Interrelationships of mucopolysaccharide and collagen in connective tissue remodelling

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SUMMARY

The effects of the release of normal distractive forces from the rabbit's tendo-achilles by distal tenotomy and proximal sciatic neurectomy alone, and in combination, have been studied. The results demonstrate that:

1. Complete release of distractive forces from the tendon is followed by changes in collagenous architecture and an increase in cell size and cell population, particularly at the musculo-tendinous junction.
2. The changes in the resident cell population and the cells migrating into the tendon from the musculo-tendinous junction and perivascular spaces are associated with the production of increased quantities of mucopolysaccharide and the disaggregation of collagen fibres in the vicinity.
3. In the initial stages this increased mucopolysaccharide appears to be predominantly unsulphated material - probably hyaluronic acid, but with the reapplication of tension which follows spontaneous repair of the divided tendon, this is replaced by a sulphated polymer which appears to be associated with the reaggregation of collagen fibres at this stage.
4. The implications of this biphasic production of mucopolysaccharide, and its effect on the stability and integration of collagen, are discussed.
5. During this study it was found that the Masson's trichrome stain appears to be a precise indicator of the state of tension of collagen fibres within the tendon, and other connective tissues.

INTRODUCTION

A temporal and spatial correlation has been observed between the disaggregation of collagen fibres and the appearance of increased quantities of mucopolysaccharide material in the repair of split skin donor sites (Flint, 1971), in precancerous changes in the dermis (Dobson & Griffin, 1962; Prodi & Maltoni, 1957), and in many hormone-dependent situations (Gersh, 1950; Storey, 1957; Bryant, Greenwell & Weeks, 1968). The role of mucopolysaccharides in this collagenolytic process has not been clearly defined, nor has it been established whether the mucopolysaccharide material is the cause or the result of collagen disaggregation. Observation of the same phenomenon in areas of dermal hypertrophic scarring which were softening after release of tension, indicated that morphological changes in a dense collagenous structure could be induced by release of distractive forces (Flint, unpublished observation).

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As tension is known to influence collagen deposition and stabilization (Abercrombie, 1957; Buck, 1953; Harkness, 1961), the effect of selective removal of the forces which normally maintain tension within the tendo-achilles has been studied, to assess the role of mucopolysaccharides in connective tissue remodelling.

**MATERIALS AND METHODS**

Fifty-six mature albino rabbits were randomly divided into four equal groups. Animals in each group were subjected to one of four unilateral operative procedures designed to disturb the normal distractive forces on the tendo-achilles. In Group A the tendo-achilles was completely divided just above the level of its insertion into the calcaneum. In Group B the distal limb muscles were paralysed by complete sciatic neurectomy in the thigh. In Group C tenotomy was combined with neurectomy. In Group D tenotomy was followed by immediate suture of the tendon gap by nylon figure-of-8 suture.

Controls were provided by the normal contralateral hind limbs, and by the tendons of tibialis posterior which remained intact on the operated side in all animals, although denervated in Groups B and C.

One animal from each group was killed at 1, 3, 5, 7, 10, 12, 14, 18, 21, 28, 32, 35, 42 and 56 days postoperatively. After removal of the skin, whole-leg specimens were fixed in 10% formalin and 0.5% cetylpyridinium chloride for at least 3 weeks. Samples of muscle, musculo-tendinous junction, tendon, tendon gap and insertion were embedded in paraffin, sectioned at 5 µm and stained with haematoxylin and eosin, Masson’s trichrome, Verhoeff’s Elastic, and Gordon and Sweet’s silver stain, using standard histological techniques (Culling, 1963). Aldehyde fuchsin (Halmi & Davies, 1953), PAS (periodic acid-Schiff) (Pearse, 1949), colloidal iron (Mowry, 1963) and alcian blue at pH 1 and 2.5, were used alone or in combination (Spicer, Horn & Leppi, 1967) to distinguish acid mucopolysaccharides and differentiate carboxyl and sulphated polymers.

**RESULTS**

At each postoperative stage, differences were observed between all four groups of animals (Table 1). Tendo-achilles tenotomy induced marked cellular and extracellular change throughout the length of the tendon and musculo-tendinous junction. Tendon suture performed immediately after tenotomy diminished or prevented these changes until the unprotected suture subsequently parted. Tendons of muscles paralysed by sciatic neurectomy, although apparently relaxed, became taut during passive dorsiflexion of the foot and showed little histological change from the controls. The most marked changes occurred in tendons which had been completely freed from distractive forces by a combination of proximal muscle paralysis and distal tenotomy. Changes were observed in the staining reaction, cellular components, collagen and ground substance of the affected tendons.
Table 1. **Histological changes in tendon**

<table>
<thead>
<tr>
<th>Distinctive forces</th>
<th>Tendon in normal leg and control tendon (tib. post.)</th>
<th>A. Tenotomy</th>
<th>B. Paralysed</th>
<th>C. Tenotomy/paralysed</th>
<th>D. Tenotomy/paralysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen staining with Masson’s trichrome</td>
<td>Red</td>
<td>Green with patches of red</td>
<td>Red</td>
<td>Green</td>
<td>Red with patches of green</td>
</tr>
<tr>
<td>Cellular changes</td>
<td>Nil</td>
<td>+, in tendon and muscle</td>
<td>Nil in tendon. + change in muscle</td>
<td>++, in tendon and muscle</td>
<td>+ --, patchy in tendon</td>
</tr>
<tr>
<td>State of collagen</td>
<td>Normal</td>
<td>Disaggregated, +</td>
<td>Normal</td>
<td>Disaggregated, ++</td>
<td>Patchy disaggregation, + --</td>
</tr>
<tr>
<td>Acid mucopolysaccharide</td>
<td>Sparse. Only around cells</td>
<td>+</td>
<td>Sparse. Only around cells</td>
<td>++</td>
<td>In repair zone, ++</td>
</tr>
</tbody>
</table>

- Minimal
- Easily demonstrable
- Gross
1. Changes in staining with trichrome stains

In contrast to the collagenous connective tissue of normal tendons which retains the red fuchsin component of trichrome stains (Fig. 1), tendons which had been divided stained green with the Masson's trichrome stain (Figs. 2 and 3). Those paralysed but not divided and the control tibialis posterior tendons still retained the red fuchsin stain. Tendons which had been divided and sutured immediately, stained mainly red, but tendons which had parted after suture, or parts of tendon which had escaped transfixion by the suture, showed partial green staining (Fig. 4). In tendons which had been completely relaxed, by sciatic denervation and distal tenotomy, green staining was apparent on the first postoperative day and became widespread throughout the tendon during the next few days. Adjacent undivided tendons of tibialis posterior, or tendon bundles still held by their fascial attachments, retained the red stain.

2. Changes in cellular components

In divided and paralysed tendons marked changes in cellularity, cell morphology and tendon structure occurred not only at the site of injury, but also throughout the length of the tendon and particularly at the musculo-tendinous junction. These changes were particularly obvious in areas which stained green with the trichrome stain, but were absent in undivided tendons or parts of tendon which still retained the red component of the trichrome stain. Tendons relaxed by tenotomy showed noticeable increase in cell size and population within two days (Figs. 7 and 9). Resident tenocytes apparently became larger and their cytoplasm more distinct. Mitotic activity of these enlarged cells supplemented the increase in cellularity produced by young connective tissue cells and fibroblasts from the musculo-tendinous junction and perivascular spaces. Increased

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**Figures 1-6.**

Fig. 1. Musculo-tendinous junction of intact tendon, from control limb, fixed in situ under normal tension, to show the red staining with Masson's trichrome stain. × 125: Agfachrome 50L.

Fig. 2. Musculo-tendinous junction of tendo-achilles, from Group C, previously relaxed by tenotomy and proximal neurectomy and fixed in situ, showing green staining with Masson's trichrome. × 62.5: Agfachrome 50L.

Fig. 3. Tendon, from Group C, incompletely relaxed showing residual flecks of red-staining collagen with Masson's trichrome. × 62.5: Agfachrome 50L.

Fig. 4. Sutured tendon, from Group D, staining mainly red but staining green in relaxed areas. × 62.5: Agfachrome 50L.

Fig. 5. Mucopolysaccharide stained blue with colloidal iron surrounding relaxed collagen bundles and coating the surface of others. 7 days postoperative, Group C. × 125: Agfachrome 50L.

Fig. 6. Mucopolysaccharide, blue with colloidal iron, coating and separating collagen fibres. × 312: Agfachrome 50L.
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Images 1 to 6 depict various stages of tissue remodelling, showcasing the interplay between mucopolysaccharides and collagen in the process of tissue regeneration.
Fig. 7. Musculo-tendinous junction of tendon, from Group C, which has been divided and paralysed, showing increased cellular activity and early changes in collagenous architecture.

Fig. 8. Normal musculo-tendinous junction from a control limb. Sparse cellularity of the tendon, except in the narrow zone at the musculo-tendinous junction.

Fig. 9. Increase in cell population, cell size and mitotic activity at proximal end of relaxed tendon from Group C.
Figs. 10, 11. Higher-power views from the same field as Fig. 9 showing enlarged cells and mitotic activity in a large tenocyte. Early separation of collagen fibres is apparent.

Fig. 12. Young connective tissue cells at musculo-tendinous junction in relaxed tendon from Group C.
cellular activity was also observed in the interfascicular connective tissue of the relaxed muscle. Within the first week after operation the greater part of the affected tendon, and particularly the proximal part at the musculo-tendinous junction, was densely populated with fibroblasts and young connective tissue cells (Figs. 9–12). Considerable mitotic activity was observed.

3. Change in extracellular component

The increases in cellular activity and cell size were constantly associated with loss of normal tendinous architecture and fragmentation of collagen bundles into finer randomly orientated fibrils (Figs. 13, 14, 15). Breakup of the collagen fibres was accompanied by a great increase in the concentration of acid mucopolysaccharide in the lytic areas as demonstrated by the colloidal iron and alcian blue techniques. In the early stages the stained material coated and separated the larger collagen bundles (Figs. 5, 16, 17, 18) but with increasing collagen disaggregation large amounts of acid mucopolysaccharide material, staining blue with colloidal iron, was observed between, and apparently separating, the fragmented and dissociated fibres. Some areas showed predominance of mucopolysaccharide with almost complete loss of normal collagen fibrillar staining (Figs. 6, 14, 15). This pronounced disorganization was apparent throughout the length of the tendon, being particularly marked in the proximal half, and musculo-tendinous junction some 3–5 cm away from the site of the original tendon section.

In tendons sutured immediately after tenotomy there was less intratendinous cellular response and collagen disaggregation, except in those areas of the tendon which appeared to have escaped transfixion by the tendon suture and which stained green with the Masson's trichrome (Fig. 4).

With the later spontaneous tendon repair and restoration of continuity, about 2–3 weeks postoperatively, the cellular and extracellular changes were reversed. The cells became orientated in the line of tension and developed the morphology of large fibroblasts. This cellular change was accompanied by the reappearance of fine collagen fibres laid down in the same tension lines around the cells, and a change in the type of mucopolysaccharide detected by histological staining.

4. Mucopolysaccharides

During the period of collagen disaggregation large quantities of mucopolysaccharide were detected between the fragmented collagen bundles by colloidal iron staining (Figs. 5, 6, 14, 15). Differential staining by alcian blue at pH 1 and 2-5 and alcian blue/aldehyde fuchsin and alcian blue/PAS sequences suggested that at the time of disaggregation of the collagen fibres much of this mucopolysaccharide was hyaluronic acid, demonstrated by alcian blue staining at pH 2-5 but not at pH 1. With the later re-establishment of tension and the realignment of fibroblasts following spontaneous repair, a sulphated acid mucopolysaccharide which showed affinity for aldehyde fuchsin and alcian blue pH 1, was found in increasing amounts. This change was associated with increased
Fig. 13. High-power fields of relaxed tendon from Group C stained with Verhoeff’s Elastic showing dissociation of collagen fibres into smaller fibrils, some spanning cystic spaces containing mucopolysaccharide.

Fig. 14. Area of polysaccharide accumulation, shown here as black, in relaxed tendon from Group C. Photographed with orange-red filter.

Fig. 15. Identical area photographed with yellow Y 3 filter. Collagen fibres appear as fine threads coated with polysaccharide.
Fig. 16. Wavy, disaggregated collagen in relaxed tendon from Group C, 7 days post-operative. Same area as Fig. 5.

Fig. 17. High-power view of area marked in Fig. 16 showing diffuse coating of polysaccharide. Photographed with yellow filter.

Fig. 18. High power view of part of segment marked in Fig. 17 refocused to show web-like coating of polysaccharide. Fine fibres of collagen and polysaccharide span and surround cystic spaces. Photographed with orange-red filter.
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deposition of orientated collagen fibres which gradually reaggregated throughout the latter half of the 8-week period. At the end of this time the mucopolysaccharide concentration had markedly decreased, but the looseness of the collagen aggregation and the larger cell population made it still possible to differentiate the areas of tendon which had passed through a remodelling process from those parts which had remained intact throughout.

DISCUSSION

These experimental studies have demonstrated that complete removal of distractive forces from the tendo-achilles induces extensive cellular and extracellular changes, particularly in the musculo-tendinous junction and proximal half of the tendon 4–5 cm from the site of tenotomy. These changes are minimized or prevented by the reapplication of tension by suturing the tendon immediately after tenotomy.

Trichrome stains and state of collagen

Previous studies (Craik & MacNeil, 1965) demonstrated that the staining affinity of dermal collagen with various trichrome procedures could be changed by strongly stretching the skin, indicating that collagenous connective tissue which has been held under tension stains with trichrome stains differently from that which has not. Observations made during these present experiments show that whereas the collagen of normal tendon stains red with the fuchsain component of the Masson’s trichrome sequence, tendon from which the distractive forces have been removed shows an increasing affinity for the light green counter-stain. This change in staining reaction can be prevented by re-suturing the tendon immediately after tenotomy, but becomes apparent if there is subsequent separation of the suture. This staining technique appears to provide a remarkably precise indicator of the state of tension within collagen fibres prior to fixation.

Relation of tension to morphological changes

The different staining potential of collagen made it possible to differentiate those parts of the tendon which were relaxed from those parts which were still held under tension. The observed changes in cell size and cell population were most evident in, and almost solely restricted to, those areas that were relaxed, as were the subsequent collagen disaggregation and mucopolysaccharide formation. It was not possible to determine whether this change in cell morphology was induced directly by release of longitudinal distractive forces, or transverse compressive forces, or whether it was induced by related but indirect factors, such as increased vascularity and changes in oxygen tension, which, in other situations, are known to affect bone and collagen deposition and reabsorption (Buck, 1953; Charnley, 1965; Harkness, 1961). It was, however, possible to show that collagen disaggregation occurred only in areas in which there were
prior changes in cell size, shape, mitotic activity and cell population, and Masson staining, and did not occur in areas in which tenocytes and cell population were within normal limits.

*Cellular activity, mucopolysaccharide formation and collagen dissaggregation*

Whilst routine connective tissue stains indicated that there was a causal relationship between cellular proliferation and collagen disaggregation, histo-chemical methods which demonstrated a concomitant increase in the concentration of acid mucopolysaccharides in these areas encouraged a re-appraisal of the role of these polymers in collagen fibre stability. The colloidal iron stain, as modified by Mowry (Mowry, 1963), indicates the presence of acid mucopolysaccharide material. Normally, very little polysaccharide material is seen, histologically, in tendon except around individual tenocytes. Between 7 and 14 days after release of tension the quantity of this material gradually increased until, by 14 days, there was in the relaxed areas often more polysaccharide material than collagen. In the areas of mucopolysaccharide accumulation, there were associated decreases in collagen fibre size, fibre aggregation and collagen concentration as judged histologically. There appeared to be almost an inverse relationship between the concentration of polysaccharide and the concentration and aggregation of collagen. Differential staining with alcin blue at pH 2-5 and 1 and PAS/alcian blue, and alcin blue/aldehyde fuchsin sequences suggested that hyaluronate staining at pH 2-5 but not at pH 1 was the dominant polysaccharide during the period of disaggregation, but that later, during the phase of collagen reorganization, when the distractive forces were reapplied by spontaneous union, the polysaccharide material was predominantly a sulphated polymer staining with aldehyde fuchsin and alcin blue at pH 1, presumably a chondroitin or dermatan sulphate.

Although these staining methods indicated that there were differences in quality and type of mucopolysaccharide produced at different phases of the remodelling cycle, it is of course impossible to be dogmatic about this until they are corroborated by micro-chemical estimations. However, extrapolation of information from other biological situations supports the concept of this biphasic polysaccharide production seen histologically. Studies in wound healing show a similar biphasic response with an initial production of hyaluronate, followed later by chondroitin sulphate during the phase of collagen aggregation (Dunphy & Udupa, 1955; Dunphy, Udupa & Edwards, 1956; White, Shetlar & Schilling, 1961). Tissue culture studies also indicate that young connective tissue cells from many sources growing in free conditions during their exponential growth phase produce hyaluronate as did the new connective tissue cells in this experiment (Grossfeld, Meyer & Godman, 1955; Morris, 1960; Morris & Godman, 1960), but later revert to chondroitin sulphate and collagen production when their growth becomes confluent (Green & Goldberg, 1963, 1964), if
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confined in a diffusion chamber (Priest & Priest, 1964), or external stresses are applied (Prockop, Pettengill & Holtzer, 1964).

This biphasic production observed during normal wound healing has been presumed to be a part of the integrated synthetic cycle leading to collagen formation. However, demonstration of increased quantities of mucopolysaccharide during the process of collagenolysis, not only in these experiments but also in the dermis following exposure to ultra-violet light (Nakamura & Johnson, 1968), in the healing of split-skin donor sites (Flint, 1971), and in the cervix, foetal membranes and pelvic ligaments as they soften before parturition (Harkness, 1956; Harkness & Harkness, 1956), suggests that some mucopolysaccharides, particularly unsulphated polymers, may be associated more with collagen fibre dispersal than with the development or close aggregation of fibres during collagen synthesis.

The various sulphated and non-sulphated polymers are known to have different potentialities for binding and aggregating collagen fibres (Wood, 1960; Toole & Lowther, 1967; Mathews, 1965, 1967). The different physical characteristics of the polymers are reflected in variations in the morphological structure of tissues with which they are associated (Meyer, 1969). The constant association of hyaluronate with very fine collagen fibres and loose connective tissue stroma (Fessler, 1960) suggests that dense collagen fibre aggregation and hyaluronate are almost mutually incompatible under physiological conditions.

It is known that the production of a highly-sulphated chondroitin polymer will, during the process of corneal repair, induce thick fibre aggregation, with a resultant loss of corneal transparency (Anseth, 1965). Conversely, in the experimental situation described here, the accumulation of hyaluronate in the relaxed areas is apparently associated with the separation of collagen fibres into smaller fibrils. It is suggested that the accumulation of large amounts of ‘collagen-dispersing hyaluronate’ in an area which is normally bound strongly by chondroitin or dermatan sulphate, will upset that part of the collagen fibre binding which is dependent on the highly charged sulphated polymer (Jackson, 1953; Mathews, 1965), leaving the separated fine collagen fibrils and tropocollagen units still bound by their inherent electrostatic bonds (Partington & Wood, 1963; Veis, Bhatnagar, Shuttleworth & Mussell, 1970).

These small units could be more readily dispersed by relatively low concentrations of the various collagenolytic and proteolytic enzymes already demonstrated (Gross, 1965; Dingle, 1965; Woessner, 1965; Houck, de Hesse & Jacob, 1967).

Production of more highly charged sulphated polymer by the connective tissue cells would reverse the collagenolytic process allowing collagen fibre reaggregation. The knowledge that such a biphasic production of mucopolysaccharide is already known to occur supports the concept that variations in type and quantity of mucopolysaccharide production by connective tissue cells may be implicated in the regulation of the state of their collagenous environment.
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The author is now a Senior Fellow of the Medical Research Council.

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