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Identification and Characterization of Wound-Associated Soluble Substances

Elizeu Alvaro Pascon

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IDENTIFICATION AND CHARACTERIZATION OF WOUND-ASSOCIATED SOLUBLE SUBSTANCES

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A THESIS
SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF DENTAL SCIENCE AT THE UNIVERSITY OF CONNECTICUT 1984
IDENTIFICATION AND CHARACTERIZATION OF WOUND-ASSOCIATED
SOLUBLE SUBSTANCES

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THE UNIVERSITY OF CONNECTICUT
1984
TO LEILAH, LUMENA, ELIZEU, JR., AND FABIANA
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INTRODUCTION

The purpose of this study was to identify, characterize, and partially purify certain highly soluble glycoproteins from experimental wounds in rabbits. An attempt was made to determine whether the changing concentrations of such substances could be used to objectively monitor the repair process.

Soluble glycoproteins derived from open wound granulation tissues have not been studied previously; but, comparable substances have been identified in extracts from polyvinyl sponge-induced inflammatory granulomas. In granulomas, these substances experience predictable changes in concentration as the tissue ages.

In the present work, soluble glycoproteins were extracted from open wound granulation tissues at increasing postwound intervals and were monitored and characterized by methods used previously for the study of granuloma-derived granulation tissues. The major purification technique involved gel filtration using Sephacryl SF 200. Crude and purified preparations were characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF), amino acid analysis, glycoprotein susceptibility to a monospecific clostridial collagenase, and measurement of soluble collagen, N-acetyleneuraminic acid (NANA), and protein. The use of these
techniques allowed the investigator to: 1) establish if wound-derived soluble substances could be used to monitor repair over a given interval, and 2) determine if open-wound granulation tissue profiles resemble those from other tissue sources (i.e., inflammatory granulomas). If soluble glycoprotein profiles could be confirmed as having characteristic configurations for wounds of specified age, they would have value as objective reflections of healing status.
Background

Normal healing of open full-thickness wounds of mammalian integument occurs by the process of repair rather than by true regeneration. As a result, the tissues of healed open wounds are neither structurally nor functionally identical to those of the pre-injury state. The inevitable scarring associated with normal healing can produce severe impairments. Efforts at directing repair so as to limit scarring and contraction or to facilitate re-epithelialization have been hampered by lack of objective methods for assessing the effects of new agents or techniques on healing processes. This deficiency is made more apparent when healing occurs abnormally; aberrations in wound repair are relatively common. Keloids and hypertrophic scars, for example, are debilitating derangements to which open wounds are particularly susceptible. In addition, failure of wounds to re-establish surface integrity and functional activity due to infection, nutritional deficit, systemic disease, or drug therapy has been well-documented. By the time such abnormalities become clinically evident, effective therapeutic intervention may be precluded. If wounds that are at risk of healing abnormally could be identified during incipient aberrancy, and if early treatment could be
initiated, a return to normal patterns of repair might be accomplished. Objective criteria for monitoring wound repair would also allow the effects of various wound treatments to be more appropriately evaluated.
Reasons for Studying Buffer-Soluble Glycoproteins from Open-Wound Granulation Tissues

Numerous methods have been suggested for enhancing the rate or quality of wound repair, but few methods have found application in clinical practice. This disparity may result from using subjective clinical criteria for monitoring the healing process and from inevitable investigator bias. Attempts have been made at more objectively quantitating healing in experimental animals by measuring rates of re-epithelization, wound contraction, connective tissue reconstitution, collagen synthesis, glycosaminoglycan (GAG) production, and determination of fibroblast growth kinetics; but, because species variation is significant, the relevance of such research to human open-wound repair remains unclear. Certainly, methods of analysis which require surgical removal of wound tissues are rarely suitable for studying healing in humans. This investigation is an experimental animal study which evaluates the contention that highly soluble substances are present in open wound tissues and can be used to accurately monitor the healing process.

The existence of wound-derived soluble glycoproteins whose concentrations change as a function of postwound interval has never been established; but, similar substances
have been reported for granulation tissues of non-wound origin (i.e., inflammatory granulomas).\textsuperscript{1-5} The close resemblance between granulation tissue derived from inflammatory granulomas and that from open, full thickness skin wounds,\textsuperscript{26} justifies the speculation that a similar assortment of glycoproteins would be identified.

Buffer-soluble granuloma glycoproteins were originally studied to assess changes in cellular function mediated by exposure of cells to extracellular matrix constituents.\textsuperscript{1} When rat granuloma tissue extracts were added to cultures of matrix-free embryonic chick tendon cells, a dramatic suppression of collagen and other protein synthesis occurred.\textsuperscript{1,2} In addition, the secretion of collagen into the medium was diminished and the normal tendency of tendon cells to cluster during incubation was inhibited.\textsuperscript{1,2} Another biological effect was a 10-fold drop in phagocytic index when peritoneal macrophages were exposed \textit{in vitro} to extracts prepared from 14- and 42-day polyvinyl sponge granulomas.\textsuperscript{3} Subsequently, efforts at isolating and identifying the active components of such extracts revealed that granuloma age was accurately reflected by highly specific gel filtration patterns.\textsuperscript{2} The characteristic changes in elution profiles observed as granuloma tissues matured led to speculation that soluble glycoproteins play an active role in directing tissue development.\textsuperscript{2}
Soluble Extracellular Granulation Tissue Constituents as Regulatory Molecules

Feedback regulation of reparative tissue development has been identified as an important function for the soluble glycoproteins of inflammatory granulomas.\(^2\) After crude extracts of granuloma granulation tissues were shown to inhibit collagen synthesis and secretion,\(^1\) subfractions were isolated which exhibited inhibition at extremely low concentrations (\(10^{-6}\) M).\(^2\) Among the many soluble extracellular substances that might function in this capacity, two categories of molecules, NANA-containing glycoproteins and glycosaminoglycans (GAG), have received particular attention.

NANA was originally implicated as a regulator of cell function when normal and transformed cells were shown to have different total sialoprotein contents.\(^28\) Changes in the synthesis/secretion of collagen and other proteins as well as alterations in cell surface attachment mechanisms, cellular aggregation phenomena, lymphocytic circulation, expression of cellular antigenicity, and sensitivity of smooth muscle cells to serotonin-induced contraction have all been envisaged as being NANA-dependent.\(^29\) A neuraminidase-sensitive glycoprotein involved in collagen synthesis and in phagocytosis has also been described.\(^34\) Such research has been used to support the conclusion that NANA-containing glycoproteins exert potentially important...
influences on the changing developmental state of granulation tissues.²

GAG have also been envisaged as being important for modulating cellular behavior in developmental³⁵-⁴² and in wound healing²¹-²³ systems. In developmental models such as newt limb regeneration ³⁵, chick embryo cornea formation,³⁶ limb bud and axial chondrogenesis,³⁷ and chick heart and brain development,³⁸,⁴⁰-⁴² the unsulfated GAG, hyaluronic acid, has been found to facilitate cellular division and migration while directly suppressing differentiation.³⁵,³⁶,³⁹ Subsequent hyaluronate removal by tissue hyaluronidase causes a cessation of migration and permits aggregation and differentiation to proceed in the proper sequence for tissue organization.³⁵,⁴² In healing open wounds, identical changes in GAG composition occur.²¹-²³ In addition, a wound associated hyaluronidase whose activity correlates with the onset of cellular differentiation has been identified.²¹,²² These studies support an analogy between reparative and developmental systems and lend credence to the hypothesis that the synthesis and degradation of hyaluronic acid help to control cellular migration, proliferation, differentiation, and synthesis.³⁵-⁴² Possible mechanisms to explain hyaluronate's effects have been advanced by Ivaska.⁵

Predicted changes in the concentrations of readily extractable glycoproteins of developing granulation tissues
were used in this study to monitor the progression of normal repair in experimental wounds. Since soluble glycoproteins from open-wound tissues have not been studied previously, an analogy between the open wound and the granuloma systems needed to be verified. This study sought to confirm that soluble glycoproteins could be isolated from open wound granulation tissues and that time-dependent changes in soluble glycoprotein composition would occur.
MATERIALS AND METHODS

Wounding

Forty 2.0-3.0 kg New Zealand white rabbits (Oryctolagus cuniculus) were caged separately and maintained on a Purina laboratory Rabbit Chow HF 5326 diet (Ralston Purina) with food and water taken ad lib. For wounding and harvesting procedures, rabbits were anesthetized by intramuscular injection of ketamine HCl (Bristol), 35 mg/kg and xylazine (Haver-Lookhart), 5 mg/kg and flanks were shaved and depilated with Nair (Carter-Wallace Inc.) Bilateral flank wounds, 6 cm x 6 cm were made in all animals (Fig. 1). Skin and superficial fascia were excised to the level of the panniculus carnosus muscle with resected skin being frozen and retained. Flank dressings consisted of petrolatum gauze, gauze roll, and a light plaster cast.

Sampling

A differential wound topography permits recognition of two distinct zones.\textsuperscript{20-22} Most of the open defect contains a provisional tissue referred to as "central granulation tissue". Along the wound's periphery, between central granulation tissue and the original unwounded skin, a bipartite zone exists which is composed of a deep layer of granulation tissue and a superficial sheet of migrating
epithelium. This bi-layered region constitutes "whole edge tissue". All samples were harvested from the central granulation tissue region and were free of peripheral epithelium. Sampling was performed on postwound days 3, 7, 9, and 14. For each animal, the tissues from the left and the right wounds were pooled and extractions subsequently performed. Excision of underlying panniculus carnosus muscle was avoided at the time of harvesting.

Grafting

Skin grafting is a common therapeutic maneuver believed to produce a significant alteration in patterns of wound repair. Two animals were, therefore, studied for which any changes in soluble glycoprotein profiles induced by the application of full-thickness autogenous skin grafts could be defined. These animals received a unilateral 6 cm x 6 cm flank wound that was dressed as described above. On the seventh day after wounding, the opposite side was wounded and the freshly harvested skin applied as a full-thickness graft to the week-old wound. The deep surface of the graft was defatted to expose clean dermis, this procedure greatly enhances the success of grafting. Abdominal skin was avoided as a source of graft material because resultant closure of such donor sites creates undesirable tensions on the flanks and might retard the natural course of contraction. Petrolatum gauze, gauze roll, and plaster cast
were again used as dressings. On the fourteenth day after original wounding (7 days after grafting), grafts were removed and subjacent granulation tissue harvested; results of previous work show that translocation of granulation tissue to grafts is minimal. The donor site was allowed to heal for a total of 14 days and then its granulation tissue harvested as described for open wounds.

**Tissue Preparation, Storage, and Extraction**

Harvested tissues were frozen in liquid nitrogen, lyophilized, milled and then stored at 4°C until required. Extracts were prepared by dissolving 100 mg of milled tissue powder in 4.0 ml of 0.05 M Tris (hydroxylmethyl) aminomethane-HCl (Tris-HCl), 0.005 M CaCl₂, pH 7.6, buffer and gently stirred at 4°C for 16 hrs. as previously described. Samples were then centrifuged at 14,700 X g for 5 min., insoluble pellets discarded, and the supernatants saved for analysis. An identical extraction was used for normal skin. Normal rabbit serum was analyzed after dialysis against the extraction buffer. Data for normal skin and serum were compared with those obtained for granulation tissues.

All samples were studied by means of gel filtration, sodium dodecyl sulfate (SDS)-polyacrylamide gel
electrophoresis (PAGE), isoelectric focusing, amino acid analysis (for fractions of sufficient purity), and measurement of soluble collagen, hydroxyproline, N-acetyleneuraminic acid (NANA), and protein.
Analytical Techniques

Gel Filtration

Gel filtration was performed by introducing a 500 ul aliquot of each crude extract to a 1.6 cm x 90 cm column of Sephacryl SF 200 (Sigma) which had been equilibrated with the extraction buffer. Elution was performed with the same buffer at a flow rate of 0.66 ml/min. Effluent was monitored at 280 nm with a Buchler Fracto-Scan UV monitor; 3.2 ml fractions were collected. Peak fractions were dialyzed against cold distilled water, lyophilized, and stored for later analysis.

Elution profiles were described on the basis of peak elution volumes. Changes in the profiles were expected to reflect the changing developmental state of the granulation tissue.

Electrophoresis

Crude extract supernatants as well as peak fractions obtained by gel filtration were electrophoresed on 1% SDS-5.6% polyacrylamide gels as described by Fairbanks, Steck et al. Staining for protein was performed with 0.05% coomassie blue and for carbohydrate with a periodic acid Shiff (PAS) procedure. All gels were photographed and then scanned with a Corning model 760 computing densitometer. Protein standards consisting of lysozyme,
\( \beta \)-lactoglobulin, ferritin, ovalbumin, bovine serum albumin, and \( \beta \)-galactosidase were used. Electrophoretic patterns allowed molecular weights, approximate carbohydrate contents, and relative purity to be determined. Densitometric scans of gels were compared for protein migration distances and for differences in densitometer-computed band areas.

**Isoelectric Focusing (IEF)**

Isoelectric focusing was performed as described by Righetti and Drysdale\textsuperscript{45} using a 40% ampholine solution (Bio Lyte 3/10) having a gradient of pH 4-9. Staining was performed with coomassie blue and all gels were then scanned. Using pI calibration standards (Pharmacia), isoelectric points over the range pH 4-9 were determined for purified extract subfractions. Since previous work has established the isoelectric points and molecular weights of soluble glycoproteins of inflammatory granulomas,\textsuperscript{3} such data provided another basis for comparing extracts from open wound granulation tissues and from granulomas. Changes in isoelectric patterns occurring as a function of postwound interval would, conceivably, be useful for monitoring the emergence and/or disappearance of various proteins as wound granulation tissue matures and remodels.
Amino Acid Analysis

Those fractions that could be purified sufficiently so as to yield a single band by SDS-PAGE were subjected to amino acid analysis. Analyses were performed after hydrolysis at 110°C in 6 N HCl for 24 hrs. in evacuated nitrogen-flushed tubes by the University of Connecticut Health Center Amino Acid Analysis Facility.

Amino acid compositions of wound-derived soluble glycoproteins were used in conjunction with SDS-PAGE and IEF in an attempt at protein identification. Comparisons were made between experimental substances and glycoproteins of known composition.

Collagen Determinations

Soluble, collagen-derived peptides present in crude extract supernatants or in isolated subfractions were identified by evaluating extract protein susceptibility to collagenase. Hydroxyproline content was also measured.

Samples were treated with a purified monospecific clostridial collagenase (EC 3.4.24.3) (Millipore) and resulting alterations in SDS-PAGE patterns identified. Samples were divided into fractions of 250 ul; each received 162.6 µg of N-ethylmaleimide (NEM) to inhibit nonspecific protease activity. Half of the samples were then treated with 23.2 µg of active collagenase and the remainder with the same amount of heat-inactivated collagenase. All
reaction mixtures were incubated at $37^\circ C$ for 2 hrs. Collagenase-mediated changes in electrophoretic patterns were monitored using the SDS-PAGE technique for collagenous polypeptides of Noelken, Wisdom et al. $^{46}$

Hydroxyproline levels were determined for all samples by the alkaline hydrolysis of Huszar, Maiocca et al. $^{47}$

**Effects of Extraction Period**

To assess the effects of varying the duration of wound tissue extraction periods on gel filtration elution profiles and on gel electrophoretic patterns, extract supernatants derived from wound granulation tissue aged 3, 7, 9, and 14 days were incubated in the Tris-HCl extraction buffer at $4^\circ C$ for 4, 8, 12, and 16 hours respectively. Any differences arising in the profile or electrophoretic pattern for a given tissue reflected the influence of extraction duration.

**Inhibition of Non-Specific Protease Activity**

For each of the extract supernatants derived from the wound tissues described above, N-ethylmaleimide (NEM) was added to a final concentration of 2.5 mM for the purpose of inhibiting non-specific protease activity. Resultant elution profiles and electrophoretic patterns were then studied and compared with NEM-untreated extracts.
Measurement of NANA, and Protein

NANA levels were estimated by the method of Aminoff.\textsuperscript{48} Samples and standards contained in a volume of 0.5 ml were incubated with 250 ul periodate reagent at 37\(^{\circ}\) C for 30 min. Sodium arsenite (200 ul) and thiobarbituric acid reagent (2 ml) were added and samples and standards heated at 100\(^{\circ}\) C for 7.5 minutes. Acid-butanol (4 ml) was added, mixtures centrifuged, and NANA-chromophore read in the butanol layer at 549 nm.

Lowry protein was measured using the modified folin-phenol reagent method reported by Peterson.\textsuperscript{49}

Statistical Methods

Data for assays of tissue samples pooled according to postwound interval or graft status were expressed as means \pm one standard error of the mean (S.E.M.). Statistical significance was determined by applying t-tests for independent variables to the difference between means. Significance was assigned at a level of probability \(\leq 0.05\). For gel filtration, PAGE, and IEF experiments, representative elution profiles and gels are presented.
RESULTS

Gel Filtration

A time-dependent change in the elution profile of supernatants derived from rabbit skin granulation tissue was observed (Fig. 2). Two major peaks (A and C) were seen for each day studied, but the relative proportion of the profile attributable to a given peak varied. Peak A was the most prominent constituent of days 3, 9, and 14, and possessed an approximate molecular weight of 93,000 (Fig. 3). This peak was markedly diminished on postwound day 7, but increased progressively throughout the second postwound week. A minor peak (E) was observed in less mature granulation tissue (postwound days 7 and 9) and was particularly evident in grafted granulation tissue (Figs. 2 and 4). Peak C had an elution volume consistent with a population of substances of differing molecular weights. This possibility is suggested by the eventual emergence of 3 discrete peaks in the C position at 14 days.

SDS-PAGE

SDS-PAGE patterns of tissue extracts exhibited a surprising degree of similarity regardless of postwound day (Fig. 5). At least five comparable bands were identified for each extract, however, densitometric scanning demonstrated important differences (Fig. 6). Although
band 3 was invariably the major component, its relative prominence was markedly diminished on day 7 when it comprised only 27% of the scan (Fig. 7). Subsequently, the percentage of the scan attributable to band 3 progressively increased, reaching a maximum on day 14 of 59.1% (Fig. 7). When Sephacryl SF 200 Peak A was isolated and electrophoresed, a single homogenous band was identified which corresponded to band 3 of the crude extracts (Fig. 5).

The other SDS-PAGE bands (1, 2, 4, and 5) constituted relatively low percentages of the scan on each postwound day. Band 5 peaked on postwound day 7 when it comprised 15.4% of the scan. Bands 1, 2, and 4 were essentially unaffected by increasing postwound intervals (Fig. 7). When gels were stained for carbohydrate, all the coomassie-staining bands were PAS positive.

Protein and N-Acetylneuraminic Acid (NANA) Determinations

The described changes in elution profiles and SDS-PAGE patterns were accompanied by increasing concentrations of extractable protein throughout the healing process (Table I). NANA levels were uniformly low on all postwound days except day 7 when the concentration of extractable NANA was 4- to 6.5-fold greater than at any other postwound interval (Table I). Isolated Peak A material was found not to possess significant amounts of NANA.
Collagen Determinations

Total Collagen Content: An estimate of total collagen content was made for uncentrifuged crude extracts by measuring the release of collagen cleavage products after treatment with purified, monospecific clostridial collagenase. Release of cleavage products increased in direct proportion to collagenase concentration until a plateau was reached at 8.9 ug/mg dry tissue (Figure 8). Collagen measured in this manner was found to be negligible at postwound day 3, but increased thereafter, peaking on day 9 and remaining elevated until day 14 (Fig. 8). Although grafting appeared to diminish the collagen content of subjacent granulation tissue, the difference between grafted and ungrafted tissue samples was found to be statistically insignificant (p \leq .05).

Collagenase Treatment of Extract Supernatants: No differences in electrophoretic patterns were observed between samples treated with active or heat-inactivated clostridial collagenase. Similarly, SDS-PAGE of samples incubated with N-ethylmaleimide (NEM) to inhibit non-specific protease activity were indistinguishable from those that were NEM-untreated.

Hydroxyproline determinations of extract supernatants were also performed. For the periods studied, variably low levels of hydroxyproline were extracted (Table I) that did
not reflect total wound collagen content (Fig. 8).

**Effects of Extraction Period and Non-Specific Proteases**

Under the conditions of the experiments (4°C), neither varying the extraction time over a range of 4-16 hours nor inclusion of NEM in extraction mixtures produced any detectable change in gel filtration profiles or electrophoretic patterns.

**Isoelectric Focusing and Amino Acid Composition:** Only Peak A (Band 3) could be isolated with sufficient purity to justify IEF and amino acid composition determination. The isoelectric point was found to be 6.9; the amino acid composition is shown in Table II.
DISCUSSION

The elution profiles of open wound granulation tissues changed as a function of postwound interval. The pattern of change was dramatic for Sephacryl SF 200 Peak A. Peak A was present in high concentrations on postwound day 3, was markedly suppressed on postwound day 7, and then progressively increased during the second postwound week (days 9 and 14).

When isolated Peak A glycoprotein was studied by gel electrophoresis, it was identified as a single, homogeneous protein corresponding to the third band in electrophoretic patterns of crude extract (Fig. 5, compare gels 1-13 with gel 14). By performing densitometry on each gel, the proportion of the pattern attributable to Band 3 could be determined at increasing postwound intervals (Figs. 6 and 7). By this method, Band 3 was shown to vary over time in a manner identical to that seen for Peak A by gel filtration, i.e., initially high levels of Peak A on day 3 were depressed on day 7 and progressively increased on days 9 and 14 (compare changes in Peak A, Fig. 2 with changes in band 3, Fig. 7).

The cause of Peak A/Band 3 (PA/B3) depression at the end of the first postwound week is unknown, but the observation that NANA was increased on day 7 may be significant. NANA-containing glycoproteins are believed to
exert numerous important effects upon cellular function. They undergo marked changes in concentration as tissues age, and they may function in the feedback regulation of granulation tissue development. Purified NANA-containing glycoproteins have been shown to suppress protein and collagen synthesis/secretion at very low concentrations (10^{-6} M). Since Bands 1, 2, 4, and 5 were not diminished on day 7, the nature of PA/B3 depression may be rather specific, whatever its ultimate cause. Importantly, these studies assessed protein content, not rate of synthesis, so subtle changes in protein synthesis and/or secretion might exist on other postwound days that would not be recognized by the methods employed.

Impressive changes in Peak C were also observed; Peak C gradually increased in quantity and eventually separated into three well-defined peaks (Fig. 2). This phenomenon may indicate a time-dependent structural modification such as aggregation; the concomitant formation of smaller, Peak C-derived remnants may also occur. The inability to distinguish differences in Peak C patterns by SDS-PAGE over time might result from reduction of disulfide bonds by dithiothreitol and disruption of Peak C into its essential, non-aggregated components. In any event, the emergence of several discrete peaks in the C position of 14-day elution profiles implies possible value for Peak C as a monitor substance.
The identity of PA/B3, Peak C and the other soluble glycoproteins remains unknown. Since electrophoresis patterns were not altered when extracts were treated with collagenase, none of the major bands appear to be collagen-derived peptides. The absence of hydroxyproline from PA/B3 and the entirely different pattern of time-dependent change observed for PA/B3 when compared to total wound collagen argue against collagen as a major soluble constituent.

Soluble glycoproteins from open-wound granulation tissues have not been studied previously, but similar substances of non-wound granulation tissue origin have been reported. These earlier works may provide insight into the nature of the materials described by this study. Bole, Jourdain et al. isolated a saline soluble glycoprotein from 14- and 42-day polyvinyl sponge granulomas in rats by chromatographic fractionation of extracts on DEAE-cellulose and Bio Gel P-150. A protein peak identified as DEAE-cellulose C/Bio Gel P-150 II exhibited chromatographic and electrophoretic characteristics similar to PA/B3, but was much smaller (MW=48,500). The material isolated by Bole, Jourdain et al. significantly depressed phagocytosis by peritoneal macrophages in culture. Similarly, Aalto and Kulonen described a 0.3 M Tris-HCl, 0.0015 M CaCl₂, pH 7.8, extract from viscose sponge-induced inflammatory granuloma tissue that suppressed the synthesis
of protein and collagen by embryonal chick tendon cells. Subsequently, a 0.5 M Tris-HCl, 0.02 M EDTA, 0.075 M boric acid extract of rat granuloma tissue was identified that had the same effect and that possessed a gel filtration pattern which accurately reflected granuloma age.\textsuperscript{2} When Aalto, Potila et. al.\textsuperscript{2} subjected these 7- and 21-day granuloma extracts to Sephadex G-200 gel chromatography, they identified three peaks (designated Sephadex G 200/I, II, and III) that appear identical to Sephacryl SF 200 Peaks A, B, and C obtained by the present analysis (Fig. 2). Sephadex G 200/I and PA/B3 occupy similar chromatographic elution positions and both increased with advancing age.\textsuperscript{2}

Isolation of Sephadex G 200/I followed by DEAE-cellulose anion exchange chromatography\textsuperscript{2} produced two protein peaks which eluted at 0.15 M NaCl, a major protein subfraction (DEAE 1) and a lesser protein subfraction (DEAE 2). Although high levels of NANA were associated with Sephadex G 200/I, NANA was confined exclusively to the lesser subfraction (DEAE 2). The major subfraction (DEAE 1) was totally free of NANA and appears to be comparable to PA/B3.

Since the specific identification of the soluble glycoproteins derived from open wound granulation tissue or from rat granulation tissue has not been accomplished, the possibility of a serum origin for such substances must be
considered. The probability of simple serum contamination of tissue samples seems small since such contamination would not explain the changing proportions of soluble constituents observed as the wounds matured. In addition, the predominant serum protein, albumin, is free of carbohydrate and possesses a substantially lower molecular weight (63,900) than the predominant PAS-positive glycoprotein identified in wound extracts (PA/B3 MW=93,000). Naturally, PA/B3 could represent a higher molecular weight serum component such as α or β macroglobulins or immunoglobulins, or even albumin functioning as a carrier and bound to some other glycoprotein. Certainly, the amino acid composition and isoelectric point of PA/B3 is rather albuminoid. In any event, a serum origin for this substance would in no way invalidate its use in monitoring the progression of normal repair given a consistent relationship between the healing process and PA/B3 concentration. The selective uptake of serum components by maturing wound tissues could provide a basis for the observed effect.

The major change provoked in gel filtration patterns by the application of skin grafts appears to be an accentuation of Peak B. This phenomenon might be the result of a retention of less mature tissue characteristics since Peak B was more prominent in tissues harvested from younger wounds (Days 7 and 9). In any event, the limited graft study of
this project supports the contention that a method known to dramatically alter the normal healing process (i.e., grafting) also produces distinct changes in glycoprotein profiles.
CONCLUSIONS

1) The profiles of soluble substances identified in this study varied in a characteristic manner as a function of postwound interval. Such profiles have apparent value as objective reflections of healing status.

2) One soluble substance, Peak A/Band 3 (PA/B3) was identified as a single, non-collagenous PAS-positive protein, having a molecular weight of approximately 93,000 and an isoelectric point of 6.9.

3) Collagen was not a significant component of soluble extracts.

4) Increased levels of N-acetylneuraminic acid on day 7 may be related to an observed suppression of PA/B3.

5) Peak C substances also varied over time and may have value as monitor substances.

6) Full thickness, autogenous skin grafting (a procedure known to dramatically alter wound healing) provoked distinct changes in soluble substance elution profiles.
LITERATURE CITED


FIG. 1

A. Flank wound boundaries.
B. Excision of skin and superficial fascia.
C. Open wound at the level of the panniculus carnosus.
D. Incised granulation tissue.
FIG. 2

Elution profiles of soluble wound extract constituents on Sephacryl SF 200 as a function of time. Normal skin; 1.8 mg, 3-day granulation tissue; 2.0 mg, 7-day granulation tissue; 2.5 mg, 9-day granulation tissue; 2.9 mg, 14-day granulation tissue.
Elution Volume (ml)

Relative Absorbance, 280 nm

Normal Skin
3d GT 1.8mg
7d GT 2.0mg
9d GT 2.5mg
14d GT 2.9mg
FIG. 3

Molecular weight determination of Peak A/Band 3 (PA/B3) material.
Lysozyme
β-Lactoglobulin
Pepsin
Ovalbumin
Bovine Serum Albumin
PA/B3
β-Galactosidase

Kav

Molecular Weight × 10^3
FIG. 4

Elution profiles of 2.9 mg, 14-day open-wound granulation tissue and 3.8 mg, 14-day grafted granulation tissue.
FIG. 5

SDS-PAGE of wound tissue extracts.

A. Gels 1-4, 3-day granulation tissue;
   Gels 5-6, 7-day granulation tissue;
   Gels 7-8, 9-day granulation tissue;
   Gels 9-10, 14-day granulation tissue (open);
   Each gel contains 25-30 ul of crude tissue extract.

B. Gels 11-13, 14-day granulation tissue (grafted);
   Each gel contains 25-30 ul of crude tissue extract.

C. Gel 14, isolated Sephacryl SF 200 Peak A;
   Gels 15-16, isolated Sephacryl SF 200 Peak C;
   Gels 17-19, overloaded crude extracts derived from 7-, 9-, and 14-day open-wound tissues.
FIG. 6

Representative densitometric scan of 9-day central granulation tissue extract electrophoresed on SDS-polyacrylamide.
FIG. 7

Time-dependent changes in electrophoretic banding pattern of crude wound tissue extracts. Abscissa: Postwound interval; Ordinate: Percentage of scan attributable to a given band.
Postwound Interval

Percentage of Scan

- Band 1
- Band 2
- Band 3
- Band 4
- Band 5

(graft)
FIG. 8

Total wound collagen content in grafted wounds as a function of time. Due to the large standard errors for open and grafted wounds at 14 days, the apparent difference between these wounds on postwound day 14 was not statistically significant.
WOUND COLLAGEN CONTENT

Post Wound Day

mg protein Released

open

grafted
<table>
<thead>
<tr>
<th>POSTWOUND DAY</th>
<th>LOWRY PROTEIN (mg/ml extract)</th>
<th>NANA (ug ± SEM)</th>
<th>HYDROXYPROLINE (ug/mg dry tissue ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.52 ± .02</td>
<td>0.20 ± .04</td>
<td>0.34 ± .08</td>
</tr>
<tr>
<td>7</td>
<td>3.89 ± .29</td>
<td>0.78 ± .02</td>
<td>0.35 ± .08</td>
</tr>
<tr>
<td>9</td>
<td>5.00 ± .38</td>
<td>0.12 ± .04</td>
<td>0.55 ± .12</td>
</tr>
<tr>
<td>14</td>
<td>5.76 ± .04</td>
<td>0.22 ± .03</td>
<td>0.44 ± .10</td>
</tr>
<tr>
<td>14 (Graft)</td>
<td>7.55 ± .22</td>
<td></td>
<td>0.62 ± .12</td>
</tr>
</tbody>
</table>
TABLE II

Amino Acid Analysis\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Sepharcll SF 200</th>
<th>Peak A/SDS-Page Band 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu 115</td>
<td>Val 61</td>
<td>Tyr 45</td>
</tr>
<tr>
<td>Lys 111</td>
<td>Gly 57</td>
<td>His 38</td>
</tr>
<tr>
<td>Leu 107</td>
<td>Arg 56</td>
<td>Iso 32</td>
</tr>
<tr>
<td>Asp 90</td>
<td>Thr 51</td>
<td>Meth 8</td>
</tr>
<tr>
<td>Pro 66</td>
<td>Ser 51</td>
<td>Hyl -</td>
</tr>
<tr>
<td>Ala 62</td>
<td>Phen 50</td>
<td>Hyp -</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Residues/1,000 Residues