PROTEOGLYCAN–COLLAGEN INTERACTIONS. STRUCTURE IN THE AMORPHOUS GROUND SUBSTANCE

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Glycosaminoglycans occur in vivo outside the collagen fibrils. In this perifibrillar space specific interactions between proteoglycans and the collagen fibrils lead to the formation of an organized structure. Different tissues were examined by an electron histochemical method and it was concluded that the single proteoglycans have specific loci of association with the type I collagen fibril and are arrayed orthogonally in respect to the fibrils.

Connective tissue collagen fibrils develop and function in intimate contact with proteoglycans (PGs).* There has long been an interest in the possibility of interactions of a specific nature between the two. It has been speculated that such interactions might influence or even control two of the most important events in connective tissue history: the increase in size, and the calcification of collagen fibrils. Although many attempts have been made to demonstrate interactions in vivo, the evidence was at most suggestive, until recently, following the development of adequate ultrastructural techniques. The clearest indications that the primary structures of the participants was important in deciding the strength of their interactions came from in vitro experiments, particularly those in aqueous solution using light scattering[1]. However, the close apposition of tropocollagen molecules in fibrils presents completely new, stable arrays of amino acid residues, extending across many collagen molecules, providing vastly increased opportunities for specific PG binding, as compared with those offered by independent single molecules in solution. The most direct and meaningful way of investigating these possibilities utilizes appropriate tissues. The results are then of functional, as well as structural significance.

Although upwards of 10 different collagen types are known or postulated, the greater part of the body collagen consists of one gene product, the collagen type I. Several major connective tissues, e.g. bone, tendon, skin, sclera, and cornea contain very little of any other type of collagen, and therefore the interaction of PGs with type I collagen are of major interest.

* Abbreviations: CSA chondroitin sulphate, DS dermatan sulphate, HA hyaluronate, KS keratan sulphate, PG proteoglycan, GAG glycosaminoglycan.

EXPERIMENTAL

Chondroitin sulphate, dermatan sulphate, and hydroxyproline were assayed by the methods listed in Scott and Hughes[2]. Staining for proteoglycans was by the Cupromeronic blue-CEC method[3] as described in ref.[4]. Enzyme digestions by keratanase and chondroitinase ABC were according to Scott and Haigh[4]. Fresh tissues were from rabbit or rat, used immediately for ultrastructural work, or frozen at −20°C before biochemical analyses. Bovine skin, bone and rat femur were demineralized by the method of Scott and Kyffin[5] and processed for electron microscopy as described in ref.[9].

RESULTS AND DISCUSSION

The tissue we chose first was tendon, since not only is it mainly type I collagen, it is morphologically simple, has an uncomplicated function, and in some cases and in some species it calcifies.

We applied the biochemical morphometric approach to 3 simple, mutually exclusive models. The soluble polymer could be *a*) entirely within the collagen fibril, *b*) entirely between the fibrils, or *c*) entirely at the surface of the fibrils. In *a*), tissue concentrations of collagen and GAG should change in parallel during development, while in *b*) they should be inversely related, since the more collagen the less space there is for GAG. The ratio of GAG to collagen in *c*) would be that of the surface area of the cylindrical collagen fibril ($2\pi rl$) to that of its volume ($\pi r^2 l$), where r is the radius and l the length of the cylinder. The ratio of tissue concentrations, collagen: GAG, would be proportional to r (refs[2,6]).

The results from chick and calf tendon[2] (Fig. 1) showed that the ratios of the concentrations of HA and CSA to that of collagen both diminished sharply, as the collagen concentration rose. This rules out possibility *a*), above, that these GAGs

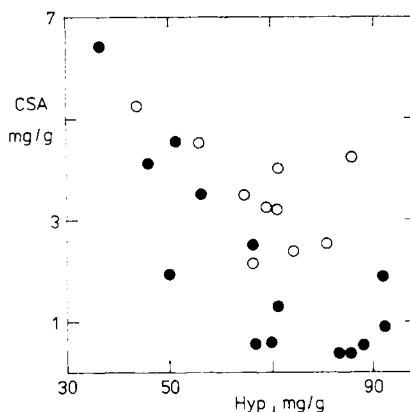

FIG. 1

Plot of tissue concentrations of chondroitin sulphate (CSA) against that of hydroxyproline (Hyp) determined on calf and chick flexor tendons; ○ chick, ● calf. The concentrations are in terms of dry weight. There is a very pronounced downward trend in this relationship, particularly in the case of calf tendon. The chick data alone are not as significant, although showing a similar trend. Compared with hyaluronate, measured on the same samples[2], the CSA data show greater scatter. This may be due to heterogeneity in the CSA-containing PGs, as compared with the chemically homogeneous hyaluronate

were present inside the collagen fibrils, and strongly suggests that they are present outside the fibrils, (possibility *b*)). These arguments assume that the distribution of GAGs inside or outside the fibrils remained relatively uniform during development, unaffected by the appearance or disappearance of other biopolymers. Since exactly similar patterns of changing CSA, HA, and collagen concentrations were observed[2] in cow flexor digitorum and rat tail tendons which develop in utero and after birth, respectively, compared with in ovo, in the case of the chick, it follows that the physiological environment of the tendon is secondary, and the observations are likely to apply to the development of tendon collagen fibrils wherever they are found.

DS does not follow the trends of HA and CSA, but it does fit relationship *c*), in which the glycosaminoglycan is assumed to be present solely and uniformly at the fibril surface[6]. HA and CS do not fit this relationship[2]. The DS data from three different species fall on the same line, implying a constant stoichiometry between the DS–PG and the fibril surface both throughout development and throughout much of recent evolution. Such constancy implies a very important function for DS–PG (ref.[6]).

Table I summarizes the results obtained by biochemical morphometry. No structural model can be proposed from these relationships. For this, it is necessary to have an ultrastructural method of visualizing PG. We developed an electron histochemical method for PGs, based on the dye Cupromeronic blue[3,7] with the particular aim of showing whereabouts vis-a-vis fibrils and cell membranes. The application of this reagent to rat tail tendon showed regular electron dense filaments arrayed orthogonally to the collagen fibrils. Subsequent uranyl acetate staining demonstrated the $a-e$ banding pattern of the collagen fibril, showing that the orthogonal PG filaments were mainly located at the *d* band, with a few at the *e* band[8]. The filaments were resistant to hyaluronidase, and this, together with the biochemical analyses on the whole tissue referred to above, indicated that they must be DS-rich. The method of biochemical analysis, requiring recovery of the whole glycosaminoglycan polymer,

TABLE I

Position of glycosaminoglycuronans with respect to collagen fibrils of developing tendon

Glycosaminoglycuronan	Hyaluronate	Chondroitin sulphate	Dermatan sulphate
Inside the fibrils	no	no	no
Between the fibrils	yes	(yes)	no
On the fibril surface	no	(yes)	yes

permitted the attribution of this iduronate-rich DS to the "small" DS–PG, rather than to the co-occuring "large" PG, ref.[6]. DS–PG was then shown to be associated with type I collagen fibrils at the *s* and *e* bands in skin, sclera (Fig. 2) and cornea[4]. Orford and Gardner[9] found a similar distribution on some collagen fibrils of articular cartilage. Since cartilage contains DS (ref.[10]) and some type I collagen[11], there is good reason for postulating that DS–PG will always be found at the gap zone (*d* and *e* bands) of the type I collagen fibril. The converse is not true. The "small" DS–PGs of different tissues and from different species are remarkably similar in their amino-acid compositions[12] suggesting a high degree of evolutionary conservation, and this might be expected if their main role is to associate with the *d* or *e* bands of type I collagen fibrils, in view of the highly conserved nature of much of the collagen structure. Pearson and Gibson[12] found that the "small" DS–PG was more difficult to extract from the tissue in salt solution; compared with the "large" DS–PG, and this, too, supports our contention that the former is bound to the collagen fibrils, whereas the latter is not.

Cornea contains not only DS but also KS, and the specific demonstration of corneal DS–PG depended on the digestion of the tissue with keratanase, prior to staining for PG. The identity of the remaining gap-zone-associated PG was confirmed by digestion with chondroitinase ABC, by which it was specifically removed[13].

Chondroitinase-digested cornea still retained orthogonally arrayed PGs but at the *a* and *c* bands, at the step to the gap zone. Keratanase digestion removed them,

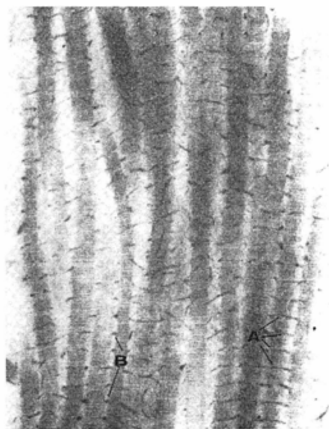

FIG. 2

Rabbit sclera, stained with Cupromeronic blue in 0·3M-$MgCl_2$ and counterstained with uranyl acetate (180 000x). This section shows orthogonally transfibrillar peripheral proteoglycan (PG) filaments A. Where the fibril is cut on a diameter, the perifibrillar PG filaments are seen in cross section (e.g. B). A tangential cut leaves almost the complete hoop of PG filament (A). The filaments are spaced apart by one D period.

thus confirming that they were KS–PG. Cornea untouched by either chondroitinase or keratanase showed PG filaments at both the gap and the step zones, i.e. at four separate binding sites, the *a*, *c*, *d*, and *e* bands (Fig. 3).

By far the largest part of the type I collagen in the body is in the bone, but the application of our techniques to mineralized tissues presents problems. The mineral must be removed to permit unequivocal attribution of electron density to stained PGs – but the process of removal must not distrub the localisation of PG. We developed a method employing non-aqueous solvents, with or without formaldehyde as a fixative, which totally prevented the loss of GAG from the tissue[5,14]. Normal analytical and ultrastructural methods could then be applied to the demineralized organic matrix. The analyses showed that there was little DS – less than 10% of total GAG, most of which was CSA, and there was no KS (ref.[4]). It is therefore particularly relevant that there was little or no observable stained PG at any of the transfibrillar loci, the *a*, *c*, *d*, and *e* bands, which had been occupied in the cornea. This result reinforces the conclusion that each PG has its specific locus of association with the type I collagen fibril, and if the relevant PG is not present, that locus will not be occupied. One PG cannot substitute for another.

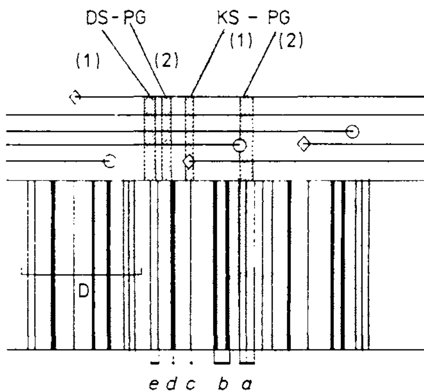

FIG. 3

Map of proteoglycan (PG) locations along the type I collagen fibril. Diagram of the *a*—*e* banding pattern within the D period of collagen type I fibril (lower portion) shown against arrangement of collagen molecules in quarter stagger (upper portion). ◊ amino terminal, ○ carboxyl terminal. The locations of PGs are displayed across the quarter stagger diagram, to correlate with the *a*, *c*, *d*, and *e* banding pattern. The DS–PG and KS–PG groupings are based on staining of keratanase or chondroitinase ABC digested tissue or hyaluronidase digestion plus biochemical morphometric analysis of tendon, skin etc. DS–PG 1, DS–PG 2, KS–PG 1 and KS–PG 2 are the proteoglycans so designated by Gregory et al.[16]. The numbers are bracketed to indicate that they are allocated to their bands on a different basis to those of the DS or KS group as a whole, and are tentative[13]. The DS–PGs are located in the gap zone in all the soft connective tissues so far examined, and the KS–PGs are at the "step" from the overlap to the gap zone (in the cornea)

The pictures nevertheless show a considerable amount of PG, mainly oriented parallel with the axes of the collagen fibrils – a finding which proves that CS–PG is an integral part of the bone structure, and not just remnants or islands of the cartilagenous precursor[4].

Since the gap zone has been implicated as the site of nucleation of calcification[15], it is of interest that DS–PG occupies this region in the non-calcifying, type I collagen--rich connective tissues, and that the gap zone is unoccupied by any PG in bone. It is tempting to speculate that DS–PG plays a part in inhibiting the calcification of type I collagen in soft connective tissues.

The demonstration of two binding sites on the collagen fibril for each kind of PG (DS and KS implies either (i) two corresponding sites on each PG molecule, or (ii) two types of PG molecule, each with one site specific for one region of the fibril surface. Since rabbit corneal KS–PGs and DS–PGs each contain two PGs, varying in the protein core[16], (ii) is the preferred explanation[13].

A third possibility, that both bands in each pair (i.e. the *d* and *e* pair and the *a* and *c* pair) contain an identical binding site implies that each pair of PGs also have an identical binding site. This possibility cannot be ruled out a priori, in view of the many repetitive sequences along the collagen molecule – and the presence of identical structures in the GAG side-chains of each pair of KS–PGs and DS–PGs would satisfy the second requirement. However, in principle, it should lead to equal occupancies of each band of the pair, and this is not the case for the DS–PG of cornea, sclera or tendon[13]. The simplest explanation is that each binding site is specific for one PG. On this basis, one can speculate that DS–PG 1 binds to the *d* band, since it is present at about 5 times the level of DS–PG 2 in cornea, and the *d* band is much more frequently occupied by PG than is the *e* band. The attribution of KS 2 to the *a* band is based on the parallels between the relative concentrations of KS 2 to KS 1 (i.e. 2 : 1, Gregory et al.[16]) and the relative masses of Cupromeronic blue-stained-PG at the *a* and *c* bands, obtained by X-ray diffraction using a Synchotron source.

These arguments lead to the proposed map of PG binding sites, shown in Fig. 3. The presence of two separate KS–PGs and two DS–PGs in cornea is well established. Our arguments would require that the DS–PGs in skin, tendon, and sclera should also contain two kinds of PG, varying in their protein core, and the literature contains observations suggesting that this is so, at least in the case of tendon and skin (see ref.[13] for a discussion).

It is possible now to see that, far from being the amorphous ground substance of classical anatomy, the perifibrillar space is structured. This structure is organized and tied down by specific interactions between PG and the collagen fibril.

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