Isolation of collagen stimulating factors from healing wounds

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SUMMARY Five factors (collagen stimulating factors) have been isolated from healing murine skin wounds which stimulate prolyl hydroxylase activity and collagen synthesis in mouse fibroblasts *in vitro*. These factors stimulate general protein synthesis to a much smaller extent. Collagen stimulating factors are detectable in wounds three days after healing begins and disappear after six days when healing is complete. These data indicate that these factors may modulate collagen production during wound healing.

A prominent feature of the wound healing process in skin is the deposition of new collagen in the wound space. The regulatory mechanisms responsible for this local increase in collagen production are not understood. Recently it has been shown that factors (collagen stimulating factors) are present in precirrhotic liver, but not in normal liver, which have the ability to produce a marked stimulation in collagen polypeptide synthesis and prolyl hydroxylase when added to cultured fibroblasts.¹⁻³ It has been postulated that these factors may play a role in the regulation of increased collagen production in chronic liver disease.³

In the present paper evidence is presented showing that similar collagen stimulating factors are also present in healing wounds and it is suggested that these may be important in the control of collagen production in skin wounds.

Material and methods

All chemicals and reagents were purchased (and/or purified) from sources cited earlier.³

Skin wounds were produced in CFLP mice. The animals were anaesthetised with ether, the back shaved and a single wound (2 cm long) was made with a scalpel on each side of the back, parallel to, and approximately 1 cm from the dorsal spine. The incision extended to subcutaneous fat. The wound

edges were brought together using black silk sutures or metal clips. Animals in all experiments were killed by cervical dislocation and the wound excised together with 2-3 mm of adjacent skin.

Prolyl hydroxylase activity was measured in wounds at various stages of healing as an index of fibroblast function.⁴ Immediately after excision the wounds were homogenised at 4°C in 10 volumes of Tris-HCl (pH 7.2) containing 0.25 M sucrose, 10⁻⁵ EDTA, 10⁻⁴ M dithiothreitol and 50 µg/ml of phenylmethylsulphonyl fluoride on a Silverson "Mixer-Emulsifier" at full power for one minute. Enzyme activity was assayed in duplicate in the crude homogenates by the tritium release method using a 3,4,³H-proline-labelled substrate produced in chick embryos.⁵

Collagen stimulating factors were isolated from wound homogenates after centrifugation by chromatography on G25 Sephadex (fine) essentially as described previously³ with one modification; the wound tissue was homogenised on a Silverson "Mixer-Emulsifier", for one minute at full power.

Collagen stimulating factors in column effluents were detected by their ability to stimulate both collagen polypeptide synthesis⁵ or prolyl hydroxylase activity in L-929 fibroblasts. For the collagen synthesis assay L-929 cells (mouse fibroblasts) were plated at 2 × 10⁶ cells/50 cm² flask and grown for 40 h at 37°C in supplemented minimum essential medium containing 10% fetal calf serum.³ The results are expressed as DPM collagenase degradable protein per 5 × 10⁶ cells or as a ratio of collagen synthesis in treated as compared to control cultures.

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Fig. 1 Prolyl hydroxylase activity in wounds at various intervals after their production.

Fig. 2(a) Chromatogram of collagen stimulating factors from 3 day wounds on G25 Sephadex (wound weight = 2.7 g). Aliquots (2 ml) of the fractions indicated were assayed for their ability to stimulate prolyl hydroxylase activity in fibroblast cultures. Column buffer does not stimulate this enzyme above values found in untreated cultures. The average hydroxylase activity of control cultures is the base line value shown.

Fig. 2(b) Chromatogram of collagen stimulating factors from 3 day wounds on G25 Sephadex (wound weight = 4.7 g). Fractions were pooled as indicated below this Figure and assayed for their ability to stimulate collagen synthesis in fibroblast cultures. The stimulation produced in cultures treated with these pools is expressed as a ratio of that found in 2 control cultures to which nothing or column buffer was added. Column buffer itself has no significant effect on these cultures. Aliquots (2 ml) of each pool (or buffer) were used in this and all subsequent experiments. Collagen synthesis in cultures treated with each pool is expressed as a ratio of collagen synthesis in control cultures. Each pool has been designated collagen stimulating factor (CSF) 1-5 as shown.

Stimulation of prolyl hydroxylase activity by fractions from G-25 columns was measured in mouse fibroblast cultures plated at 10^6 cells/50 cm² flask and grown as described earlier.³

Results

As a measure of the collagen biosynthetic activity in wounds, prolyl hydroxylase was measured at various stages of healing. The tissue concentration of this enzyme begins to rise two days after wounding, reaches a maximum on day 4 or 5 and is back to normal skin levels by day 8 (Fig. 1).

Fig. 3(a) Molecular sieve chromatograph (G25 Sephadex) of a normal skin homogenate (skin weight = 5.2 g). Fractions were pooled as indicated and 2 ml aliquots of each assayed for their ability to stimulate prolyl hydroxylase in fibroblast cultures. Hydroxylase stimulation is expressed as a ratio of that found in control cultures.

These results indicate that fibroblastic activity is established in wounds 2–3 days after their production. Three day wounds, therefore, were chosen as the most likely source of material in which collagen stimulating factors might be present. When 3 day wounds are homogenised, passed over G25

Fig. 3(b) This is the same chromatogram as shown in Fig. 3(a) but 2 ml of each pool were assayed for their ability to stimulate collagen synthesis in fibroblasts. The data are expressed as a ratio of control cultures.

Sephadex and the column effluent monitored for its ability to stimulate prolyl hydroxylase in fibroblast cultures, five peaks of activity were found (Fig. 2a). To determine whether the activity peaks identified by their ability to increase enzyme activity in fibroblasts (Fig. 2a) were also capable of stimulating collagen synthesis in the same cells, the fractions comprising the five peaks of activity shown in Fig. 2a

Table 1 Stimulation of collagen and total protein synthesis by collagen stimulating factors from 3 day wounds

Addition*	Collagen		Total Protein	
	Synthesis (DPM $\times 10^{-3}/5 \times 10^6$ cells)	Stimulation	Synthesis (DPM $\times 10^{-3}/5 \times 10^6$ cells)	Stimulation
Nil	6.8	1	132	1
Buffer	4.6	1	96	1
CSF 1	16.5	2.9	251	2.2
CSF 2	13.9	2.4	221	1.9
CSF 3	29.5	5.2	373	3.3
CSF 4	21.6	3.8	182	1.6
CSF 5	21.6	3.8	290	2.5

* 2 ml of each factor, isolated from 3 day wounds by gel filtration, were added to replicate cultures and their effect on collagen and total protein synthesis measured after a 3 h incubation period. Each culture was pulsed with $15 \mu\text{Ci}$ of ^3H -proline 60 min before harvesting. The stimulation of collagen and protein synthesis with each factor is derived by dividing the value found in cultures treated with each factor by the average value of untreated cultures and those treated with column buffer.

Fig. 4 (a) and (b) Temporal appearance of collagen stimulating factors in wounds at various stages of healing. Wound tissue (approximately 5g) was excised at the times indicated and collagen stimulating factors isolated by gel infiltration under identical conditions. Immediately after their isolation 2 ml of each pool was added to replicate cultures, incubated for 3 h and collagen synthesis measured in treated and control cultures. The stimulation of collagen synthesis in treated as compared to control cultures was then calculated. The degree of stimulation of collagen synthesis produced with 2 ml of each pool, isolated from wounds of different duration and normal skin (zero time) is expressed as collagen stimulating factor activity. The stimulation produced with each pool was approximately linear with the quantities used and also with time.

were pooled separately. Aliquots of each pool or buffer were added to cultures and the rate of collagen synthesis measured after a total incubation period of three hours. As shown in Fig. 2b all of the pools derived from 3 day wounds stimulate collagen synthesis in fibroblast cultures. Pool 3 was the most effective producing a 5.2 fold stimulation of collagen synthesis when compared to control cultures; the four other pools produced a 2.4–3.8 fold increase in collagen synthesis.

For comparison with wound tissue, normal shaved mouse skin was homogenised, passed over G25 Sephadex and pooled fractions assayed for their ability to stimulate prolyl hydroxylase. As shown in Fig. 3a, pool 2 from normal skin stimulates collagen prolyl hydroxylase 2.6 fold compared to control cultures but all other pools had no activity. Aliquots of each pool from the chromatogram shown in Fig. 3a were also examined in the collagen synthesis assay. As shown in Fig. 3b, none of the pools from normal skin stimulates collagen synthesis in vitro including pool 2 which stimulated enzyme activity. Indeed, pools 1–3 had a depressant effect on collagen synthesis compared to control cultures to which buffer alone, or nothing was added. It should be noted that the original starting weight of normal skin used was comparable to the amount of wound tissue employed above. Furthermore, the amount of material eluted from this column with absorbance at 280nm was similar to the wound columns shown in Figs. 2a and 2b.

It is conceivable that the increase in collagen

synthesis produced by treating cultures with the various chromatographic fractions from 3 day wounds is only a reflection of a general stimulation of protein synthesis by these fractions. As shown in Table 1, these fractions do increase the incorporation of ^3H -proline into total cell layer protein. The incorporation of isotope into collagenase degradable protein ranged from 5.2 and 3.8 fold while the increase of incorporation into total protein was 3.3 and 1.6 fold respectively.

Since prolyl hydroxylase activity in healing wounds was observed to be at a maximum at day 4 and 5 after injury and 3 day wounds were used as the source of collagen stimulating factors, it was judged that the temporal appearance of these factors should be examined. Wounds of varying duration were used between one and six days and collagen stimulating factors isolated as before. Fig. 4 shows the stimulation of enzyme activity in each pool up to 6 days after wounding. This demonstrates that the concentration of each factor increased by day 2 and reaches a maximum on the day 3. There is a considerable decline on day 4 and by day 6 collagen stimulating factors could not be detected.

Discussion

Increased collagen deposition is a feature common to many pathological situations. Knowledge of the regulatory mechanism governing this process is rudimentary. It is clear, however, from the present and earlier studies,¹⁻³ that factors can be isolated

from healing wounds and collagen formative liver disease whose properties suggest that they may have a regulatory function in the control of collagen production in both of these conditions. That is these factors not only stimulate the activity of prolyl hydroxylase in fibroblasts but as shown here they also stimulate collagen polypeptide synthesis in fibroblast cultures. The temporal appearance of these factors in wounds also adds weight to the argument that they have a regulatory function in collagen synthesis. Collagen stimulating factors are increased significantly at day 2 just as fibroblast activity appears in the wound space. They reach a maximum at day 3 when fibroblast function is also rising but is not at a maximum. Thus the maximum concentration of these factors occurs immediately prior to maximum fibroblast activity, a fact which makes biological sense if they participate in the regulation of collagen synthesis in wounds.

The chemical nature of these factors is unknown. It has been reported that bradykinin and the prostaglandins E_1 and $F_1\alpha$ stimulate proline and lysine hydroxylation (30–40%) in an isolated chick tibia system.⁶ Experiments in this laboratory, however, failed to demonstrate any stimulating activity of bradykinin on collagen polypeptide biosynthesis and prolyl hydroxylase in our system. Although it has not yet been possible to check whether prostaglandins affect collagen synthesis in our system, the fact that at least two collagen stimulating factors are trypsin-sensitive^{1,2} indicates a peptide bond dependency; it would seem unlikely, therefore, that these factors are prostaglandins because the latter contain no peptide bonds.

These factors not only stimulate collagen synthesis but also general protein synthesis. The latter, however, is raised to a much smaller extent (see Table 1). It is conceivable that the effect on protein synthesis is due to the induction of other intracellular enzymes necessary for the synthesis of collagen itself and perhaps other connective tissue components.

The data presented in this paper and elsewhere³ indicate that collagen stimulating factors, which do not influence fibroblast number, and are therefore unrelated to lymphokines previously described,⁷ may play a role in all collagen formative disorders.

References

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