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Phospholipid Changes during Fracture Healing (40988)[1]

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Abstract. The changes in the phospholipid content of healing rat fracture callus were shown to resemble those occurring during endochondral ossification. Specifically, the calcium–acidic phospholipid–phosphate complexes (Ca–PL–PO_4) which have been associated with the onset of mineralization in other tissues, are most abundant in the Day 11 fracture callus (19% of total lipid) where mineral is accumulating most actively. The Ca–PL–PO_4 content then gradually decreases to the value observed for metaphyseal and diaphyseal bone (6% of total lipid). This study provides further proof that the Ca–PL–PO_4 complexes are associated with the early stages of mineralization, and demonstrates another manner in which fracture healing in the rat resembles endochondral ossification.

Healing fracture callus is known to undergo changes which morphologically (1, 2) and biochemically (3–7) resemble those occurring during endochondral ossification. Among the biochemical events that occur during the formation of calcified cartilage and primary spongiosa are alterations in the phospholipid composition of the tissue (8) probably related to the formation of calcium–acidic phospholipid–phosphate complexes (Ca–PL–PO_4) (9). The Ca–PL–PO_4 complexes that are formed *in vivo* are most likely membrane components (10, 11). They are capable of inducing *in vitro* hydroxyapatite formation (12) and are believed to be involved in the initiation of *in vivo* calcification (11, 13). The purpose of this study is to compare the time-dependent changes in phospholipid composition in healing fracture callus with the zonal phospholipid changes characteristic of endochondral ossification.

Materials and methods. The right tibias of 215- to 250-g male Sprague–Dawley rats were fractured so as to produce maximum fracture callus formation (7). An anterior transverse cut was made halfway through the tibia of Ketelar-anesthetized rats using a dental drill fitted with a saline-bathed serrated circular bit. The cut was made at a point 3–4 mm proximal to the distal tibia–fibula junction. Lateral flexion of the tibia manually completed the fracture through the tibia and adjacent region of the fibula. Three sutures were used to close the skin incision and no immobilization was provided for the fracture. Clinical and radiographic osseous stability was apparent at 28 days. Groups of 12–15 animals were sacrificed with an overdose of anesthesia at 7, 11, 14, 17, 21, 28, and 42 days after fracture. In the case of the 7- and 42-day calluses 28 animals were used to furnish sufficient material for analysis. At sacrifice, the center portion of the callus, 3 mm in length, freed of the existing cortical bone, was removed along with control diaphysis from the contralateral tibias and metaphyseal bone from the operated and contralateral femurs. Material from all animals at a given healing stage was pooled, frozen in liquid nitrogen, lyophilized, and ground in a liquid-nitrogen-cooled colloid mill. Tissues were washed in 0.05 *M* tris (hydroxymethyl)-aminomethane–HCl (Tris, pH 7.4) until the wash water was hemoglobin free (9). After relyophilization, samples were subjected to replicate (a minimum of three and a maximum of nine) analyses for ash weight,

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hydroxyproline content, DNA content, and a total lipid content.

Ash weight was determined following drying of 5- to 20-mg samples at 100° for 24 hr and ashing at 600° for an additional 24 hr. The hydroxyproline content of 2- to 5-mg samples was determined by the method of Hutterer and Singer (14) after hydrolysis in 6 *N* HCl for 20 hr at 120°. The DNA content of 10- to 20-mg samples of tissue was measured using a modification of the method of Burton (15). The ground tissue was decalcified in three 2-ml volumes of cold (0–4°) 0.5 *M* $HClO_4$. Magnetic stirrers were utilized to create the necessary agitation, and the supernate was decanted after centrifugation at 2000 rpm (4°). The decalcified pellet was put through a repeated sequence consisting of sonication in 0.5 *M* $HClO_4$, incubation at 70°, and centrifugation to separate supernate from pellet. The DNA content of the three combined perchlorate extracts was then determined.

Total lipids were extracted with sonication from 80 to 100 mg of callus or bone, using a total of 30 ml of glass-redistilled chloroform:methanol (2:1, v/v) and 10 ml of 0.05 *M* Tris (pH 7.4) (16). This method has previously been shown to release all lipids from calcified tissues without need for prior demineralization (16, 17). The total lipids were washed three times with 10 ml of 0.05 *M* Tris (pH 7.4) that had previously been saturated with chloroform:methanol (2:1, v/v). Total lipids were then dried under nitrogen, stored 48 hr under vacuum, and then weighed on a microgram balance.

$Ca-PL-PO_4$ complexes were separated from the noncomplexed lipids by repeated extraction to constant weight with 3:1 (v/v) ethanol:ether. The phospholipid content of the noncomplexed lipids, as well as that of the complexed lipids, was determined colorimetrically (18) following separation of phospholipid classes by two-dimensional thin-layer chromatography (19). Prior to chromatographic separation, $Ca-PL-PO_4$ complexes were dissociated in formic acid and partitioned between chloroform:methanol and 1 *N* HCl (2:1:1, v/v/v) (16). The aqueous phase was analyzed for Ca (20) and inorganic phosphate contents (21).

Each biochemical parameter listed above was measured a minimum of three times for control diaphyseal and metaphyseal bone and for all callus samples from 11 to 28 days. Insufficient callus was provided by the 7- and 42-day samples for triplicate determinations of all parameters in spite of using 28 pooled animals per point, and thus the 7- and 42-day callus data were not evaluated statistically. Significant differences between the content of the callus and control bone were sought at all other healing times. *F* tests were used to test the significance of populations of different size and when different groups were located, Student's *t* tests were applied to determine the significance of parameters with comparable variance.

Results. The time course of fracture healing was characterized by a continuous rapid increase in mineral content (Fig. 1), and an initial increase in collagen hydroxyproline (Table I) as the tissue changed from hematoma to mesenchymal to chondroid and to chondroid–osteoid (7, 22). Thereafter, the collagen content approached the value of control bone as the mineralized callus entered the remodeling stage (Day

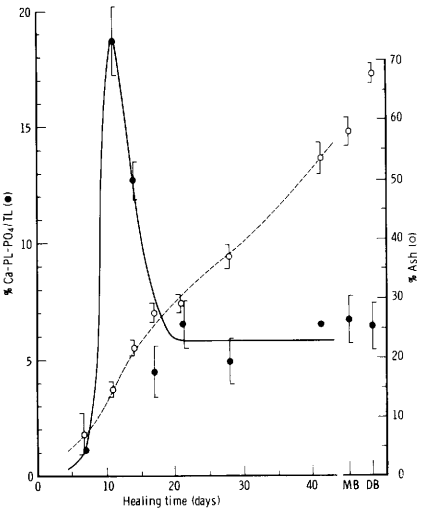

FIG. 1. The time-dependent changes in the mineral content of the fracture callus (open circles) and the percentage of total lipid pool (TL) that is complexed acidic phospholipid $Ca-PL-PO_4$ (closed circles). Error bar shows standard deviation. Control data are shown for metaphyseal bone (MB) and diaphyseal bone (DB).

TABLE I. HYDROXYPROLINE AND DNA CONTENTS[a] OF RAT FRACTURE CALLUS AND CONTROL BONE

	OHPRO (μg/mg ± SD)	DNA (μg/mg ± SD)
Callus (Day)		
7	30 ± 3**	20 ± 5**
11	63 ± 6*	22.9 ± 0.7*
14	57 ± 10***	21 ± 1*
17	58 ± 7*	20 ± 2*
21	38 ± 6*	18 ± 6***
28	40 ± 2*	19 ± 6***
42	27 ± 1	
Bone		
Metaphyseal	21 ± 3	5.2 ± 0.6
Diaphyseal	28 ± 3	6.5 ± 2

Note. Statistics: Callus values (n = 3) compared to diaphyseal bone values (n = 6) using Student's t test with 7 degrees of freedom.

[a] μg/mg total tissue weight.

* $P \leq 0.0005$.

** $P \leq 0.002$.

*** $P \leq 0.01$.

42). The DNA content of the callus was relatively constant during the first 4 weeks of fracture healing (Table I). These trends are seen when the data are expressed per total dry tissue weight, as in Table I, or when expressed per demineralized dry weight. Corrected for the presence of mineral, the hydroxyproline content per demineralized dry weight at Days 11, 14, and 17 was 75 ± 4 μg/mg, at Days 21, 28, and 42 days it decreased to 58 ± μg/mg. The constant amount of DNA is similarly seen when the data are expressed per demineralized dry weight; 29 ± 3 μg/mg for all calluses. This is significantly greater ($P \leq 0.001$) than the 20 ± 2 μg/mg found in control bone.

The percentage of total lipids that are involved in complexed acidic phospholipid formation is maximum at 11 and 14 days and decreases rapidly to the level of control bone by 21 days. This trend is seen in Fig. 1, where the Ca–PL–PO_4 to total lipid ratio is presented, as well as in Table II, where the measured Ca–PL–PO_4 to whole tissue ratio and calculated Ca–PL–PO_4-to-DNA ratios are presented. DNA content was chosen as a denominator since its value was unchanged through the course of fracture healing. For the second ratios comparisons were not made to control bone since the DNA content of the bone was markedly reduced.

Comparisons between callus and control values for all parameters were made relative to the mean value for control diaphyseal bone for all experiments. It should be noted that the Ca–PL–PO_4 content of the control bone was not invariant, but was slightly higher (10.8 ± 2.3% of the total lipid) at 7 days contrasted for example, with 6.9 ± 0.3 and 6.7 ± 2.9% of the total lipid at 14 and 28 days, respectively. Thus the general trend was for the Ca–PL–PO_4 content of control bone to be slightly higher during the early stages of fracture healing. However, since these increases in the early time periods were not statistically significant, the mean from all experiments was used for comparison with the callus data.

At Day 7, when the callus was mainly hematoma, the total lipid content was greater than that of control bone. Thereafter, the total lipid content of the organic matrix of the fracture callus was invariant and slightly less (7.4 ± 1.2% of the demineralized dry weight) than that of control bone (9.1 ± 0.61%). This suggests that in the case of fracture callus, the total lipid content is a good denominator for use in comparing the Ca–PL–PO_4 contents at different healing stages from Days 11 to 42. Thus, the peak in Ca–PL–PO_4 content is seen at 11 days when total lipid content (Fig. 1) is used as the denominator. This

TABLE II. Ca–PL–PO_4 RATIOS[a] IN HEALING RAT FRACTURE CALLUS AND CONTROL BONE

	Ca–PL–PO_4/ tissue	Ca–PL–PO_4/ DNA
Callus (day)		
7	1.3	65
11	12 ± 6**	522 ± 27
14	5.8 ± 0.8***	276 ± 50
17	2.6 ± 1.3	130 ± 28
21	3.7 ± 1.1*	206 ± 11
28	2.7 ± 0.7	142 ± 85
42	1.8	
Bone		
Metaphyseal	2.3 ± 0.2	442 ± 20
Diaphyseal	2.7 ± 0.5	415 ± 21

[a] Weight ratio of Ca–PL–PO_4 to total tissue mass and to tissue DNA content (μg/mg) ± SD (n = 3 for callus, n = 6 for bone). Comparisons based on Student's t test show callus different from diaphyseal bone at * $P \leq .01$, ** $P \leq .005$, *** $P \leq .0005$.

peak at 11 days is also seen when whole tissue mass (Table II) and total lipid phosphorus are used as denominators. The latter ratio indicates that in the early stages of callus mineralization a greater percentage of the total lipid phosphorus is associated with calcium and inorganic phosphate than is seen at later stages or in control bone.

The composition of the Ca–PL–PO_4 complexes did not differ significantly with time and thus only representative values are given in Table III. Averaging the data at all time points, of the entire Ca–PL–PO_4, 51 ± 5 mole% is Ca, 9 ± 4 mole% is inorganic phosphate, and the remainder is lipid phosphorus. The acidic phospholipids phosphatidyl serine and phosphatidyl inositol accounted for 97.2 ± 6% (n = 10) of all the phospholipid phosphorus in the complexed lipids, with 80.8 ± 0.8% coming from phosphatidyl serine.

Representative data for the overall composition of the phospholipids of the callus are shown in Fig. 2. Throughout the course of healing the only statistical difference in the phospholipid content or composition occurred at 11 days when the percentage of phosphatidyl serine was increased. No other significant differences were seen throughout the course of fracture healing, thus data from 11, 17, and 28 days are presented as representative.

TABLE III. MEAN COMPOSITION[a] OF COMPLEXED ACIDIC PHOSPHOLIPIDS IN REPRESENTATIVE FRACTURE CALLUS AND CONTROL BONE

	Mole% ± SD (n = 3)		
	Ca	P_i	P_{org}
17-Day callus	51 ± 2	11 ± 4	35 ± 6
28-Day callus	54 ± 2	12 ± 5	30 ± 10
Metaphyseal bone	47 ± 2	9 ± 4	44 ± 2
Diaphyseal bone	50 ± 2	7 ± 2	46 ± 4

[a] Ca–PL–PO_4 was dissociated in formic acid and partitioned between 1 N HCl and chloroform:methanol. The Ca and inorganic phosphate (Pi) content of the aqueous layers were determined in triplicate for each sample. One determination per sample was made of the organic phosphorus content, P_{org}, of the organic phase. For each callus or bone sample, three individual aliquots of tissue were examined.

Discussion. This study has shown that the changes in Ca–PL–PO_4 content that occur in rat fracture healing are similar to those observed during endochondral ossification in the bovine fetal epiphyseal plate (8, 9). In both cases, the maximum amount of complexed acidic phosphate lipids, independent of the denominator by which they are expressed, occurred where rapid mineralization is commencing (11 days in

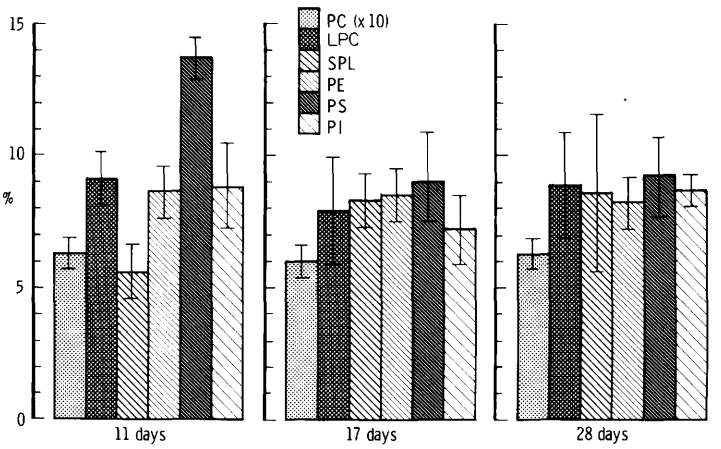

FIG. 2. Representative data showing the phospholipid composition (percentage lipid P corresponding to given lipid class per total lipid phosphorus) of healing fracture callus as a function of time. The only significant difference ($P < 0.05$) is the percentage of total phospholipid pool corresponding to phosphatidyl serine (PS) at 11 days. PC = phosphatidyl choline, PI = phosphatidyl inositol, PS = phosphatidyl serine, PE = phosphatidyl ethanolamine, LPC = lysophosphatidyl choline, SPL = sphingomyelin.

healing fracture callus and in the lower half of the bovine epiphyseal plate). At 11 days, the rat fracture callus changed from the hematoma-filled, low-ash (7%), 7-day callus, to the 20% mineralized cartilagenous tissue. In the lower half of the hypertrophic zone of the growth plate, cartilage calcification commenced and the ash weight increased similarly from about 12 to 30%.

The best parameters for measuring $Ca-PL-PO_4$ content appear to be a weight ratio based on total lipid content or a molar ratio based on quantitation of the amount of lipid phosphorus in the complexed and noncomplexed lipid fractions. Thus, at Day 11, 20 mole% of the lipid phosphorus is associated with Ca and inorganic phosphate in $Ca-PL-PO_4$ complexes, as compared with 9–11% at later stages of healing and in control bone. The increased $Ca-PL-PO_4$ content of the control bone just after fracture may be related to the increased mineralizing activity that has been noted by others (23) in the nonoperated bones after fracture.

The biochemical changes seen in the growth plate (11) and in healing fracture callus include increases in the relative proportion of acidic phospholipids at the time when mineralizing is commencing. This increase, and the concomitant increase in $Ca-PL-PO_4$, may be due to the increase in the number of extracellular matrix vesicles (24) associated with callus calcification (25, 26). These extracellular matrix vesicles are known to be rich in the acidic phospholipids (27, 28) and to contain Ca–acidic phospholipid–phosphate complexes (10).

Our earlier studies suggested that the complexed acidic phospholipids might be one of the factors responsible for the initiation of *in vivo* mineralization. The prevalence of the $Ca-PL-PO_4$ complexes in cartilagenous areas where mineralization is being initiated, specifically in the bovine epiphyseal growth plate (9) and in healing rat fracture callus, as shown in this study, gives further credence to this thesis.

In terms of the overall composition of the matrix of the callus, it is of interest to note that the DNA content of healing rat fracture callus is comparable to that of rabbit and bovine articular cartilage (9, 29) and higher than that of rabbit, bovine, and rat bone (9, 29). Mankin (29) observed that the DNA content of articular cartilage was higher in younger, as opposed to more mature, animals. Thus, it is not surprising that in this young, developing hypercellular cartilage of fracture callus, the DNA content is high. The collagen (hydroxyproline) content of the matrix during the first periods of healing corresponds to that observed by others (30) in the epiphysis, and at later stages in primary spongiosa, but never reaches the values observed in control (diaphyseal) bone. Thus, the matrix of the callus, though much more heterogeneous (4) than the matrix of the growth plate, undergoes changes, as the callus becomes mineralized, that are quite comparable to those observed within the growth plate.

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Volume **164**, No. 4 (1980), in the article, "Increased Infectivity of Oncogenic Herpes Viruses of Primates with Tumor Promoter 12-*O*-Tetradecanoylphorbol-13-Acetate (40901)," by D. V. Ablashi, Z. H. Bengali, M. A. Eichelberger, K. S. Sundar, G. R. Armstrong, M. Daniel, and P. H. Levine, pp. 485–490:

Through a printer's error, Figure 3 was omitted from the article. For the convenience of the reader the figure is reprinted below.

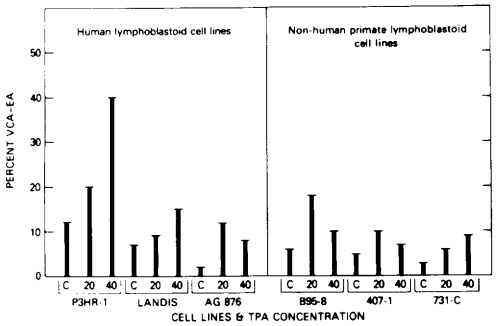

FIG. 3. Comparison of stimulation of antigens by TPA in primate lymphoblastoid cell lines producing Epstein–Barr virus. C = no treatment. TPA concentration, 20 or 40 mg/ml; cell concentration, 5×10^5; and cell viability, >80%.