Contact-Mediated Bone Resorption by Human Monocytes in vitro

Abstract. *Human circulating monocytes in tissue culture are capable of resorbing devitalized adult and fetal bone. An important component of this process is the adhesion of the cells to the mineralized substrate and the localized removal of matrix from beneath the attached cells. The process appears to involve both release of lysosomal enzymes onto the substrate and intracellular accumulation (transport) of resorbed matrix.*

There is an increasing body of evidence which indicates that osteoclasts are derived from some hematopoietic precursor cell carried within the blood vascular system (*1*, *2*). Although the identity of this precursor is not known with certainty, the fact that the monocyte can transform into a foreign body giant cell resembling the osteoclast in morphology, number of nuclei, and lysosome enzyme content suggests strongly that this mononuclear cell is the progenitor (*3*). In addition, the differentiated monocyte, the macrophage, has been shown to have the capacity to directly remove both mineralized and nonmineralized skeletal matrices under some circumstances in vivo (*4*). These observations prompted us to investigate in vitro the morphological and physiological response of the circulating monocyte to bone matrix.

Since this work was initiated, evidence was reported that monocytes will, without physical contact with the substrate or notable morphological change of the cells, resorb fetal bone in tissue culture (*5*). The results reported here, however, provide evidence that these cells will adhere avidly to a calcified tissue substrate; assume some, but not all, of the morphological features of osteoclasts; and resorb adult bone matrix in vitro. Furthermore, our observations indicate that an intracellular accumulation of matrix may be a component of this resorption process.

Monocytes and lymphocytes were isolated from freshly collected human blood by using a Ficoll-Hypaque gradient (*6*) and suspended in serum-free α-MEM (minimum essential medium) at a concentration of 1×10^4 to 1×10^6 monocytes per milliliter. Portions (0.1 ml) of the cell suspension or cell-free medium (control cultures) were pipetted onto a substrate containing particles of either human cancellous bone obtained at autopsy or adult rat long bones that had been labeled with ^{45}Ca 14 days before the rats were killed. The particles were prepared by washing the bone samples in 0.9 percent NaCl, drying them at 40°C, and grinding the matrix into a coarse powder. The bone powder was then passed through a stainless steel sieve to select particles to 10 to 105 μm in diameter. These particles were then sterilized with ultraviolet light and incorporated into thin layers of rat tail tendon collagen spread on the bottom surface of Aclar plastic disks (*7*) or sterile microtiter wells. As an additional measure of the resorptive activity of monocytes, cultures were also prepared with ^{45}Ca-labeled, intact calvaria of 21-day-old fetal rats as the substrate.

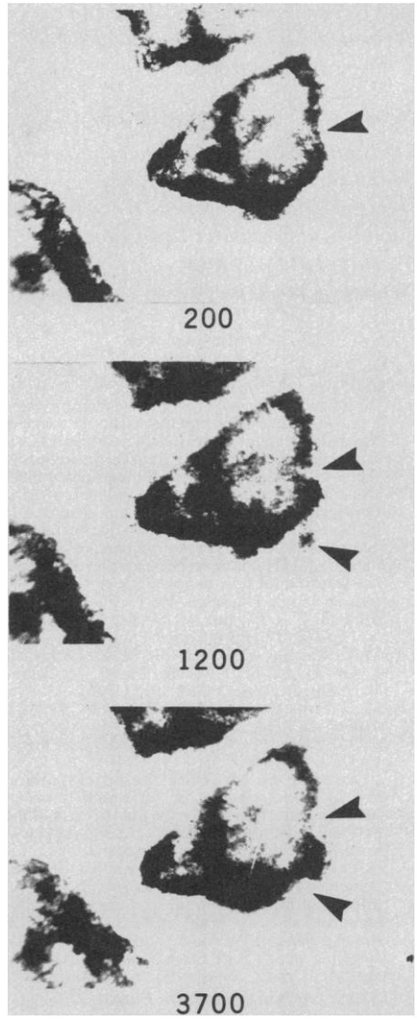

Fig. 1. Three frames from a time-lapse motion picture showing the removal of matrix from a particle of human adult bone by human monocytes in vitro. The cells are not visible in these reproductions. Notice the removal of a bone fragment from the parent piece in frame 1200 and the marked reshaping of the surface that has occurred by frame 3700. The time interval between frames 200 and 1200 was 33.3 hours; between 1200 and 3700, 83.3 hours. The cell concentration was ~ 1×10^5 (× 100).

After 1 hour of incubation (37°C; 10 percent CO_2), the original medium was replaced with fresh nutrient solution to which 10 percent fetal calf serum was added. The cultures were fed every third or fourth day and were maintained for periods of up to 15 days. The type and extent of bone resorption were determined by (i) phase-contrast, time-lapse microcinematography, (ii) transmission electron microscopy and acid phosphatase histochemistry (*8*), and (iii) the appearance of matrix isotope in the medium.

Table 1 shows the results of two experiments in which matrix resorption was monitored by isotope release. The data from both experiments indicate that ^{45}Ca release was significantly greater in cultures containing cells than in those without cells. Further, the monocytes were at least as effective in degrading adult bone matrix as they were in resorbing 21-day fetal rat calvaria. Finally, in the experiment with adult bone matrix, the greatest degree of isotope release occurred in cultures containing 1.6×10^6 cells. In cultures containing many more or many fewer cells, isotope release was significantly less. This result suggests that there is an optimal ratio of cells to matrix for resorption.

The results of time-lapse cinematography are well correlated with the net bone resorption documented by radioisotope release. The films clearly demonstrate that monocytes bind more readily to bone matrix than they do to the surrounding collagen substrate. In fact, despite the considerable mobility shown by the attached cells, they seldom, if ever, leave the bone surface. The films also show that the matrix is altered as a consequence of cellular activity. Some of these alterations were the result of the cleavage of a smaller piece of matrix off a larger parent piece (Fig. 1, frame 1200). In other cases, the matrix seemed to be lost by a "melting-away" process similar to that described for osteoclasts in vitro (*9*). The latter phenomenon sometimes resulted in the formation of "resorption bays" (Howship's lacunae) (Fig. 1, frame 3700).

The electron microscopy and histochemistry were also consistent with a mechanism of resorption which operates best when the cells are in intimate con-

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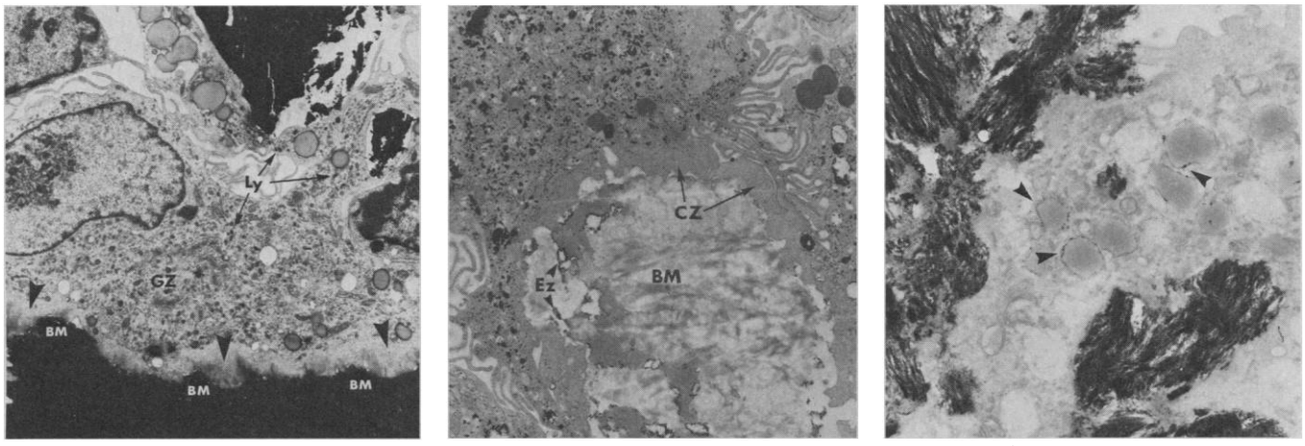

Fig. 2 (left). Nondecalcified thin section showing the formation of a distinct clear zone (arrowheads) in the interface between a monocyte and the bone matrix (*BM*). The cytoplasm also shows a well-developed Golgi zone (*GZ*) and numerous dense-core lysosomes (*Ly*). The sample was collected after 6 days of incubation. Stained with uranyl acetate and lead citrate (× 3900). Fig. 3 (center). Electron micrograph showing the attachment of two monocytes to a piece of bone matrix (*BM*). Notice the clear zones (*CZ*) at sites where the cells contact the substrate; also notice the accumulation of electron-opaque acid phosphatase reaction product (*Ez*) on the matrix surface. The matrix was demineralized in EDTA before the histochemical reaction; the section is unstained (× 6290). Fig. 4 (right). Unstained, nondemineralized section of monocyte resorbing bone matrix. The arrowheads indicate the intracellular accumulation of crystalline, electron-opaque material around the lipid vesicles (× 8600).

tact with the matrix. For example, the monocytes, which often become multinucleated in culture, form extensive "clear zones" at points where the cells are juxtaposed to the matrix (Fig. 2). These zones, which are characteristic of osteoclasts, are specializations of peripheral cytoplasm that have been associated with the development of cell-to-cell and cell-to-substrate adhesions (*10*). In osteoclasts, the fine particles or threads of material which accumulate in clear zones contain an actinlike protein (*11*).

The cultured monocytes also show polarization of organelles with respect to the bone matrix which is suggestive of a site-specific concentration of cellular activity. This impression is strongly supported by acid phosphatase staining, which shows this lysosomal enzyme concentrated in the portions of the cell that contact the matrix surface as well as on the matrix itself (Fig. 3). The latter observation is also indicative of lysosomal enzyme release. Last, and again suggestive of the importance of cell-matrix contact, some of the adherent cells also showed an intracellular, circumvesicular accumulation of crystalline, electron-opaque material similar to that observed in the adjacent bone (Fig. 4). A comparable intracellular localization of acid phosphatase activity was also occasionally observed.

These results show that human monocytes are capable of resorbing mature bone. In contrast to the study of Mundy *et al.* (*5*), which stressed the ability of the cells to act at a distance in promoting fetal bone degradation, our study suggests a strong contact-related component of monocyte-mediated resorption. In fact, it is the ability of the monocyte to adhere to and resorb bone at specific sites that is most reminiscent of the osteoclast. Although the cultured monocytes possess, or acquire in culture, a number of the morphological characteristics of osteoclasts, we have not yet observed well-developed ruffled membranes. As this morphological feature is the anatomic sine qua non of the functioning osteoclast (*2, 10, 12*), its identification is requisite to the conclusion that the monocyte is capable of osteoclastic transformation.

Table 1. Release of ^{45}Ca from prelabeled devitalized adult rat bone matrix (A) and freeze-thawed, 21-day fetal rat calvaria (B). Samples were collected for counting after 7 days of culture in (A) and after 6 days of incubation in (B). The results are expressed as the ratio of isotope released in the presence of monocytes to that released in their absence. The data in (A) are derived from 12 replicate cultures at each cell concentration. Those in (B) are taken from 20 paired cultures (with and without cells) in which the substrate in each pair was a half calvarium taken from the same fetal animal. Statistical significance was determined by using Student's *t*-test, and comparing the percentages of isotope released from bone cultured with and without monocytes; N.S., not significant.

Cells per culture	Isotope release ratio	Statistical significance
	(A) Adult rat bone	
1.3×10^7	1.56	N.S.
6.5×10^6	2.28	$P < .025$
3.3×10^6	2.07	$P < .001$
1.6×10^6	2.57	$P < .001$
8×10^5	1.61	$P < .025$
4×10^5	1.89	$P < .025$
2×10^5	1.50	N.S.
	(B) Fetal rat calvaria	
1×10^6	1.62	$P < .025$
2.5×10^5	1.53	$P < .01$

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Cyclic Adenosine Monophosphate Production by Embryonic Chick Cells

Abstract. *Cells dissociated from 1-day-old chick embryos produce a pulse of cyclic adenosine monophosphate (cyclic AMP) when stimulated with cyclic AMP. There is a stimulus threshold concentration of about 10^{-8} molar cyclic AMP and an upper limit, above which the response is suppressed, of about 6×10^{-6} molar. The response occurs within 5 seconds of stimulation and corresponds to an average pulse size in the range of 10^7 molecules per cell.*

A microelectrode releasing small amounts of cyclic adenosine monophosphate (cyclic AMP) continuously can divert the axis of a 1-day [Hamburger and Hamilton (*1*) stages 4 and 5] chick embryo (*2*). Cells dissociated from such an embryo agglutinate in suspension; the agglutination is enhanced by added cyclic AMP and its dynamics have been interpreted as implying that it is controlled by a propagated cyclic AMP signal (*3*). It therefore seemed important to establish whether cells dissociated from the chick embryo do, indeed, produce cyclic AMP in response to stimulation by cyclic AMP. We report here the results of direct assays performed to settle this point.

Fertile White Leghorn eggs were incubated for 1 day at 38°C. The embryos, at Hamburger and Hamilton stages 4 and 5, were removed and dissociated into single cells by agitation in $10^{-4}M$ EDTA in chick Ringer solution (*3, 4*). The cells so obtained have been described previously (*4*). The cells were incubated, in suspension in Ringer solution, at 38°C for 1 hour, having been adjusted to the required cell density after a sample was counted in a hemocytometer. Stimulation with cyclic AMP was accomplished, at 38°C, by adding 0.2 ml of cell suspension, with an Eppendorf pipette, to 1.8 ml of cyclic AMP solution in Ringer solution. The mixture was vortexed to ensure complete mixing and samples (500 μl) were transferred to test tubes, which had been preheated in boiling water to inhibit further cellular responses. The cells were removed by centrifugation at 4500*g* for 15 minutes since we had found that their nonspecific cyclic AMP binding capacity interfered with the subsequent assay. Samples were taken at times up to 40 seconds after stimulation; each experiment was performed in triplicate. For high stimulus concentrations the samples were diluted with Ringer solution to bring the added cyclic AMP concentration to $10^{-7}M$, within the preferred range for assay. The assay used depended on the capacity of human erythrocyte ghosts to bind cyclic AMP with high specificity. Ghosts were prepared by the method of Fairbanks *et al.* (*5*) and the assay was performed as described by Hesse *et al.* (*6*). The technique is essentially that of isotope dilution wherein tritiated cyclic AMP, bound to the ghosts, is displaced by cyclic AMP in the sample to be assayed, and the amount displaced is compared with amounts on a calibration curve for the batch of ghosts used. The input cyclic AMP concentration is subtracted from the total found, giving the amount produced by the stimulated cells.

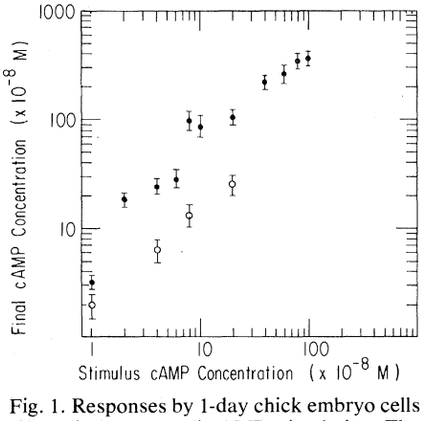

Fig. 1. Responses by 1-day chick embryo cells (filled circles) to cyclic AMP stimulation. The cell density was 7.7×10^6 per milliliter. Samples, in triplicate for each point, were collected 40 seconds after stimulation. A cyclic AMP phosphodiesterase (PDE) inhibitor, IBMX (3-isobutyl-1-methyl xanthine), was present at a concentration of $10^{-5}M$. Diminished responses were found after incubation for 40 seconds without IBMX, suggesting the presence of an extracellular PDE (*3*). Open circles show responses at room temperature.

Figure 1 shows the results of a typical experiment. Cyclic AMP production was caused by stimulus concentrations over a range of 10^{-8} to $10^{-6}M$. A control experiment, in which samples of cells were added to Ringer solution only, showed no cyclic AMP release; the graph shows a much reduced production by cells at room temperature (22° to 23°C). The amount of cyclic AMP produced per cell may be readily calculated; it reached a maximum of 2×10^8 molecules for a stimulus concentration of $10^{-6}M$. For a stimulus of $8 \times 10^{-8}M$, which produced the greatest input/output ratio, the response corresponded to 6×10^7 molecules per cell. In further experiments we found that no response could be detected, with our assay, for stimulus concentrations above $6 \times 10^{-6}M$, and that all the cyclic AMP was released within 5 seconds of stimulation, the shortest time we could employ reliably. No responses were found to dibutyryl cyclic AMP over a concentration range of 10^{-8} to $10^{-5}M$.

These results show that cells from the early chick embryo can produce cyclic AMP very rapidly, when stimulated with cyclic AMP, and thus provide further evidence for a role of cyclic AMP in intercellular communication during development. While the results are consistent with rapid extracellular release of cyclic AMP, this must be established by further experiment. Nonetheless, our results show that there are interesting parallels with signaling in the cellular slime mold *Dictyostelium discoideum* (*7*) and suggest that responsiveness to low concentrations of extracellular cyclic AMP might be a usually unnoticed but nonetheless widespread phenomenon in metazoan cells and tissues. Indeed, Whitfield *et al.* (*8*) came to a similar conclusion when examining the effects of cyclic AMP concentrations between 10^{-8} and $10^{-6}M$ on mitogenesis in rat thymic lymphoblasts.

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