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Lack of Lipid Involvement in Nonosseous Tissue Repair (41704)

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Abstract. Calcium–acidic phospholipid–phosphate complexes (Ca–PL–PO_4) have been implicated in the onset of tissue mineralization in healing fracture callus as well as in normal osteogenesis. Although these complexes are not found in nonmineralizing normal tissues, the possibility that they are more involved in healing phenomena than in actual mineralization cannot be excluded. The present study confirms that Ca–PL–PO_4 complexes are only involved with osseous repair. In healing skin at 5, 8, and 11 days after wounding, Ca–PL–PO_4 concentration was not significantly elevated (percentage complexed lipid P/total lipid P: 3.7, 2.4, and 3.8, respectively) relative to the value found for normal skin (2 ± 2%), although the total lipid phosphorus content at 8 and 11 days was increased.

In healing fracture callus (1), calcifying bacteria (2), and in endochondral ossification (3), significant increases in levels of calcium–acidic phospholipid–phosphate complexes (Ca–PL–PO_4) accompany the onset of mineralization. These complexed acidic phospholipids initiate *in vitro* hydroxyapatite formation (4) and may provide an *in vivo* nucleation site for hydroxyapatite. However, the available data do not exclude a role for Ca–PL–PO_4 complexes in subsidiary processes of a more generalized tissue regeneration and repair.

Concentrations of Ca–PL–PO_4 in normal marrow, adipose tissue, muscles, and ear cartilage are negligible in comparison to mineralizing tissue (5). In the present study, healing of soft tissue is analyzed for Ca–PL–PO_4 content. Scar formation after removal of a patch of skin from the dorsum of rats is used as a model of nonosseous repair since the surgery is reproducible, facile and has low mortality. Also, skin shares significant regenerative capabilities with bone (6) and is an established model for biochemical studies of connective tissue (7).

Materials and Methods. Twenty-four mixed-sex, 225 to 250-g Sprague–Dawley rats anesthetized with 25 mg Ketalar each, were weighed, their backs shaved, and a 2 cm × 2-cm piece of skin removed down to the fascia under sterile conditions. At 5, 8, and 11 days postinjury, the rats were sacrificed via heart puncture. One specimen of scar tissue at each time point was sectioned and stained for histological analysis, and the remaining scar tissue used in chemical analyses. Normal skin was obtained from the backs of five 225 to 250-g Sprague–Dawley rats in a similar manner, and used as controls. Skin samples were frozen in liquid nitrogen, lyophilized, and ground in a liquid nitrogen-cooled colloid mill. The tissue was washed in 0.05 M NH_4HCO_3, pH 7.8, repeatedly, until the wash water was hemoglobin free (Ames, Hemotest). After relyophilization, samples were subject to ash weight analysis, and to replicate total lipid and hydroxyproline determinations.

Total lipids were extracted from 40- to 500-mg tissue samples. Preliminary analyses revealed that the Ca–PL–PO_4 content of the scar was so low that tissue samples in excess of 100 mg were required for analyses. In order to obtain this quantity of tissue, scar samples were pooled for lipid and mineral determinations, while the larger normal skin samples were analyzed separately. The samples for lipid analyses were homogenized five times for 1 min at maximum speed on a Virtis 45 homogenizer (Brinkmann, N.Y.). Fifteen milliliters of 2:1 (v/v) glass redistilled chloroform:methanol and 5 ml 0.01 M ammonium bicarbonate was sonicated 10 times, 1 min/time. Total lipids were washed three times with 10 ml of chloroform:methanol (2:1, v/v) saturated buffer. These lipids were then dried under N_2, lyophilized, and weighed on a microgram balance.

Ca–PL–PO_4 was separated from noncomplexed lipids (NCLs) by repeated extraction to constant weight with ethanol:ether (3:1, v/v). The lipid phosphorus content of Ca–PL–PO_4 and of NCLs was determined after formic

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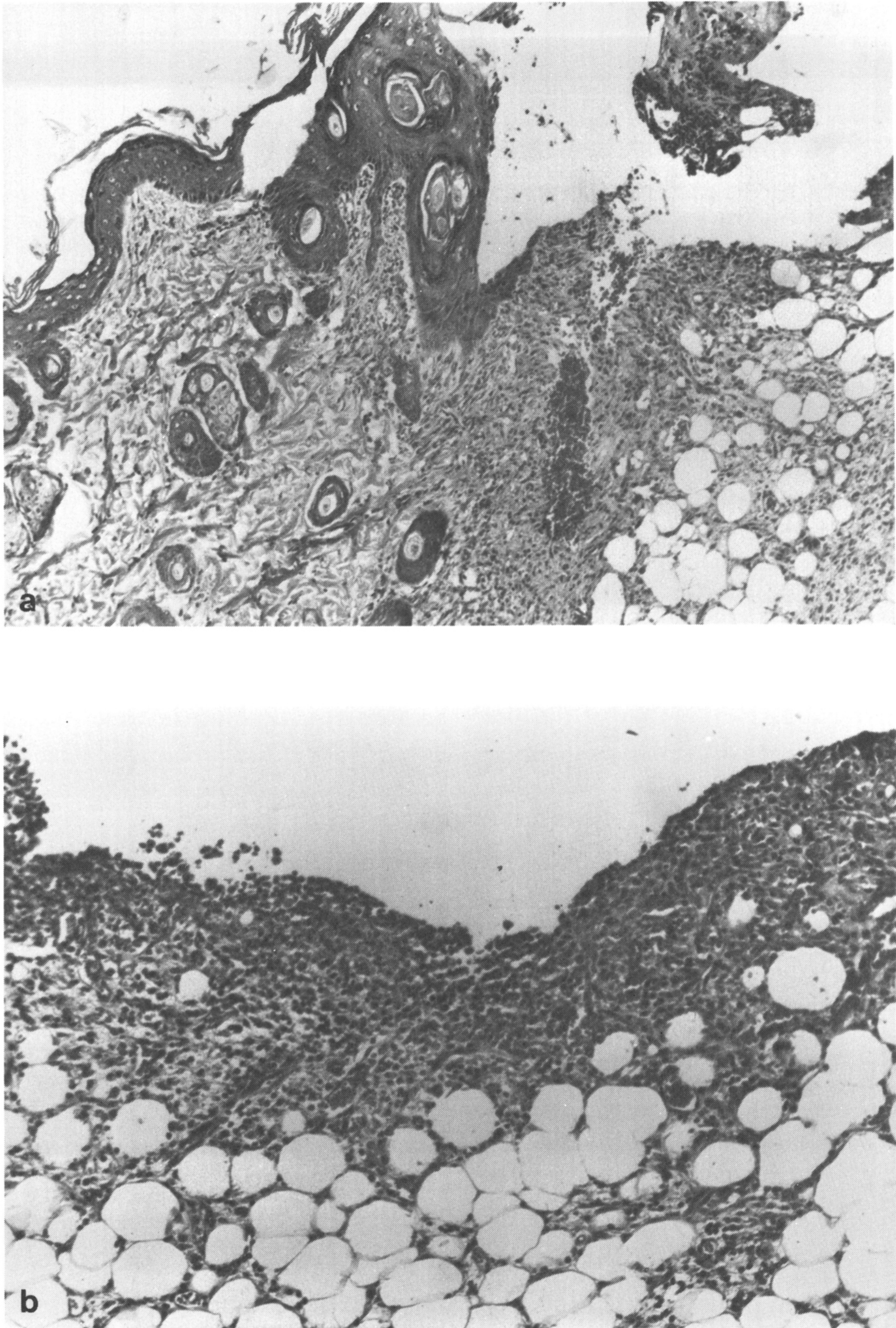
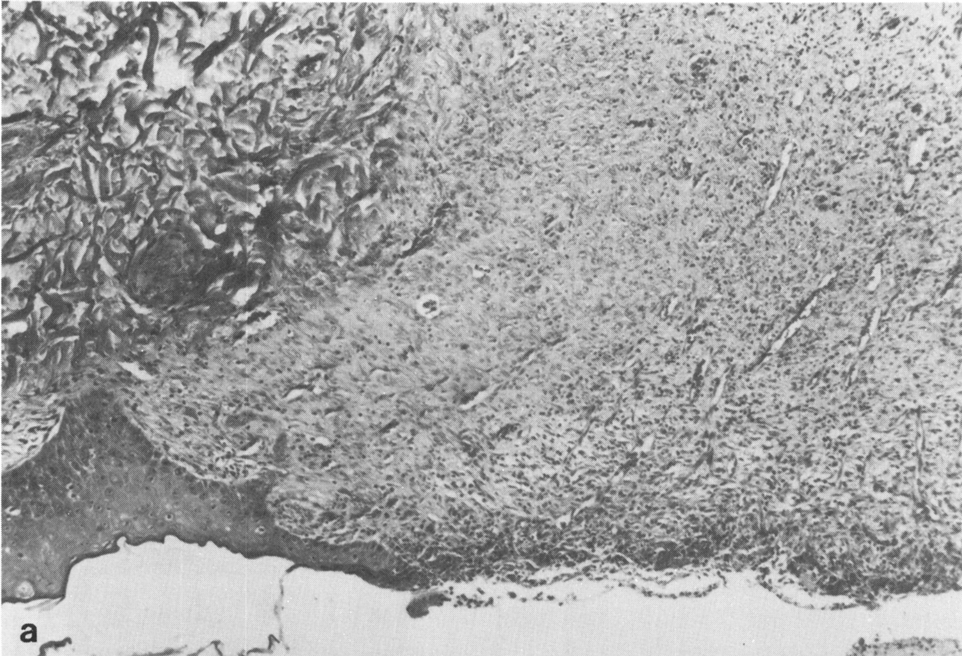


FIG. 1. (a, b) Day 5. (a) Wound with ulceration and mixed acute inflammation (H&E, ×19). (b) Higher view of ulcerated zone showing mixed acute and chronic inflammation (H&E, ×75).



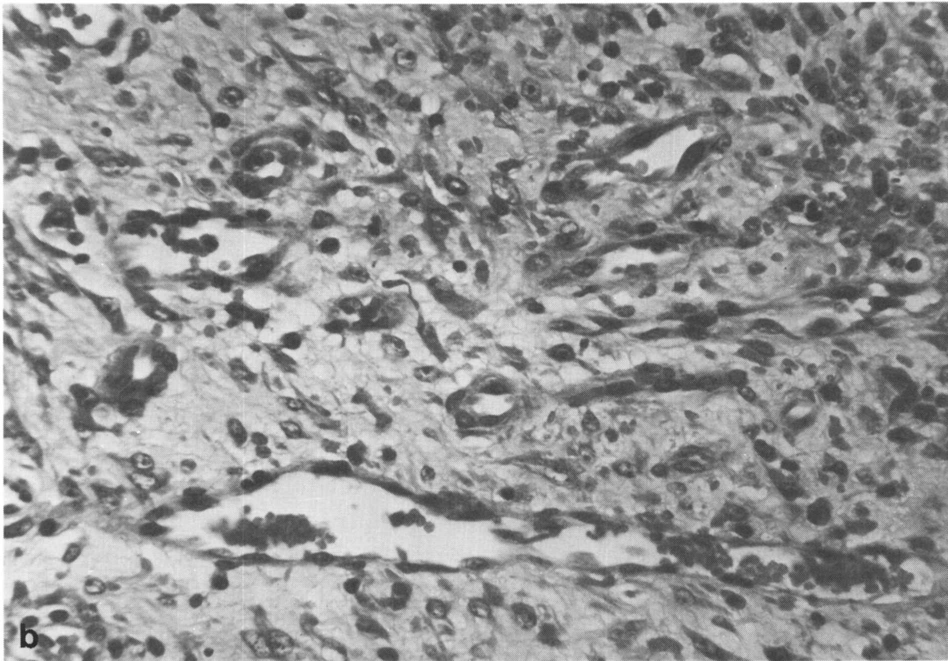
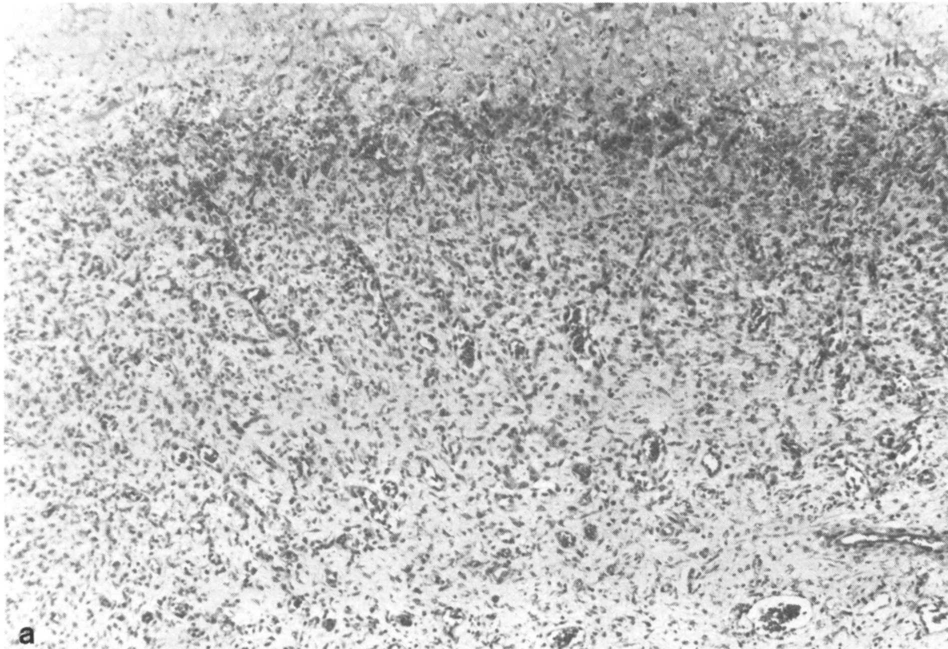


FIG. 2. (a, b) Day 8. (a) Edge of wound (H&E, ×19). (b) Higher view of ulcerated zone shows abundant capillaries and fibroblasts with patchy chronic inflammation consistent with granulation tissue (H&E, × 75).



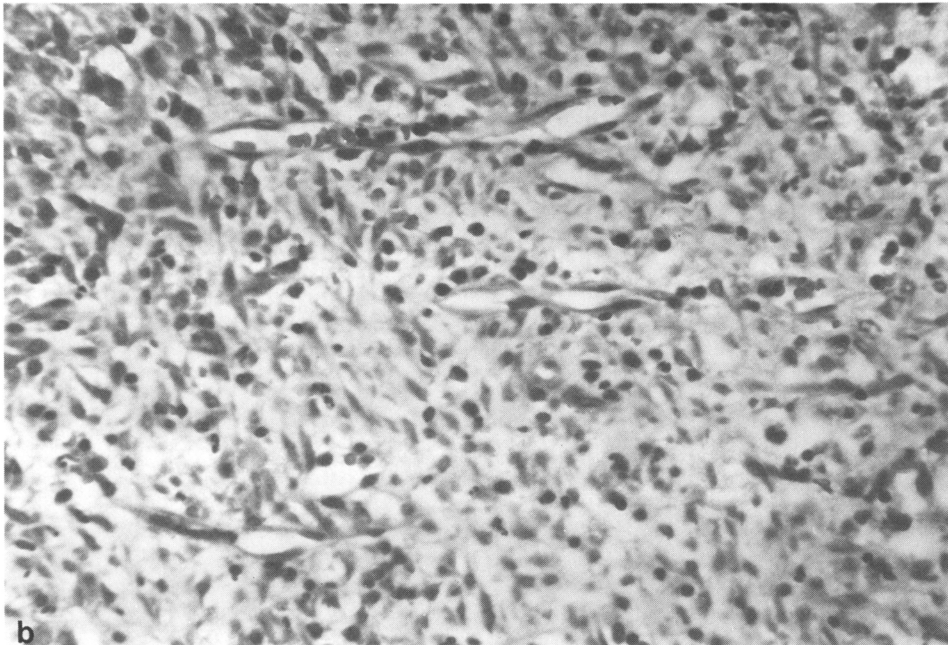


FIG. 3. (a, b) Day 11. (a) Ulcerated wound tissue. Dense scarring is evident beneath the ulcer. Note loss of skin appendages (H&E, ×19). (b) Higher view of scarred area showing dense fibrous connective tissue and little chronic inflammation (H&E, ×75).

acid dissociation of the Ca–PL–PO_4, and partitioning between chloroform:methanol and 1 N HCl (2:1:1, v/v/v). Colorimetric quantitation of organic phosphate content was then performed according to the method of Fiske and Subbarow (8).

Hydroxyproline, an indicator of collagen content, was assayed in 0.5- to 3.75-mg samples according to the method of Hutterer and Singer (9) following tissue hydrolysis in 6 N HCl for 20 hr at 120°C. Ash weight was determined by drying 50- to 60-mg samples to constant weight (100°C, 24 hr) and subsequently ashing at 600°C for an additional 24 hr. Due to limited sample size only one determination of ash content was made at each time point.

Means and standard deviations were calculated for replicate determinations on pooled scar samples, and for replicate determinations of individual normal skin samples. Significant differences between the content of the healing tissue and the normal skin were calculated using the Student's t test, based on 2 df. Within each group, spurious results were rejected only if the criteria of the Q test (10) were met.

Results. Histological inspection of the wound site at each time point reveals the expected time course of healing (11–13) (Fig. 1–3). Acute and chronic inflammation was present at Day 5. At 8 days, capillary buds and fibroblasts were visible, and a scar had formed by Day 11.

TABLE I. COLLAGEN AND LIPID CONTENT OF HEALING SCAR TISSUE[a]

Tissue	Collagen	Total lipid	Lipid P
5 Day	8 ± 3 (N = 6)	80 ± 50 (N = 3)	0.25 ± 0.05 (N = 3)
8 Day	12 ± 2[b] (N = 6)	140 ± 90 (N = 3)	0.61 ± 0.19[b] (N = 3)
11 Day	21 ± 5[b] (N = 6)	160 ± 60 (N = 2)	0.41 ± 0.07 (N = 3)
Normal skin	49 ± 19[b] (N = 18)	150 ± 40 (N = 3)	0.25 ± 0.05[b] (N = 3)

[a] Means ± SD for (N) determinations on pooled samples. For normal skin, results come from three animals, where $N < 3$ third value rejected based on Q test. Collagen hydroxyproline = μg/mg dry tissue. Total lipid = μg/mg dry tissue. Lipid phosphorus = μg lipid P/mg dry tissue.

[b] Significantly different from previous time point, $P \leq 0.05$.

TABLE II. COMPLEXED ACIDIC PHOSPHOLIPID CONTENT OF HEALING SCAR TISSUE

Tissue	CLP (%) / TLP	Ca-PL-PO_4 (μg/mg) / Tissue
5-day scar	3.7 ± 0.7	2.1 ± 0.6
8-day scar	2.4 ± 0.8	1.7 ± 0.4
11-day scar	3.8 ± 0.8	2.8 ± 0.2
Normal skin	2 ± 2	1.2 ± 0.4

Note. CLP/TLP—phospholipid content of Ca-PL-PO_4 complex as percentage of total lipid phosphorus (means ± SD, for three determinations of pooled scar tissue, and, for means of three determinations of three separate normal skins). No significant differences exist between individual time points and normal skin, assuming 2 df.

Collagen hydroxyproline increased continuously, but in the short span of the study, did not reach levels of normal skin (Table I). The total lipid content did not vary significantly during the course of wound healing (Table I). The lipid phosphorus content, in contrast, was increased both at 8 and 11 days.

The Ca–PL–PO_4 content of the wound did not differ from that of normal skin. This can be seen in Table II where the Ca–PL–PO_4 concentration is expressed in two ways: as a function of total lipid phosphorus content, and as a function of dry tissue weight. It should be noted that ash weight determinations revealed the absence of mineral in the scar tissues (mineral content was 0.9 ± 0.3% across all stages of healing and normal skin).

Discussion. Ca–PL–PO_4 content during the lag, fibroblastic, and maturation stages of wound healing does not differ from the concentration observed in normal skin. This is unlike the phenomena seen in mineralizing tissue, where during the growth and active phases of repair, significant elevations of Ca–PL–PO_4 concentration are seen (1, 3, 5). In actively mineralizing tissues, such as healing fracture callus (1) and the lower half of the hypertrophic zone of the epiphyseal growth plate (3), the Ca–PL–PO_4 concentration can reach levels more than 10 times the value seen in scar and skin. The presence of low but nonzero levels of Ca–PL–PO_4 in the skin and scar, may be explained by the hypothesis (15) that these complexes play a role in cell-membrane fusion as well as in the mineralization process.

The lack of variation in scar Ca–PL–PO_4 content occurs in spite of the increase in total

lipid phosphorus content seen at Days 8 and 11 of scar formation. The increased phospholipid content at 8 days is similar to the findings reported by Jackson (7). However, the finding of constant total lipid content is in direct contrast to Vyazmina's histological study which showed the greatest lipid content at 3 days postinjury with virtually no lipids in the healed wound at 60 days (14). The discrepancy between biochemical and histochemical lipid analysis is most likely due to an inability to stain the lipids after initial scar formation.

The experimental variability noted in this study can be attributed both to small sample size and to specimen heterogeneity. The presence of skeletal muscle in some specimens, and the inclusion of variable amounts of wound edge, where initial fibroblast and capillary proliferation occur, help to account for the variations seen. Hunt and Dunphy regard the healing ridge present in an open wound between 7 and 9 days as a simplified counterpart of a fracture callus (13). At least in terms of Ca–PL–PO_4 complexes, which in the more intricate process of fracture healing show marked increases in the early stages of repair, we confirm their observation.

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