit distinctive differences. Corneal stroma (A, AFM, scan size=1.2μm, and C, SEM, field width 4μm) appears almost unaffected in its architecture; its interfibrillar spaces and proteoglycan filaments are not unlike those of Figures 2/A and 2/C. Tendon (B, AFM, scan size=2μm, and D, SEM, field width 4μm), by contrast, is now completely depleted of proteoglycans; its collagen fibrils, only covered by sparse debris, show more clearly their 67nm axial banding.

Leonard, 1993; Fratzl & Daxer, 1993), while the inter-fibrillar spacing, which is more sensitive to dehydration (Fratzl & Daxer, 1993), is reduced by some 25% with respect to the values reported in normally hydrated stroma. Proteoglycans are extremely abundant and appear irregularly distributed along the fibrils, apparently due to the presence of multiple (but partly vacant) binding sites.

Digestion with chondroitinase ABC, which removes chondroitin- and dermatan-sulfate side chains (bound to decorin) but not keratan-sulfate (bound to lumican, fibromodulin and keratocan), leaves the tissue architecture and the fibril spacing unchanged.

It is known that keratan sulfate-containing proteoglycans are highly critical to corneal transparency. A low content of keratan sulfate is observed in pathological states (Chakravarti *et al.*, 1988) as well as in corneal scars, whose return to transparency invariably accompanies the return of keratan sulfates (Fosang & Hardingham, 1996); lumican-deficient animals develop bilateral corneal opacity (Iozzo, 1999). A few years ago, it was hypothesized that glycosaminoglycan side chains interact in the interfibrillar space with other, antiparallel chains (Scott, 1995) forming rigid interfibrillar bridges, whose length would impose a constant interfibrillar distance and which would be quite consistent with our observations. Such an antiparallel interaction was subsequently observed in solutions of other polysaccharides (Cowman *et al.*, 1998; Scott and Heatley, 1999), although different interaction mechanisms are also reported (Gribbon *et al.*, 1999). Since the distinctive interfibrillar spacing of corneal stroma is maintained after chondroitinase digestion as well as in decorin-lacking animals, it must be ascribed to the keratan-sulfates.

Tendon, on the other hand, is made of collagen fibrils of large, variable diameter, ranging anywhere from 25 to 250nm (but values outside this range are not rare), closely packed into tight bundles where no interfibrillar spaces are visible. Side chains are far more scarce than in corneal stroma and show a more evident relationship with the axial period, with just one binding site per period; they also stretch out on adjoining fibrils, interweaving them into a tight, dense structure. Despite the evident saturation of their proteoglycan-binding sites, tendon fibrils always run in close contact, tightly packed in dense bundles. Chondroitinase ABC digestion destroys the tissue architecture and removes all the side chains, consistent with the notion that only dermatan sulfate-containing decorin is present in this tissue in substantial amounts.

This proteoglycan-mediated interconnection of collagen fibrils is likely to play an important functional role *in vivo*. Even if the mutual adhesion of two side chains has been estimated to be just 40 picoNewtons (Dammer *et al.*, 1995), the progressive build-up of shearing forces along the fibril can rapidly exceed the tensile strength of the fibril itself - a familiar principle in composite materials engineering. This functional role of decorin in interconnecting collagen fibrils is highly consistent with experimental observations: alpha-amylase digestion causes an evident, dose-dependent decrease of the mechanical properties of fibrous tissues (Watanabe & Komatsu, 1997) and the skin of decorin-deficient animals exhibit reduced tensile strength (Danielson *et al.*, 1997), while the addition of chondroitin-6-sulfate to a collagen gel significantly increases its Young's modulus and ultimate strength (Osborne *et al.*, 1998).

The differences observed between the layout of chondroitin/dermatan-sulfates (readily evident in tendon, where no other significant glycosaminoglycan species are present) and the keratan-sulfates (easily observable in corneal stroma once other glycosaminoglycans have been removed by chondroitinase digestion) clearly suggest different interaction patterns for these proteoglycans. The molecular machinery responsible for these interactions is, however, still insufficiently known. Glycosaminoglycan side-chains have been supposed to interact with anti-parallel chains from neighboring fibrils (Scott, 1995; Dammer *et al.*, 1995), with the fibril surface itself (Font *et al.*, 1993; Pogany *et al.*, 1994), with microfibrils of type VI collagen (Nakamura *et al.*, 1994), or again with the core protein of other proteoglycans - not to mention less pertinent structures such as fibronectin, TGF-β and C1q. It seems that, under the right circumstances, anything can interact with anything else; and all these interactions being electrostatic or hydrophobic/hydrophilic, they are highly sensitive to experimental conditions.

A highly critical point, at least in proteoglycan-rich tissues, may be the balance of order and disorder. It is noteworthy that proteoglycans in corneal stroma seem to follow a somewhat irregular pattern not only in our own SEM and AFM observations, but also with freeze-etching (Yamabayashi *et al.*, 1991), cationic dyes (Takahashi & Tohyama, 1991) and immunolocalization (Bairaktaris *et al.*, 1998). The variation of the interfibrillar spacing with hydration (Fratzl & Daxer, 1993) and the high osmotic pressure still present in

intact, healthy cornea indicate that its physiological hydration level is far less than the proteoglycans would allow, so that mutual interaction of proteoglycans is not fully exploited under normal conditions. This may explain the formation of spacers of consistent, but hydration-depending, length.

Dermatan sulfates, by contrast, may be unable to form antiparallel bridges at all, since in tendon they interact preferentially with the fibril surface.

This topic is the subject of ongoing research on other tissues. Although highly challenging from the technical standpoint, a wider understanding of these interactions would shed light on the structural biology of all connective tissues and represents a highly rewarding objective.

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