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DISSOCIATION OF ORGANIC ACID SECRETION FROM MACROPHAGE MEDIATED BONE RESORPTION

J. David Malone, M.D.[1], Arnold J. Kahn, Ph.D.[2], and Steven L. Teitelbaum, M.D.[2]

[1]Department of Medicine, Renal Division, The Jewish Hospital of St. Louis, Washington University School of Medicine, St. Louis, Missouri 63110.

[2]Department of Pathology and Laboratory Medicine, Division of Bone and Mineral Metabolism, The Jewish Hospital of St. Louis, Washington University Schools of Medicine and Dental Medicine, St. Louis, Missouri, 63110.

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SUMMARY: A prevailing concept in the literature on bone resorption suggests that the removal of calcium crystals from the bone matrix is the result of the secretion of lactic and/or citric acid. In the present study, we have reassessed this concept using an *in vitro* bone resorption system consisting of thioglycolate elicited rat peritoneal macrophages co-cultured, for up to 96 hours, with devitalized ^{45}Ca-labeled bone particles. In these combined cultures, medium lactate concentration increased linearly for the first 48 hours of culture and remained at a plateau thereafter. Medium citrate concentration, on the other hand, remained constant and at very low levels throughout incubation. In contrast to both citrate and lactate, bone resorption, measured as ^{45}Ca release, began a few hours after the onset of culture and increased at a constant rate for the balance of the 96-hour culture period. Alteration of resorptive activity by the addition of 10^{-6} M cortisol (which stimulates ^{45}Ca release) or the interposition of a filter between cells and bone (which inhibits resorption) was not paralleled by similar shifts in lactate or citrate concentration. These experiments indicate that mobilization of the bone mineral can occur in the absence of a concurrent, generalized release of lactic and citric acid by resorbing cells. On the other hand, the data do not exclude a possible role for these compounds under circumstances where they are secreted into a "closed" compartment at the cell-bone interface or, in the case of lactate, during the initial period of resorptive activity.

INTRODUCTION

Osteoclastic bone resorption is a highly complex process involving the extracellular degradation of the mineral and organic components of bone matrix. Organic matrix degradation is almost certainly the result of the combined activities of collagenolytic and proteolytic enzymes (1). Mineral mobilization, on the other hand, is much less well understood, but has been

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speculated to be the result of the localized secretion of organic acids. This view follows from the observations that bone matrix may be demineralized, in vitro, by exposure to acid (2,3) and that hormone stimulated bone resorption in organ culture is paralleled by proportional increases in the secretion of lactic and citric acids (2,3,4). The difficulty in fully accepting the second and more critical set of observations is that they were made on intact embryonic bone rudiments, i.e., on relatively large pieces of tissue containing a multiplicity of cell types in addition to the osteoclast. Consequently, it is impossible to unequivocally ascribe to one particular cell (in this case the osteoclast), both resorptive activity and the secretion of lactic and citric acids. To circumvent this kind of difficulty, we recently developed an assay system for bone resorption which utilizes a single cell population, the elicited rat peritoneal macrophage (MØ)* (5). In the present study, we have employed this system to reassess the role of organic acid secretion in the resorptive process.

MATERIALS AND METHODS

Cell Isolation. Peritoneal exudate cells were taken from 150 to 200 g Sprague-Dawley rats three days following intraperitoneal injection of 100 ul/ml Brewers thioglycolate medium (Difco Co., Detroit, MI.). The cells were washed in serum free, α-modified Eagle's Minimal Essential Medium (αMEM), buffered to pH 7.4 with 3N morpholinopropanelsulfonic acid. 5×10^5 cells were plated in the same medium in Costar multiwell dishes (well diameter 16 mm)(Costar, Cambridge, MA.) and incubated for one hour at 37 C in atmospheric conditions. The cultures were then rinsed and the medium was replaced with bicarbonate buffered α-MEM, supplemented with 100 ul/ml fetal calf serum (α-10). The cells were incubated at 37 C in humidified 5% CO_2 and air overnight, and then rinsed again to remove non-adherent cells. The percent of macrophages in the residual adherent cell population was determined by non-specific esterase staining (6) or phagocytosis of yeast (7). Generally $3\text{-}3.5 \times 10^5$ cells remained in each well, i.e., 60-70% of those plated. Ninety-nine percent of these cells were macrophages (MØ).

Determination of cell number. Adherent cell number was determined at the beginning of each experiment by lysing adherent cells in duplicate wells in hexadecyltrimethylammonium (Cetrimide Technical, J.T. Baker Chemical Co., Phillipsburg, N.J.) and counting the released nuclei in an electronic particle counter (Coulter Electronic, Inc., Hialeah, FL.).

Bone Resorption Assay. One hundred-gram Sprague-Dawley rats were injected subcutaneously with 100 μCi of $^{45}CaCl_2$ on alternate days for fourteen days prior to sacrifice. The long bones were dissected free of soft tissue, devitalized and pulverized as previously described (5). A common pool of labeled bone powder was used in all experiments. Particles of labeled bone less than 25 μm in diameter were obtained by sieving and were sterilized by

* MØ: thioglycolated elicited peritoneal macrophages

ultraviolet irradiation. Following overnight incubation of the macrophages, the medium and non-adherent cells were removed and replaced with one ml aliquots of fresh α-10 containing 115 μg of labeled bone particles. The cultures were maintained for up to four days and the percentage of total ^{45}Ca which was released into the medium was determined at various time points. The values obtained were corrected to reflect cell-mediated resorption by subtracting the percent ^{45}Ca released by bone alone incubated in α-10 for identical periods of time. Experiments involving blocking of cell-matrix contact were performed by placing sterile Amicon filters (10,000 dalton mol wt exclusion; Amicon Corp., Lexington, MA.) over the adherent cell layer prior to the introduction of the bone particles. Controls consisted of cultures in which the filters were inserted after both the cells and the particles had been introduced in the wells. Each variable in the resorption assay was performed in replicas of six. Cell number and viability were indirectly assessed at intervals during incubation by measuring the DNA content of the adherent cell fraction (8). Medium glucose was measured by the hexokinase glucose-6-phosphate dehydrogenase (G-6-PDH) method (9)(Calbiochem, La Jolla, CA.), and lactic acid by the conversion of NAD to NADH at 340 nm in the presence of LDH (10)(Sigma Chemical Co., St. Louis, MO.). Citrate was determined by the method of Mollering and Gruber (11)(Boehringer Mannheim, Indianapolis, IN.). All biochemical assays were performed in triplicate. Net medium organic acid concentration was calculated as reaction product generated from medium incubated with cells and bone, minus the values obtained from medium with bone alone. Each experiment was repeated two to four times. In individual experiments each data point represents the mean ± SEM of six, ^{45}Ca release, or three, lactate, citrate, and glucose, determinations. Interexperimental variation was consistently ~10% and comparisons between ^{45}Ca release and medium concentrations of lactate, citrate and glucose were made in the same experiments. Representative experiments are shown in the results section.
Statistical comparisons were made using Student's t-test of means and regression analysis where appropriate (12).

RESULTS

Medium organic acid concentration did not parallel MØ-mediated bone mineral mobilization. Net percent ^{45}Ca release progressively increased up

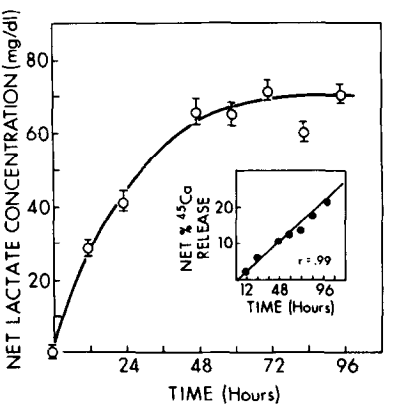

Figure 1 Lactate concentration in the medium increased up to 48 hours of culture. No further increase in medium lactate concentration was observed during the second 48 hours of culture. In the same experiment (insert) net percent ^{45}Ca release increased up to 96 h of culture (each point is the mean of six replicate wells, S.E.M. ± 5%).

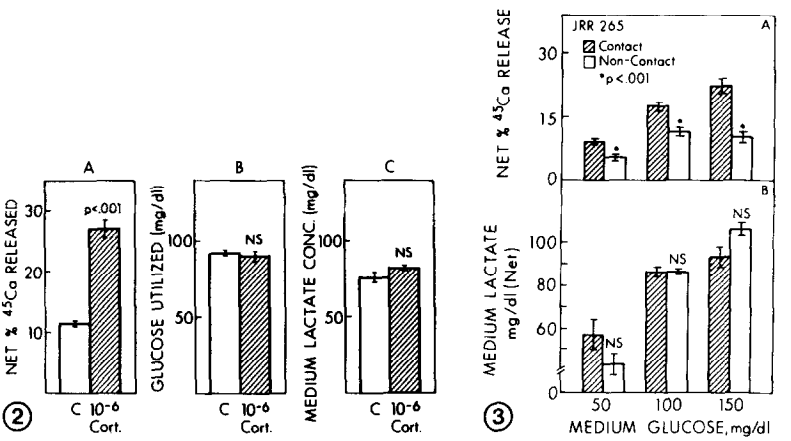

Figure 2 The addition of 10^{-6} M cortisol (10^{-6} cort) to MØ cultures was associated with a doubling of net percent ^{45}Ca released at 96 hours when compared with control (C)(Panel A). This striking increase in net percent ^{45}Ca release was not accompanied by any change in glucose utilization (Panel B) or medium lactate concentration (Panel C).

Figure 3 When contact between MØ and bone particles was blocked by the insertion of a filter there was a significant decrease in net percent ^{45}Ca release. The decrease was independent of the glucose concentration in the medium (panel A). Lactate concentration was dependent on medium glucose concentration but was not affected by the presence or absence of contact, (panel B).

to, and including 96 hours of culture (Fig. 1, insert). Conversely, medium citrate did not change (data not shown) and lactate reached a plateau at 48 hours (Fig. 1) at which time medium glucose was depleted.

We have previously shown that 10^{-6} M cortisol stimulates MØ-mediated bone resorption (13). However, a more than doubling of net percent ^{45}Ca release under the influence of cortisol was not associated with altered medium lactate (Fig. 2). Furthermore, medium citrate declined under these circumstances (2.08 mg/dl $\pm$ .02 vs 1.22 mg/dl $\pm$ .02; $p < .001$).

We have also previously demonstrated that cell-matrix contact was essential for the full expression of MØ-mediated bone resorption (5). As can be seen in figure 3(A), this was true regardless of initial medium glucose concentration. On the other hand, prevention of cell-bone contact did not affect the progressive increase in medium lactate which attended glucose supplementation (Fig. 3[B]). In fact, under circumstances where cell-matrix contact was permitted, additional glucose resulted in augmentation of both ^{45}Ca mobilization (Fig. 3[A]) and medium lactate concentration (Fig. 3B).

DISCUSSION

We noted above the problem inherent in identifying the activities of a particular cell within the context of a heterogeneous population of cells. Nowhere is this problem more apparent than in the literature relating organic acid secretion to bone resorption. Here, despite some evidence to the contrary (14), the prevalent view is that the mineral mobilization phase of the resorptive process occurs as a consequence of the localized release of lactic and citric acids (3,4,5). The difficulty, of course, is that this view was developed primarily from work done in organ culture under conditions where the source(s) and location of organic acid could not be unequivocally identified. In the present study, we have reexamined the relationship between lactate and citrate secretion and bone mineral mobilization using a culture system in which the cells (macrophages) responsible for both acid release and resorptive activity are known with certainty. Our data indicate that, under routine culture conditions, bone matrix degradation (^{45}Ca release) is paralleled by the secretion of lactic acid only for the first half of the culture period. Subsequently, resorption continues but the concentration of lactate in the medium remains unchanged. In contrast to both ^{45}Ca release and lactate concentration, citric acid levels do not vary throughout the entire culture period.

The addition of cortisol to macrophage cultures significantly enhances bone resorption (13), but this increase is not associated with any alteration in lactate levels. Moreover, medium citrate concentration actually declines in the presence of cortisol. Similarly, the interposition of a filter between macrophage and bone sharply curtails resorptive activity, but this diminution in matrix degradation is not accompanied by any change in medium lactate levels.

It is clear from these observations that macrophage-mediated resorption is <u>not</u> associated with an ongoing, proportional increase of lactic or citric acid concentration. However, the data do not exclude a possible role for either organic acid under circumstances where the acids are released into a

"closed" compartment at the cell bone interface (cf., the localized secretion of lysosomal enzymes (5)) or a role for lactate during the initial 48 hours of resorption in vitro. In fact, our observations of a concurrent increase in ^{45}Ca release and lactate production in the presence of excess glucose and cell-bone contact (Figs. 3A, B) are consistent with both these possibilities.

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