Citrate and Alkaline Phosphatase During Transformation of Fibroblasts by the Matrix and Minerals of Bone[1] (36557)

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Citric acid was discovered in bone by Dickens (1); its nature and functions are unclear although it has been suggested that citrate may be involved in bone resorption (2).

The fibroblasts of adult animals can be transformed readily into bone either by an osteogenic epithelium (3) or by acid-insoluble fractions of tooth and bone (4, 5). Fibroblasts of the rat are most prone (6) to the transformation induced by demineralized bone matrix *in vivo* or in tissue culture (7).

In the present paper we describe the changes in content of citric and lactic acids and of alkaline phosphatase in grafts following allogeneic transplantation of the acid-soluble residue of rat bone into the subcutaneous tissue of young rats. Also we report that rat bone ash in 1:1 ratio with acid-insoluble residue blocks the transformation process. Bone ash alone caused the rapid production of multinucleate osteoclastic giant cells and the grafts contained large quantities of organic acids.

Material and Methods. Male and female rats of Long and Evans strain aged 25–35 days were used exclusively. Our sample of acid-insoluble bone matrix of rat bones consisted of 30 g of dehydrated powder (74–420 μ in size) after successive extractions of bone powder with 0.5 *N* hydrochloric acid, water-saturated phenol, alcohol and ether; it was ashless and the content of Ca^{2+} was 0. Bone ash was prepared by placing rat bone powder in a muffle furnace at 650° for 18 hr; the nitrogen content of the ash was 0. Allogeneic transplants of these preparations were made by depositing 10–20 mg subcutaneously into recipient rats (6); there were 8 grafts in each recipient.

The grafts were harvested at intervals and they always appeared as discrete fused masses 40–100 mg in weight. These plaques were cleansed of adherent tissues, weighed, homogenized in ice-cold 1 *N* $HClO_4$ (for determination of organic acids) or 0.15 *M* NaCl containing 0.003 *M* $NaHCO_3$ (for alkaline phosphatase estimation). The homogenates were centrifuged at 12,000*g* for 15 min at 2° and the supernatant was saved for enzyme assays. Citric (8) and lactic (9) acids were measured by colorimetric methods and the results expressed as micrograms per gram of tissue, fresh weight. The content of alkaline phosphatase in the grafts was determined as described previously (10); 1 unit is the enzyme activity which liberates 1 μmole of *p*-nitrophenol/0.5 hr/1 g tissue fresh weight under stated conditions. Tissues for histological studies were fixed in Bouin's fluid.

Results and Discussion. In confirmation of our earlier observations (5, 6), acid-insoluble residue of bone when transplanted to subcutaneous sites in recipient rats resulted in the appearance of large amounts of cartilage by day 6, bone between days 10–12 and bone marrow by day 15. As shown in Fig. 1 there was a progressive augmentation in citric acid in grafts of acid-insoluble residue. There was a dramatic increase (nearly fivefold) in citric acid content between days 11 and 15, at which time there was histological evidence of numerous osteoblasts and osteocytes; necrosis was absent. There was a good correlation between the values of citric acid and the histologic appearance of bone in the grafts. The levels of lactic acid were

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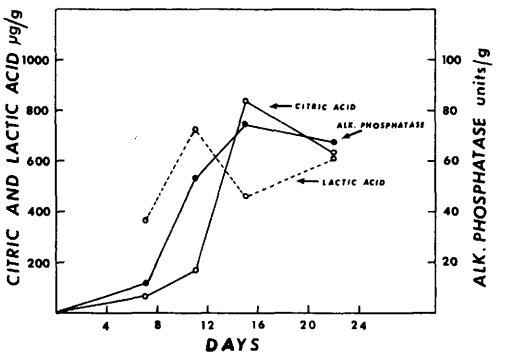

FIG. 1. Changes in citric and lactic acids and alkaline phosphatase in subcutaneous transplants of acid-insoluble residue of bone. Cartilage was present in large amounts on days 7–11. Large quantity of bone was evident by day 15.

rather variable and no general pattern was evident. The increase in citric acid was preceded by a considerable elevation of alkaline phosphatase activity—a well known index of ossification (11) during transformation of fibroblasts.

The citric and lactic acid concentrations in different skeletal tissues and teeth of normal rats age 50–54 days were determined as a reference for comparison to the bone produced in experimental grafts. The salient findings (Table I) are the abundance of citric acid in calcified tissues: femur, calvarium and tooth. It is interesting to note that the articular cartilage in comparison to the ensiform cartilage has much larger concentrations of citric acid. Also noteworthy is the fact that both bone marrow and tooth pulp (medullary soft tissues) have rather large concentrations of lactic acid and relatively smaller amounts of citric acid. The fascia from the back which essentially consists of fibroblasts has the lowest concentration of citric acid among the tissues examined.

The subcutaneous transplantation of bone ash led to the production of multinucleate giant cells; large numbers of these cells were evident between days 11 and 15 after transplantation (Fig. 2). There was no necrosis or inflammation as assessed by gross inspection and histological examination. The changes in the organic acids after bone ash transplantation are depicted in Fig. 3. Both citric and lactic acids reached a peak on day 11. Whereas the lactic acid values remain at a high level, the citric acid concentrations declined precipitously after day 15. Bone ash (Fig. 3) did not induce alkaline phosphatase in the grafts in contrast to the high values which were observed when demineralized bone matrix was transplanted (Fig. 1).

In six experiments a mixture (1:1) by weight of acid-insoluble bone residue and bone ash was transplanted subcutaneously; the grafts were harvested on day 7, 11 and 14. In all cases the osteoclastic giant cell response was observed whereas bone and cartilage were not induced. The formation of citrate-containing osteoclasts had blocked the transformation of fibroblasts and the chain reaction leading to bone and cartilage did not occur.

Bujard (12) first observed that the foreign body giant cells elicited by an injection of ground bone possessed osetoclastic properties. Irving and Handelman (13) on the basis of cytochemical properties concluded that these multinucleate giant cells produced in response to decalcified bone are comparable to osteoclasts. The present finding of high concentrations of organic acids in multinucleate giant cells to our knowledge is a novel report. It would appear that these organic acids may be involved in the solubilization of the bone minerals. Firschein *et al.* (14) have implicated citrate in the parathyroid hormone-induced bone resorption. Vaes (15) observed increased release of citrate and lactate in the medium of explants of calvarium un-

TABLE I. Citric and Lactic Acid Concentrations in Skeletal and Dental Tissues of Normal Rats.

Tissue	Citric acid (μg/g)	Lactic acid (μg/g)
Bone (femur)	1068 ± 338[a]	402 ± 120
Bone marrow	98 ± 32	852 ± 193
Tooth (incisors)	280 ± 88	142 ± 27
Tooth pulp	92 ± 37	640 ± 123
Calvarium	960 ± 401	277 ± 94
Ensiform cartilage	140 ± 24	333 ± 168
Articular cartilage	1514 ± 370	680 ± 162
Fascia	83 ± 20	411 ± 203
Tendo Achilles	103 ± 39	377 ± 155

[a] Mean values of 7–10 observations are given; ±, standard deviation.

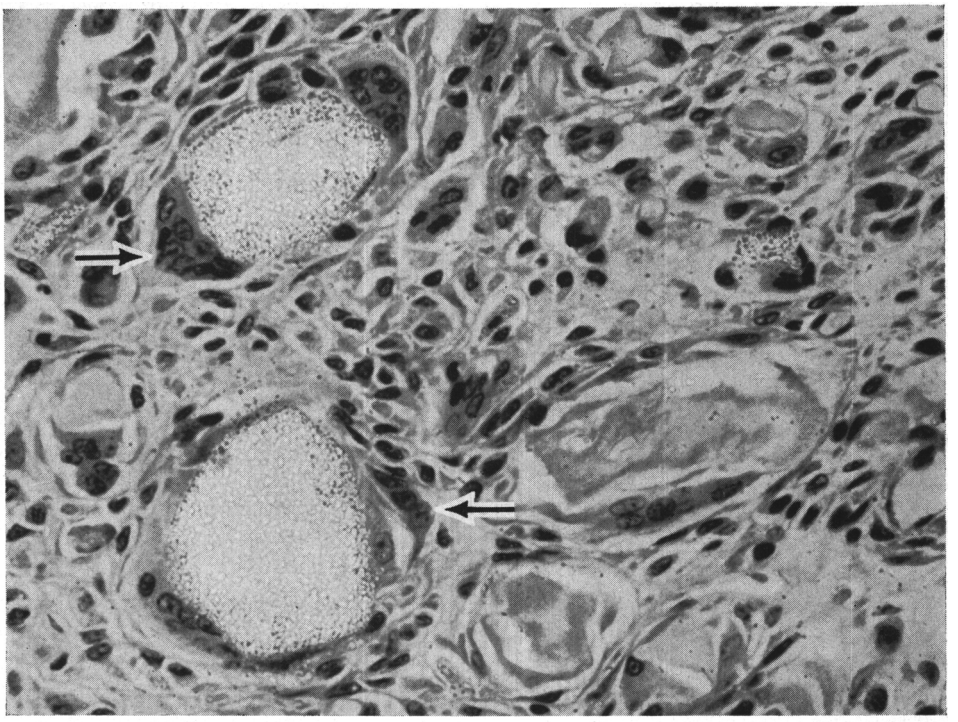

FIG. 2. Allogeneic transplant of rat bone ash to subcutaneous tissue of rat on day 11. Arrows point to multinucleate giant cells. ×360.

dergoing resorption in the presence of parathyroid extract.

Summary. Allogeneic transplantation of demineralized rat bone initiates a chain reaction in responding fibroblasts; alkaline phosphatase and, later, citric acid increase to high levels. The chain reaction is not evoked by bone minerals; instead vast numbers of multinucleate osteoclastic giant cells form around the grafts with high levels of citric acid without an increase of alkaline phosphatase.

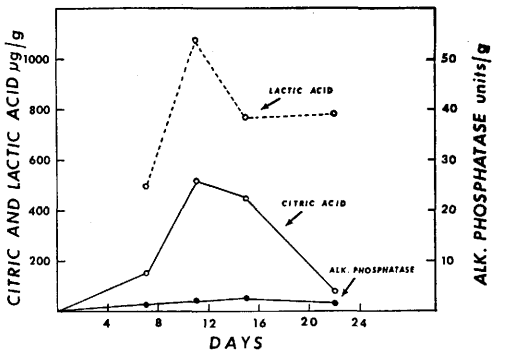

FIG. 3. Changes in citric and lactic acids and alkaline phosphatase in subcutaneous transplants of bone ash. Numerous multinucleate giant cells were present between days 11–15.

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