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Presence of Type III Collagen in Guinea-Pig Dermal Scar

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Guinea-pig dermal scar was shown to contain type III collagen, and, from densitometric analysis of gel electrophoretograms, it was shown to have a higher concentration than the surrounding dermis. This finding is consistent with the 'embryonic' nature of newly formed dermal wound tissue, reflected in increased hydroxylation of collagen lysine and the presence of dihydroxylysino-norleucine (after reduction) as the major cross-link.

We have reported briefly (Barnes *et al.*, 1975) our findings that, in the young guinea pig, the rate of collagen synthesis in the dermal scar is initially high, but gradually approaches that in the surrounding dermis over a period of about 6 months. Concomitant with the increased rate of collagen synthesis, the extent of hydroxylation of collagen lysine in the very early wound is elevated by approx. 50% (see also Shuttleworth *et al.*, 1975), but declines rapidly, reaching normal values around 3 weeks after wounding. In consequence of the fall in hydroxylation, there is a change in cross-linking pattern. Dihydroxylysino-norleucine, derived from two residues of hydroxy-lysine, is the major collagen cross-link (detected after reduction) in the early wound tissue (Forrest *et al.*, 1972). Subsequently, hydroxylysino-norleucine, derived from one residue each of lysine and hydroxy-lysine (and detected after reduction), appears as the major cross-linking species (Barnes *et al.*, 1975). The same age-related change in cross-linking pattern has been described in the human dermal scar (Bailey *et al.*, 1975a).

Precisely the same type of changes occurs in skin collagen during the early phase of growth. Thus high synthetic activity in embryonic or foetal skin is accompanied by elevated hydroxylation of collagen lysine, and dihydroxylysino-norleucine is a major reduced cross-link. The subsequent decrease in rate of synthesis is accompanied by a fall in hydroxylation (Barnes *et al.*, 1971b, 1974) and change in cross-link pattern (Bailey & Robins, 1972; Bailey *et al.*, 1973) similar to that described above.

Skin contains two species of collagen, types I and III (Miller *et al.*, 1971; Chung & Miller, 1974; Epstein, 1974). Type III collagen has been referred

to as 'embryonic' collagen since its concentration relative to that of type I appears to be appreciably higher in embryonic or foetal tissues than in more mature tissues (Epstein, 1974; Shuttleworth & Forrest, 1975). In view of the evidence outlined above, that dermal scar tissue in its early phase of development is more akin to embryonic skin than more mature dermis, which it subsequently resembles, it seemed reasonable to consider that type III collagen should be present in the early wound too and at a higher concentration than that in the surrounding dermis. Type III collagen has in fact been detected in experimentally induced granulation tissue in the rat (Bailey *et al.*, 1975b) and in human dermal scar tissue (Bailey *et al.*, 1975a). It has, however, been reported to be absent from the guinea-pig dermal scar (Shuttleworth & Forrest, 1974; Shuttleworth *et al.*, 1975). The present paper reports our own investigations on the possible presence of type III collagen in guinea-pig dermal scar and on the changes in collagen lysine hydroxylation and cross-link pattern in the developing dermal wound.

Materials and Methods

Wounding

Young female guinea pigs, Duncan Hartley albinos (body wt. approx. 250g), were wounded by removal of a rectangle of skin from each side of the body, as previously described (Barnes *et al.*, 1975). In two separate experiments, wound tissue was collected at 10 and 21 days after wounding, together with a sample of dermis from the dorsal surface. In the first experiment, four animals were used at each time-period and each received, by intraperitoneal

injection, 0.4mCi of L-[4,5-³H]lysine (The Radiochemical Centre, Amersham, Bucks., U.K.)/100g body wt., 24h before collection of tissue. In the second experiment, ten animals were used in each group, and radioactivity was not administered. Samples of wound tissue or control dermis were severed into small pieces and combined within each group in each experiment.

Hydroxylation of collagen lysine

A duplicate portion from each of the combined samples was treated with hot trichloroacetic acid for the extraction of collagen, as previously described (Barnes *et al.*, 1970). Extracts after exhaustive dialysis were digested with collagenase by the procedure of Benya *et al.* (1973). The products so obtained were examined, after hydrolysis, for lysine and hydroxylysine radioactivity, as previously described (Barnes *et al.*, 1971a). Hydroxyproline in the hydrolysate was determined colorimetrically (Bergman & Loxley, 1963).

Analysis of cross-links

Reducible cross-links were examined by reduction of the tissue with tritiated borohydride and separation of the labelled components by ion-exchange chromatography, as described previously (Robins *et al.*, 1973).

Analysis for type III collagen

The tissue was washed in 0.9% NaCl then solubilized by pepsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) digestion for 18h at 10°C, as described by Chung & Miller (1974).

Samples of the total digest were examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis before and after reduction with β -mercaptoethanol, as previously described (Sykes & Bailey, 1971; Bailey *et al.*, 1975a). Gels were analysed with a Chromoscan densitometer.

The pepsin digest was then precipitated, the precipitate redissolved in 1.0M-NaCl, pH 7.4, and the solution subjected to fractional precipitation with NaCl to permit the separation of collagen types I and III (Chung & Miller, 1974; Epstein, 1974). The step-wise addition of NaCl resulted in a series of precipitates at 1.4M-, 2.0M- and 2.4M-NaCl. Precipitates were redissolved in 0.5M-acetic acid, dialysed against the same, freeze-dried, and then analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and in some instances by CM-cellulose chromatography.

CM-cellulose chromatography. This was by the procedure of Bellamy & Bornstein (1971). Fractions within each peak were combined, desalted and subjected to amino acid analysis on the Jeol 6AH-DK automatic amino acid analyser.

Results

In the initial experiment, hydroxylation of collagen lysine in dermal scar tissue was 31% at 10 and 22% at 21 days after wounding, control skin values being 20%. Specific radioactivities (c.p.m./mg of hydroxyproline) for scar and control dermis were respectively 4966 and 1440 at 10 and 2165 and 1325 at 21 days after wounding, indicating an increase in collagen synthesis in the early wound and compatible with results obtained previously with radioactive-proline labelling (Barnes *et al.*, 1975). Examination of cross-links (after reduction) indicated that at both 10 and 21 days there were equal proportions of dihydroxylysino-norleucine and hydroxylysino-norleucine in the scar tissue. Histidinohydroxymerodesmosine was only a minor component at 10 days, but increased, although it was still less than in control skin, at 21 days. Control dermis revealed only hydroxylysino-norleucine and histidinohydroxymerodesmosine as the major cross-link components. These data are compatible with increased hydroxylation at the telopeptide site in the wound collagen.

Examination of the total pepsin digest on polyacrylamide gels revealed, in both scar tissue at 10 and 21 days after wounding and control dermis, bands corresponding to α , β and γ chains of collagen. On reduction with β -mercaptoethanol there was a marked decrease in the intensity of staining of the γ chains and concomitantly an appearance of α chains derived from type III collagen, distinguishable from the corresponding α chains of type I collagen by their lower mobility. These results therefore indicate the presence of type III collagen in both skin and dermal scar. Estimates of the relative proportions of types I and III collagens from densitometric tracings of the gels indicated that in the 10-day wound tissue type III represented 9%, in the 21-day wound 7% and in control dermis, at both time-periods, 4% of the total. These estimates are only very approximate, but they do indicate a higher content of type III collagen in the early wound compared with the surrounding dermis.

Confirmation of the presence of type III collagen in these tissues was obtained from fractional precipitation of the pepsin digests with NaCl and examination of the fractions by gel electrophoresis. Reduction with β -mercaptoethanol clearly indicated in the 1.0M-NaCl-insoluble and 1.4M-NaCl- and 2.0M-NaCl-precipitated fractions from the pepsin digest of normal dermis the loss of γ chains with the appearance of type III α and β chains. These fractions appeared to be mostly type III collagen, whereas the 2.4M-NaCl-precipitated fraction was type I collagen. Essentially similar results were obtained for the 10-day wound tissue and control skin (Plate 1).

At 10 days after wounding, wound edges are still apart. In the second experiment it was therefore

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the fractions obtained by precipitation with NaCl of the pepsin digest of 10-day scar tissue

(1)–(4), 1.0M-NaCl-insoluble and 1.4M-, 2.0M- and 2.4M-NaCl-precipitated fractions respectively; (5)–(8), as (1)–(4) but after reduction with β -mercaptoethanol. The first two fractions appeared to consist of type III, the last type I and the 2.0M-NaCl-precipitated fraction a mixture of the two collagens. Note the slightly lower mobility of α (III) and β (III) in comparison with α_1 (I) and β_{11} (I) chains respectively.

Fig. 1. Collagen cross-link patterns in wound tissue

(a) Collagen cross-link pattern (after acid hydrolysis) in 10- and 21-day wound tissue and control dermis after reduction with NaB^3H_4 ; 10-day wound tissue refers to the granuloma tissue at the wound centre. (b) Collagen cross-link pattern (after alkaline hydrolysis) in tissue synthesized at the wound edge of the 10-day dermal wound and in control dermis, after reduction with NaB^3H_4 . Identity of the glycosylated components was confirmed by acid hydrolysis and rechromatography. Note that, in the wound-edge material hydroxylysinoisnorleucine occurred mostly in the glycosylated form, whereas in control dermis there were approximately equal proportions of glycosylated and non-glycosylated forms. —, 10-day wound tissue; ----, 21-day wound tissue; ····, control tissue. $\text{O}^5\text{Lys}(\text{O}^5\omega\text{Nle})$, dihydroxylysinoisnorleucine; $\text{O}^5\text{Lys}(\omega\text{Nle})$, hydroxylysinoisnorleucine; Lys(ωNle), lysinoisnorleucine; HHMD, histidinohydroxymerodesmosine. The peak labelled Glu-Gal- $\text{O}^5\text{Lys}(\omega\text{Nle})$ (glucosylgalactosyl-hydroxylysinoisnorleucine) also contained a small amount of the aldol condensation product of two lysine-derived aldehydes.

decided to separate the wound tissue into the granulation tissue covering the open centre of the wound and what appeared to be newly synthesized skin being produced at the wound edge. Scar tissue was collected in the normal way at 21 days after wounding, since at this time the wound edges have closed and dissection as indicated above is not possible.

Examination of cross-links in the 10-day wound material indicated that the pattern previously observed, namely a high content of dihydroxylysinoisnorleucine and a low content of histidinohydroxymerodesmosine, was found only in the material from the wound centre (Fig. 1). Material from the wound edge revealed a pattern very similar

to that of control skin. This may suggest that newly synthesized collagen formed at the wound edge in response to the wound is normally hydroxylated (rather than over-hydroxylated). However, contamination with pre-existing skin at the wound edge cannot be ruled out. Alkaline hydrolysis to permit detection of glycosylated cross-links did, however, suggest some difference between normal dermis and the material designated newly synthesized skin at the wound edge. Thus, although lysyl hydroxylase may not be different, there seemed to be higher glycosylase activity in the latter tissue, since a greater proportion of hydroxylysine norleucine was glycosylated than in normal skin (Fig. 1). Examination for type III collagen was subsequently confined to the wound-centre material, because of the possibility of contamination of the wound-edge sample with normal skin. Salt fractionation after pepsin digestion gave fractions as follows: 1.0M-NaCl-insoluble and 1.4M-NaCl- and 2.4M-NaCl-precipitated. Gel electrophoresis gave results similar to those already described and indicated that the 1.0M-NaCl-insoluble and 1.4M-NaCl-precipitated fractions were type III collagen. This was confirmed by CM-cellulose chromatography of these fractions after pooling. A peak was obtained just before $\alpha 2(I)$ chains in a position expected for type III collagen (Chung & Miller, 1974). Amino acid analysis revealed a composition compatible with its identification as type III collagen.

Discussion

These studies have revealed the presence of type III collagen in guinea-pig dermal scar. An approximate analysis from densitometric tracings of gel electrophoretograms indicated a higher concentration in the scar than in the surrounding dermis, which is compatible with the 'embryonic' nature of early wound tissue, as reflected also in the elevated hydroxylation of collagen lysine and the presence of dihydroxylysine norleucine as the major cross-link (after reduction) in early wounds. The elevated concentration of type III collagen and the use of wound-centre material rules out the possibility that its detection in wound tissue was due to contamination with normal skin. The reasons for the absence of type III collagen from the guinea-pig dermal scar in the studies by Shuttleworth & Forrest (1974) and Shuttleworth *et al.* (1975) is not clear. The answer may lie in the method of wounding. In the present work a normally contracting wound was studied, whereas Shuttleworth and colleagues used an open splinted wound.

There is a rapid fall in the overall amount of lysine hydroxylation in scar tissue, which by 21 days is almost normal. Nevertheless there is still at this time a high concentration, relative to normal skin,

of dihydroxylysine norleucine, implying substantial hydroxylation still at the telopeptide site. These changes in hydroxylation and cross-link pattern are not due simply to the replacement during development of type III by type I collagen, since the hydroxylation changes have been detected in isolated $\alpha 2$ chains specific to type I collagen (Barnes *et al.*, 1971b), and the cross-link changes have been detected separately in both type I and type III collagens (Bailey & Sims, 1975). The apparent independence of hydroxylation at the telopeptide site relative to hydroxylation within the triple helix may suggest the involvement of a separate hydroxylase (Barnes *et al.*, 1974).

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