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Skeletal Muscle Injury— Molecular Changes in the Collagen During Healing*

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Summary. Changes in the collagen types and cross-linking of granulation and scar tissue in the injured site of partially ruptured gastrocnemius muscle were studied after a reproducible contusion injury to the left calf of a rat. In normal i.m. collagen the proportion of Type I collagen was considerably higher than Type III. Following injury there was a rapid increase in the proportion of Type III collagen reaching a maximum at 5 days after injury. After a further 2 days the proportion of Type I had increased significantly resulting in a decrease of the Type III/I ratio to below that of the control. However, as healing progressed there was a gradual shift back to the Type III/I ratio for normal i.m. collagen.

The collagen produced in response to an injury was initially stabilized by the stable keto-imine cross-link hydroxylysino-5-keto-norleucine, characteristic of embryonic collagenous tissues. The proportion of the stable keto-imine cross-link gradually decreased, and a reversion to the cross-link pattern of normal uninjured i.m. collagenous connective tissue occurred towards the end of the 42-day follow-up period.

The present biochemical study demonstrates that during the early phases of the repair process there is a reversion to the collagens typically present in high proportions in embryonic dermal connective tissue. This suggests that the fibroblasts have the ability to modify their product expression under varying circumstances. The study also demonstrates the importance of collagen cross-linking in determining the tensile strength of collagen fibre during the repair process.

Key words: Collagen types – Collagen cross-linking – Injury – Skeletal muscle – Healing

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Introduction

In normal muscle the collagenous tissue consists of Types I and III collagen, Type I collagen being present predominantly in the epimysium forming the outer sheath of the muscle, and in the perimysium surrounding the muscle fibre bundles. Type III collagen is confined mainly to the perimysium and the endomysium, the latter structure surrounding each individual muscle fibre [13, 15].

When a muscle is injured the connective tissue structures are disrupted, and the resulting space is gradually filled with proliferating cells and extracellular matrix components synthesized by them. During the healing process two competitive events are thus taking place simultaneously: regeneration of disrupted muscle and production of a connective tissue scar [1, 23]. The latter can inhibit complete regeneration of muscle fibres when an excessive production of granulation tissue occurs. In the treatment of muscle injuries a common complication is the re-rupture of the healing muscle associated with both early and overly vigorous mobilization of the injured limb [21, 33] presumably due to the inability of the scar tissue to resist the sudden pulls directed on it.

In the early stages of dermal wound healing and experimental granulation tissue formation there is an increase in the synthesis of Type III collagen [9, 12, 16] but as the granuloma matures, it produces predominantly Type I collagen [16]. However, in certain pathological repair processes, such as hypertrophic scars and keloids, a failure in the regulation of collagen synthesis results in a high proportion of Type III collagen, which persists several years after the injury [5]. It has been suggested that the low tensile strength of scar tissue is related to the high proportion of Type III collagen present in the early stages of healing [18], in that these fibres have a much smaller diameter than Type I fibres [36]. In all tissues the collagen fibres act as the mechanical support and as such must be highly cross-linked to provide an inextensible stress-bearing fibre [3]. The stability of the fibrillar structure must therefore also be considered an important feature in wound healing, in addition to the differences in polymorphic forms of collagen.

The two major intermolecular collagen cross-links in young tissues have been characterized as dehydro-hydroxylysinonorleucine (dehydro-HLNL) and hydroxylysino-5-ketonorleucine (HLONL) [6]. Predominance of dehydro-HLNL or HLONL varies depending on the tissue, but no correlation between structure and function of the tissue and the cross-linking has been found [3]. During embryonic development, however, there is a predominance of HLONL in foetal dermis, which also contains high proportions of Type III collagen (0.6:1 Type I to Type III [14]). In the early postnatal period there is a rapid change over to a predominance of dehydro-HLNL as the major cross-link [35], and the proportion of type III collagen is markedly decreased (4:1, Type I to Type III [14]). It has been shown that the change over from HLONL in the foetal skin to dehydro-HLNL in postnatal skin is due to elevated lysine hydroxylation in embryonic collagen [2, 10], and is not related to a change over in the ratio of the two collagen types [7]. Similarly, in the healing of a normal scar there is a change over in the cross-link pattern from the embryonic to adult type, i.e. a change in predominance of HLONL to dehydro-HLNL, whereas

hypertrophic scars retain the characteristics of embryonic cross-linking [5]. The proportion of reducible cross-links in connective tissue during the postnatal growth period is high due to the rapid turnover of collagen, but gradually decreases during ageing [35]. During this time the fibre matures, and the stability or mechanical strength increases due to the conversion of the reducible cross-links to stable non-reducible multivalent cross-links the chemical characteristics and nature of which are not yet known [32].

The present study was carried out to determine the changes in the ratio of Types I and III collagen and in cross-linking of collagen during healing of a muscle injury.

Materials and Methods

Thirty-six male Spraque-Dawley rats aged 12–16 weeks were housed in stainless steel cages and were fed ad libitum on commercial pellets and water. A constant transverse contusion injury was induced to the left calf under light ether anaesthesia using a blunt spring-loaded hammer so that the gastrocnemius muscle was partially ruptured without tearing the overlying skin [22]. The day of traumatization was designated Day 0.

Specimens were obtained after killing on days 5, 7, 14, 21, and 42 from injured muscles by dissecting the injured area of six rats in each group and the specimens from each follow-up stage were combined. The corresponding area from six uninjured gastronemius muscles were combined and served as controls.

The soluble non-collagenous muscle proteins were removed by two extractions in Hasselbach-Schneider solution and one in distilled water [20].

Estimation of Type III/I Collagen Ratio

The samples were chopped finely with a scalpel and dried on a filter paper at 4°C. For estimation of the Type I and Type III collagen ratio 20-mg portions were dissolved in 2 ml 70% formic acid containing 20 mg cyanogen bromide.

Digestion was allowed to proceed for 4 h at 30°C with constant stirring. Any residual particulate matter was removed by centrifugation (range 0.186–0.218 mg lyophilized material). The CNBr-solubilized samples were then diluted with a ten/fold volume of distilled water, and the excess CNBr and formic acid were removed by evaporation in vacuo at 30°C [31]. The CNBr peptides were dissolved in 0.5 *M* acetic acid and lyophilized (range 4.98–5.37 mg).

The collagen CNBr peptides were then dissolved to a final concentration of 5 mg/ml in a solution containing 2% (w/v) SDS, 0.125 *M* Tris-Cl pH 6.8, 10% glycerol and 0.5% (w/v) bromphenol blue by incubating at 60°C for 30 min [30]. The peptides of the experimental samples and of Types I and III reference collagens, prepared in an identical way to the experimental samples, were resolved by SDS-polyacrylamide gel electrophoresis [26, 27].

Gels were fixed and stained overnight in a Coomassie brilliant blue solution (0.1% w/v) dissolved in a mixture of methanol, acetic acid and water (5:1:5, respectively). Destaining was performed in 7.5% (w/v) acetic acid and 5% (w/v) methanol. Destained gels were scanned by quantitation of reflected light using a Joyce-Loebl Chromoscan Densitometer and photographed. Individual peptides were quantitated by tracing, cutting out and weighing the peaks as described by Light [29].

Collagen Cross-link Analysis

Cross-link determinations were carried out on the remaining portions of the samples (range 200–400 mg wet weight). They were suspended in PBS and reduced with tritiated potassium

borohydride, KB^3H_4 (Amersham, Bucks., UK; 100 mCi/10 mg; The Radio-Chemical Center UK) under standard conditions as described by Robins et al. [35]. After reduction the samples were dialyzed against distilled water, centrifuged at 10,000 *g* for 15 min, and the pellets were lyophilized. Weighed portions of the lyophilized samples (range 17.5–62.0 mg) were hydrolyzed in 6 N HCl at 110°C for 24 h in open flasks equipped with a glass tube to allow ^3H escape. The HCl was removed by evaporation in vacuo at 60°C. The hydrolysate was dissolved in a known volume of water (5 ml) from which an aliquot (0.5 ml) was taken by hydroxyproline determination (Table 1) using the method of Woessner [38], as adapted for the Technicon Auto-Analyzer [19]. The remaining volume was evaporated to dryness, dissolved in 0.1 *M* pyridine-formate buffer (pH 2.9) and submitted to ion exchange chromatography using an ion-exchange resin equilibrated in pyridine/formate buffers. The amino-acids and cross-links were separated by means of a pH gradient (pH 2.9–5.0), and the column effluent was collected in 5-ml fractions. The radioactivity of each fraction was measured and a curve of radioactivity vs. fraction number constructed. The identity of the radioactive intermolecular collagen cross-links was confirmed by comparison with authentic samples on the extended basic column of a Beckman amino acid analyser [35]. Quantitation of the individual peaks was carried out by tracing, cutting out and weighing the peaks as described by Light [29].

Results

Collagen Typing

The purified Types I and III collagen were submitted to cyanogen bromide digestion to obtain type specific marker peptides for SDS-polyacrylamide gel electrophoresis. The electrophoretic mobilities of the peptides $\alpha 1$(I) CB^8 and $\alpha 1$(III) CB^6, which were used to estimate the two collagen types [30] are shown in Fig. 1. The marker peptide $\alpha 1$(I) CB^8 for Type I collagen, (M_r 22,000), runs relatively close to some peptides, but was adequately resolved to afford accurate quantitation. The marker for Type III collagen $\alpha 1$(III) CB^6 M_r 8,000) [30] runs free from other peptides.

SDS polyacrylamide gels of CNBr peptides from control, granulation and scar tissue 7 and 42 days after injury are shown in Fig. 1. Samples were run before and after reduction with 2% w/v mercaptoethanol, the latter clearly having no effect on the mobility of the peptides. $\alpha 1$(I) CB^8 and $\alpha 1$(III) CB^6

Table 1. Recovery of muscle collagen after Hasselbach-Schneider extraction

	Total tissue after Hasselbach-Schneider extraction (mg dry weight)	OH-pro (μg)/total tissue	Collagen content (mg dry weight)	Recovery (%)
Control	21.5	2,190	15.6	72.5
5 days	17.5	1,770	12.6	72.0
7 days	38.5	3,624	25.8	67.0
14 days	41.0	5,040	35.8	87.5
21 days	48.0	4,140	29.5	61.4
42 days	62.0	5,412	38.6	62.2

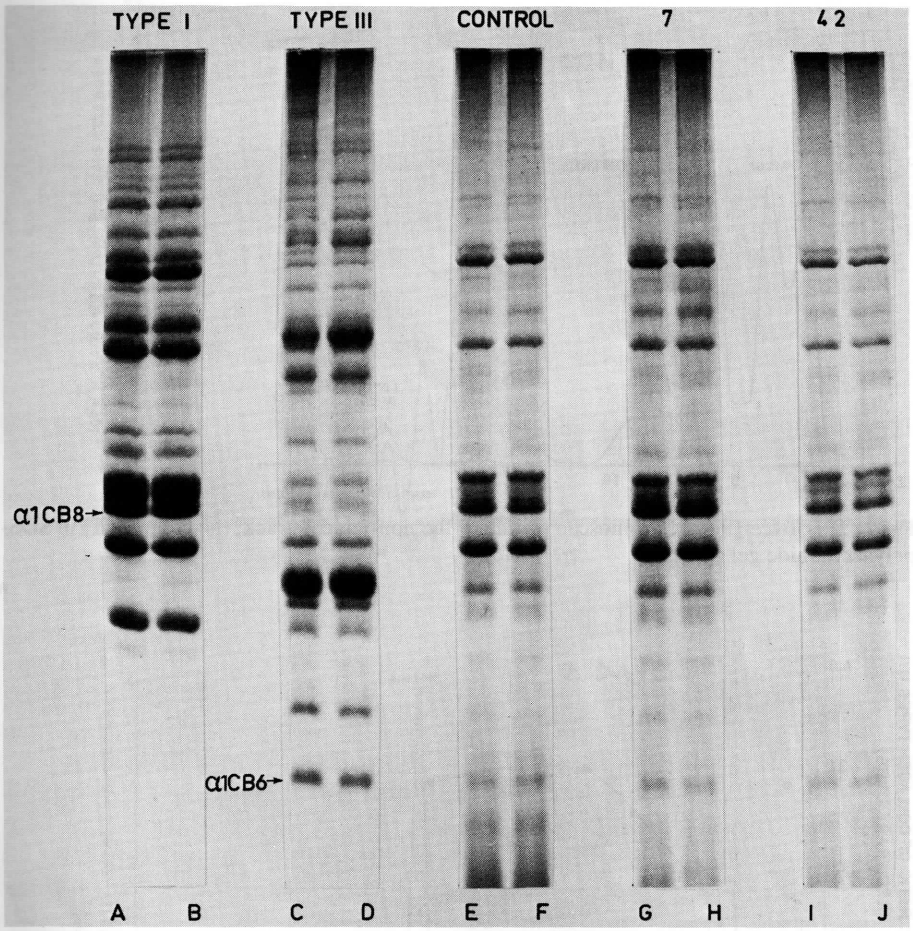


Fig. 1. SDS-polyacrylamide gel electrophoresis of CNBr peptides from pepsin-extracted rat skin Type I collagen (*A*) and (*B*) without (−) and with (+) mercapto-ethanol (*ME*), respectively. Pepsin-extracted rat skin Type III collagen (*C*) and (*D*) − and + ME, respectively. Normal i.m. collagen (*E*) and (*F*) − and + ME, respectively. Granulation tissue of the injured area seven days after traumatization (*G*) and (*H*) − and + ME, respectively. Scar tissue of the injured are forty-two days after traumatization (*I*) and (*J*) − and + ME, respectively. The control, 7- and 42-day samples were first exhaustively extracted with Hasselbach-Schneider solution and distilled H_2O to remove non-collagenous material

peptides were detected in all the samples, thus confirming the presence of Types I and III collagens and the proportions of these two peptides, obtained from the densitometric scans (Fig. 2), were used to calculate the Type III: Type I ratio.

By Day 5 the III/I ratio showed a marked increase over the control, but by day 7 the ratio had dropped to a level much below that of the control. The ratio was still low on Day 14, but by Day 21 had returned to the level of the control and thereafter remained constant (Fig. 3).

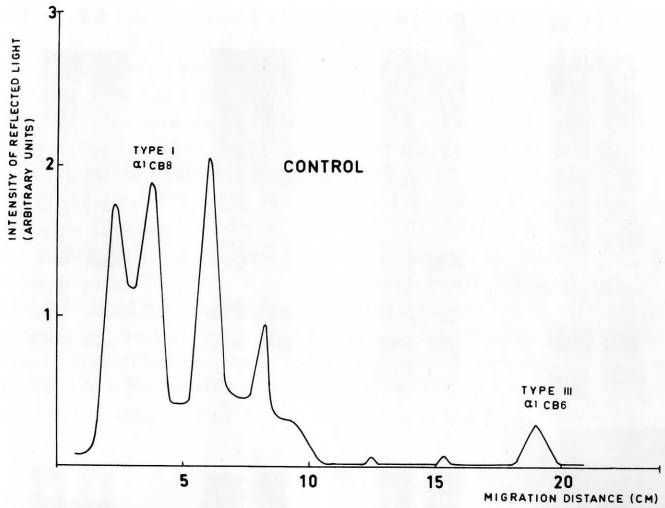


Fig. 2. The Joyce-Loebl Chromoscan profile of the nonreduced track, the control (*E*) in SDS-polyacrylamide gel of Fig. 1

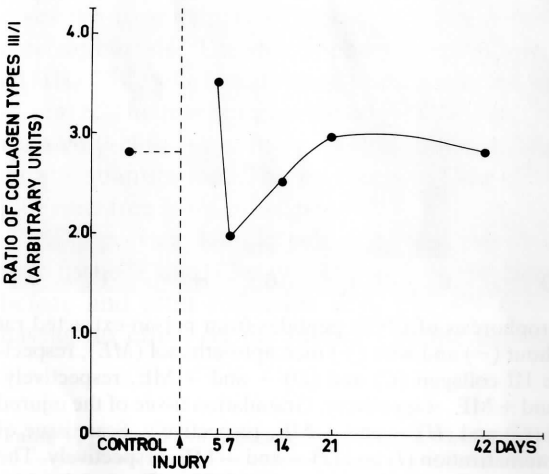
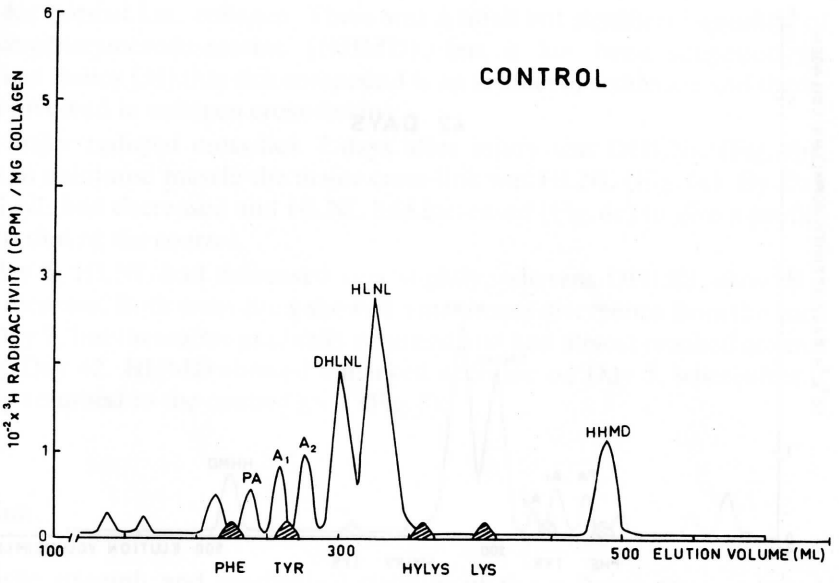


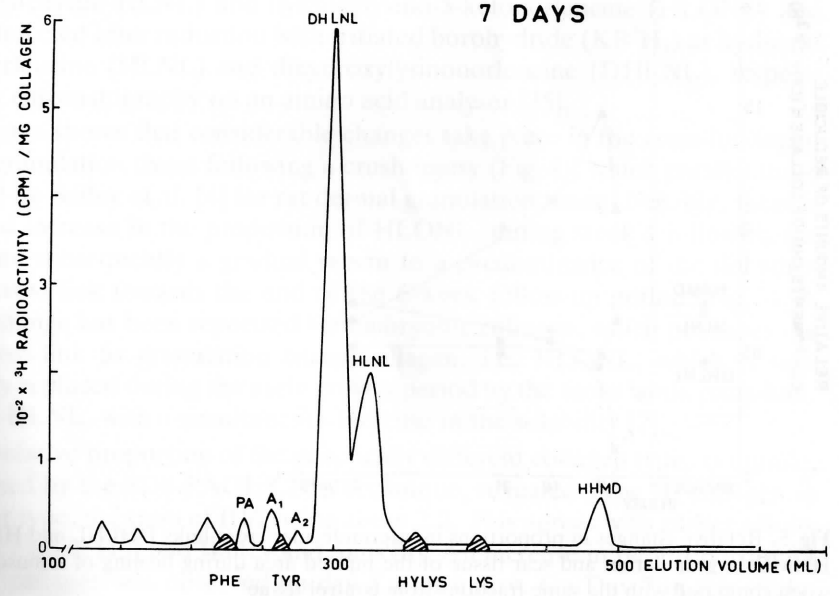
Fig. 3. Relative changes in ratio of collagen Types III/I in granulation and scar tissue of the injured area during the healing process of a muscle injury when compared with the ratio of collagen Types III/I in normal i.m. collagen (control). Densitometric scans of the two peptides $\alpha 1$(I) CB^8 and $\alpha 1$(III) CB^6 were used to calculate the Type III/I ratio

Reducible Intermolecular Cross-links

Cross-link analyses were carried out on the connective tissue from normal control muscle, granulation tissue and scar tissue 5–42 days after injury. After reduction with tritiated borohydride (KB^3H_4) the samples were hydrolyzed and submitted to ion-exchange chromatography. The elution pattern of the reducible components from the control sample was typical for normal i.m. collagen



a



b

Fig. 4. Elution patterns of acid hydrolysates of tritiated KB^3H_4 reduced collagen from (**a**) normal i.m. collagen, (**b**) granulation tissue in the injured area 7 days after traumatization and (**c**) scar tissue in the injured area 42 days after traumatization (for (c) see next page). Before reduction with borohydride the samples were exhaustively extracted with Hasselbach-Schneider solution and distilled H_2O to remove noncollagenous material. The locations of the reduced cross-links are shown relative to the usual amino acids. Reduced collagen cross-links are dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL)

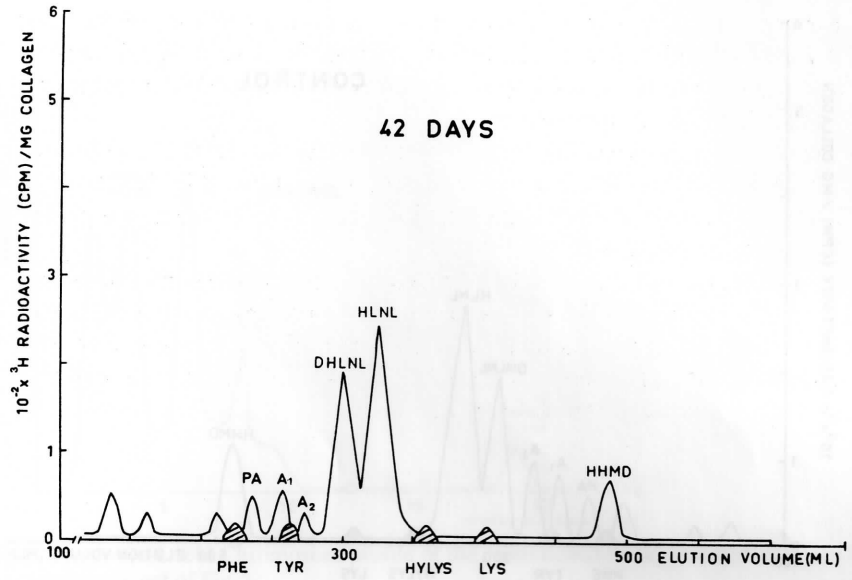


Fig. 4c

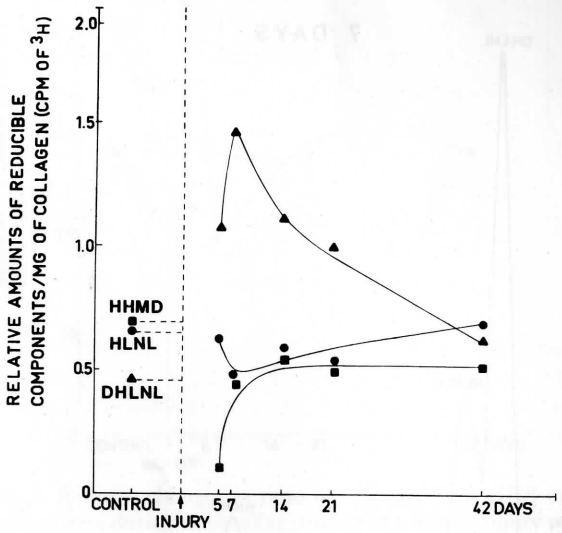


Fig. 5. Relative changes in proportions in intermolecular cross-links DHLNL and HLNL and HHMD in granulation and scar tissue of the injured area during healing of a muscle injury when compared with the same fractions from control tissue

(Fig. 4a). Fractions pA and A_1, which eluted close to tyrosine in the pyridine-formate buffer system, are glycosylated derivatives of hydroxylysine and lysine, respectively, whereas fraction A_2 is a hydrolysis artifact of hexitol lysine [35]. Dihydroxylysinonorleucine (DHLNL) and hydroxysinonorleucine (HLNL) [35] eluted between tyrosine and hydroxylysine, and showed a typical elution

pattern for normal i.m. collagen. There was a small but significant quantity of histidinohydroxymerodesmosine (HHMD), but it has been suggested by Robins and Bailey [34] that this compound is an artifact of reduction and therefore not involved in collagen cross-linking.

The major reduced cross-link 7 days after injury was DHLNL (Fig. 4b), whereas in uninjured muscle the major cross-link was HLNL (Fig. 4a). By Day 42, DHLNL had decreased and HLNL had increased (Fig. 4c) to give a profile similar to that of the control.

By day 5, HLNL had decreased very slightly, whereas DHLNL showed a marked increase. Both cross-links showed a maximum divergence from the control at Day 7, but thereafter gradually returned and had almost reached control levels by Day 42. HHMD showed a marked decrease on Day 5, whereafter it gradually returned to the control level (Fig. 5).

Discussion

The tensile strength and mechanical stability of the collagen fibre is based largely upon the formation of intermolecular cross-links between the constituent molecules [6]. The cross-links exist in vivo as dehydro-hydroxylysinonorleucine (dehydro-HLNL) and hydroxylysino-5-ketonorleucine (HLONL) and can be detected after reduction with tritiated borohydride (KB^3H_4) as hydroxylysinonorleucine (HLNL) and dihydroxylysinonorleucine (DHLNL), respectively, by chromatography on an amino acid analyser [35].

We have shown that considerable changes take place in the cross-linking of muscle granulation tissue following a crush injury (Fig. 4), which parallel those reported by Bailey et al. [4] for rat dermal granulation tissue. Notably, there is a marked increase in the proportion of HLONL, during week 1 following the injury and subsequently a gradual return to a predominance of the dehydro-HLNL cross-link towards the end of the 6-week follow-up period (Fig. 5). A similar change has been reportred for embryonic collagen, which possesses the same cross-link as granulation tissue collagen, i.e. HLONL, which is subsequently replaced during the early growth period by the more labile cross-link, dehydro-HLNL, with a simultaneous increase in the solubility [7].

The relative proportion of the genetically different collagen types in muscle, determined by the SDS-PAGE/CNBr technique, revealed Type III collagen as the major type, the ratio of III/I being about 2.7. This agrees with earlier results of Duance et al. [13] and Bailey and Sims [8]. An increase in the proportion of Type III collagen was observed on day 5 after injury. On Day 7 in this study, only 2 days after the first follow-up stage, the ratio of Type I and III collagen had changed again, this time reflecting an increase in synthesis of Type I collagen (Fig. 3). During the next 15 days the ratio of III/I slowly increased back to that of the control (Fig. 3). This initial reaction to injury is similar to the findings reported for rat dermal granulation tissue [9] human and guinea pig scars [5, 11] and cellulose sponge cellstic implants [18, 37] and also supported by indirect immunofluorescence using type-specific collagen antibodies [18, 25]. This sug-

gests that the synthesis of the two different types of collagen may take place in the same cells, as has been shown for isolated human skin fibroblasts [17].

The early increase in Type III collagen synthesis observed in the present and other studies on experimentally induced granulation tissue may thus be important in establishing the initial structure of the wound by providing a basic lattice for subsequent healing events. It is unlikely that Type III collagen contributes significantly to wound tensile strength because the greatest increase in tensile strength is not observed until the later stages of wound healing and tissue repair [24]. Moreover, the fact that Type III collagen fibrils are smaller in diameter than Type I collagen fibrils [28, 36] may account in part of the lower overall tensile strength of scar tissue. Presumably, as the tissue becomes stabilized with larger Type I collagen fibres and the rate of synthesis decreases, the dehydro-HLNL cross-link matures to the same multi-valent non-reducible cross-link formed in normal tissue. This late stabilization is consistent with the findings of Järvinen [24] in that the tissue strength of the injured muscle is not fully restored until 6 weeks after partial muscle rupture.

In this study, Day 7 represented a peak in the ratio of Type I to III collagen, referring possibly to a moment of the most active synthesis of Type I collagen. During the time period from 1 to 3 weeks the ratio of Type III/I collagen showed a gradual shift to a level close to the ratio of normal i.m. collagen. However, the proportion of Type III collagen was still high 6 weeks after injury (Fig. 3). High proportions of Type III collagen are often found in tissues which require a certain degree of plasticity, e.g. embryonic skin and smooth muscle. In granulation tissue the proportion of Type III collagen may be increased during the active phase of tissue remodelling, when flexibility and plasticity are probably required. Once the rate of turnover reduces the Type I fibres can increase in size and stabilise through the mature cross-links, and thus the original mechanical strength of the tissue is restored.

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