Changes in glycosaminoglycan content of healing rabbit tendon

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SUMMARY
Changes in the concentration of the major glycosaminoglycan compounds were measured during healing of severed Achilles tendons in rabbits. Using zone electrophoresis for separation, and densitometry for quantitation of these compounds, their concentrations could be measured in each of several portions of the tendon and its associated muscle.

Immediately after injury, the concentration of hyaluronic acid in the healing area and those portions of the tendon closely associated with it increased dramatically. An increase in the concentration of dermatan sulphate accompanied the subsequent formation of repair tissue.

Increased concentrations of glycosaminoglycan compounds were also found in the musculotendinous area remote from the injury, probably in response to relaxation of the severed tendon.

INTRODUCTION
The healing of vertebrate wounds is associated with dramatic changes in both the concentration and type of glycosaminoglycan found in the healing area (Dorner, 1968; Bazin, Delaunay, Nicoletis & Delbet, 1970). These compounds are produced by fibroblasts, which, having proliferated in the adjacent perivascular spaces, migrate into the healing area (Lindsay & Birch, 1964; Greenlee & Pike, 1971). Whilst other work on the healing tendon has described changes in the healing area itself (Buck, 1953; Munro, Lindsay & Jackson, 1970), Flint (1972) examined the histology of the entire tendon and showed major changes throughout the tendon, including staining reactions suggesting extensive increases in glycosaminoglycans at considerable distances from the healing area. The present paper examines these observations by means of recently reported methods for the separation and estimation of micro amounts of glycosaminoglycans in small tissue samples (Breen, Weinstein, Anderson & Veis, 1970).

MATERIALS AND METHODS
Achilles tendons of 6-month-old New Zealand white rabbits were used as experimental material. The sciatic nerve was exposed in the thigh of one hind limb in each animal and cut to paralyse the calf muscles. The Achilles tendon in

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Rabbits were killed at 3, 7 and 18 days after operation. The Achilles tendon and gastrocnemius muscle were removed from each limb for analysis of their glycosaminoglycan content. The newly formed granulation tissue between the distal end of the experimental tendon and the calcaneum was removed. The tendon was freed from the paratenon and then cut into portions as indicated in Fig. 1. Each tendon was cut into two equal lengths. Since the musculotendinous junction could not be separated into discrete tendon and muscle portions, a portion including both tendon and muscle, equal in length to the tendon pieces, was removed. A muscle sample was taken from that part of the gastrocnemius muscle contiguous with the musculotendinous junction.

The tissue portions were minced by hand and the fat removed by two 24 h extractions with ether:acetone 1:1 (20 ml/g of tissue). The fat-free tissue was dried and weighed. The tissue was then digested for 24 h at 50 °C with sufficient Protease Type VI (Sigma) in Tris/HCl buffer at pH 7-4 to provide 0-1 mg protease per mg fat-free dry tissue. The digest was cooled to 4 °C and trichloracetic acid was added to a final concentration of 5 %. This solution was allowed to stand for 20 min before centrifuging at 48000 g for 20 min at 4 °C. The supernatant was decanted into 3 vols. of absolute ethanol containing 0-5 % 2 m-CaCl₂, stored for 72 h at −10 °C, then re-centrifuged at 48000 g for 20 min at 4 °C. The supernatant was discarded and the precipitate washed with ether:ethanol 1:1 and then ether alone. For convenience, the ether-extracted precipitates were transferred to small ampoules as follows: the precipitate was dried, then dissolved in ca. 0-1 ml distilled water, transferred to the ampoule and lyophilized.

Electrophoretic separation and estimation of the glycosaminoglycans in each tissue sample was carried out using cellulose acetate electrophoresis (Breen et al. 1970) with a Beckman Microzone Electrophoresis Cell. Lyophilized glycosaminoglycan precipitates were dissolved in sufficient distilled water to give a glycosaminoglycan concentration of ca. 0·5 mg/ml, and 0·25 μl samples were
applied to the cellulose acetate membranes. Electrophoresis was carried out at
4 °C, using a 0-2 M-ZnSO₄ electrolyte and constant current (1 mA/cm membrane
front) for 35 min. After electrophoresis the membrane was stained, destained,
and densitometrically scanned as described by Breen et al. (1970). Glycosamine-
glycans in the tissue samples were identified by co-electrophoresis with standards
of hyaluronic acid (Sigma), dermatan sulphate (Hoffman-La Roche) and
chondroitin sulphate mixed isomers (Sigma).

RESULTS

Electrophoresis of tissue extracts

Typical electrophoretic patterns of glycosaminoglycans from the distal
portion of a control tendon and a healing tendon are shown in Fig. 2. The
glycosaminoglycans of both tissues separated on electrophoresis into two distinct
bands—one clearly defined band of hyaluronic acid, and a slightly more diffuse
band co-electrophoretic with dermatan sulphate. In no samples was there any
measurable amount of chondroitin sulphate.

Changes in the concentration and distribution of hyaluronic acid during healing

The concentration of hyaluronic acid in the different portions of the tendons
during the healing process is shown in Table 1. Within 3 days of tenotomy, the
Table 1. Changes in the hyaluronic acid concentration in portions of rabbit tendon during healing (µg hyaluronic acid/mg fat-free dry weight)

The concentration in the control distal portion is also given for the 'control' healing area. Subscript letters show confidence limits. Figures in vertical columns with no letter in common are significantly different at the 5% level.

<table>
<thead>
<tr>
<th>Healing area</th>
<th>Distal portion</th>
<th>Proximal portion</th>
<th>Musculotendinous junction</th>
<th>Muscle</th>
<th>Paratenon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.37&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>3 days postoperative</td>
<td>3.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.41&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 days postoperative</td>
<td>3.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.24&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.79&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18 days postoperative</td>
<td>4.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.68&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 2. Changes in the dermatan sulphate concentration in portions of rabbit tendon during healing (µg dermatan sulphate/mg fat-free dry weight)

The concentration in the control distal portion is also given for the 'control' healing area. Subscript letters show confidence limits. Figures in vertical columns with no letter in common are significantly different at the 5% level.

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<th>Musculotendinous junction</th>
<th>Muscle</th>
<th>Paratenon</th>
</tr>
</thead>
<tbody>
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<td>0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 days postoperative</td>
<td>0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>7 days postoperative</td>
<td>2.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.37&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;ab,e&lt;/sup&gt;</td>
<td>1.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
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<td>1.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;c&lt;/sup&gt;</td>
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concentration of hyaluronic acid in the healing area had risen markedly. A concomitant fall in the concentration of hyaluronic acid in some of the surrounding tendon portions was not significant at the 5% level. Over the next 3 weeks, the hyaluronic acid concentration in these portions rose to higher than control values. The hyaluronic acid concentration in the musculotendinous junction increased more during healing than that in the proximal tendon portion. In the latter, the concentration during healing was never significantly different from that of the control.

Changes in the concentration and distribution of dermatan sulphate during healing

The changes in the dermatan sulphate content of different parts of the tendon during healing are shown in Table 2. At 3 days the concentration of dermatan sulphate in the healing area had not changed significantly. In all tendon portions other than the healing area, dermatan sulphate was present in lower concentrations than in their respective controls. In the muscle and paratenon portions, this fall was significant at the 5% level. In the later stages of healing, the
Fig. 3. Changes in the ratio of hyaluronic acid to dermatan sulphate during the healing of rabbit tendon. The ratio for the control distal portion is used as the value for the healing area at $t = 0$. Changes in the ratio are shown for the healing area (■—■), distal portion (▲—▲), proximal portion (□—□), musculotendinous junction (○—○), muscle (▽—▽) and the paratenon (●—●).

concentration of dermatan sulphate rose markedly in the healing area, and steadily in all other portions of the tendon.

Changes in the ratio of hyaluronic acid and dermatan sulphate in healing tendon

The changes in the ratio of hyaluronic acid to dermatan sulphate during tendon healing are shown for each of the tendon areas in Fig. 3. In control tendons, the ratio was 0·45 for the tendon proper, 1·5 for the muscle and 0·95 for the paratenon and musculotendinous junction. After tenotomy, the ratio rose extremely rapidly in the healing area itself, reducing thereafter, but never to the low value of the control tendon portions. This change was mirrored by the areas most closely associated with the healing area – the distal area and the paratenon. The ratio of these compounds in the proximal portion and in the musculotendinous junction did not change substantially although a slight change was observed in the muscle tissue itself.
DISCUSSION

The changing morphology and biochemistry of regenerating tendons have been the subject of many recent investigations (Buck, 1953; Dorner, 1968; Harper, Lowery & Wray, 1970; Schmitt, Ervig & Beneke, 1970; Greenlee & Pike, 1971; Flint, 1972). From the complex (and sometimes conflicting) changes that have been reported following tenotomy, a consistent pattern of regeneration emerges. The cut tendon end, severed from its attachment to the calcaneum, retracts to form a broad stump, leaving a space between itself and the calcaneum which is partly covered by paratenon sheath. The fibrinous matrix which forms in this healing area is rapidly invaded by fibroblasts from the adjacent loose connective tissue and paratenon sheath. These cells are largely responsible for the subsequent processes of healing and repair. The results reported here have been expressed on a dry-weight basis. Substantial increases in dry weight (up to 100 %) occurred in all tendon portions during healing. The changes in glycosaminoglycan concentration that we measured therefore indicate even greater increases in the total glycosaminoglycan content of each tendon portion during healing. The use of microzone electrophoresis has now enabled us to study chemical changes in all portions of the tendon during these processes of healing and repair.

Within 3 days of the tenotomy, the concentration of hyaluronic acid produced by the fibroblasts in the healing area was 11 times that of the normal concentration in the distal portion, causing a very large change in the glycosaminoglycan ratio in this area. A fall in the concentration of both dermatan sulphate and hyaluronic acid was observed in most portions of the tendon at 3 days after operation. This could either reflect migration of fibroblasts from these tissues (becoming the fibroblasts of the healing area) or may result from metabolic changes in response to the operation. The changed glycosaminoglycan ratio which accompanies lowered concentrations in these areas at 3 days after tenotomy argues for the latter hypothesis. Certainly, the relatively rapid turnover of glycosaminoglycan molecules described by Schiller, Mathews, Cifonelli & Dorfman (1956) would allow quite rapid metabolic control of the production of these compounds.

As healing progressed further, there was a marked increase in levels of glycosaminoglycans throughout the tendon, particularly in the paratenon sheath and distal area. These changes undoubtedly reflect the close association of these latter tissues with the healing area – both develop hypercellularity as healing progresses, and the gliding space between the tendon and the paratenon sheath is obliterated in the region of the healing area (Lindsay & Birch, 1964). Increased concentrations of glycosaminoglycans in other parts of the tendon did not represent mere contamination from the healing area, since the ratios of glycosaminoglycans in these tissues did not change in the same manner as in the healing area itself.

The concentrations of glycosaminoglycans in the proximal portion of the
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tendon did not change significantly throughout the healing period, but the level of hyaluronic acid in the musculotendinous junction almost doubled during the 18 days following tenotomy. This observation confirms the intriguing histological observations (Buck, 1953; Flint, 1972) indicating changes in tissue architecture and chemistry in this portion of the tendon which is some distance from the site of the injury itself. That changes occur in areas separated from the wound by unaffected tendon suggests that there must be some physical effect (such as relaxation of tension following tenotomy) altering the metabolism of the fibroblasts there.

Undoubtedly the most interesting change that occurs during tendon regeneration is the rapid accumulation of dermatan sulphate in the later stages of healing. This restores the glycosaminoglycan ratio in the healing area to near that of normal tendon, which argues for the suggestion (Flint, 1972) that the initial part of the healing process involves production of unsulphated glycosaminoglycans to aid collagenolysis, and that the later stages require a sulphated ground substance to assist the deposition of collagen fibres. Our understanding of the biochemistry of wound healing will be much advanced when we understand the metabolic control of glycosaminoglycan production by the fibroblasts in regenerating tendons.

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REFERENCES


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