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Macrophage-derived growth factor for osteoblast-like cells and chondrocytes

(bone cells/coupling factor/bone formation)

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ABSTRACT **Rat resident peritoneal macrophages in primary culture were found to elaborate a mitogenic factor (or factors) for rat osteoblast-like cells and chondrocytes but not for skin fibroblasts. Growth-promoting activity appeared in the incubation medium within the first 20 hr of macrophage culture and was released in amounts that paralleled the number of macrophages per culture. After their proliferative response, as judged by increases in DNA synthesis and cell number, the osteoblast-like cells became enriched in alkaline phosphatase, an index of osteoblast specialization. The macrophage-derived activity was nondialyzable and heat-stable, and it was eliminated by exposure to trypsin. Inhibition of prostaglandin cyclooxygenase failed to modify its generation. Partial purification (Amicon filter concentration, gel filtration) disclosed principal peaks of activity corresponding to M_r of 43,000 and 10,000. The crude conditioned medium and the M_r 43,000-peak, but not the low-molecular-weight peak, exhibited interleukin 1 activity, as judged by the ability to stimulate the proliferation of mouse thymic lymphocytes. The macrophage-derived growth factor described herein may participate in bone remodeling and repair and in primary bone and cartilage growth.**

The mammalian skeleton is a dynamic tissue, undergoing virtually continuous turnover after its initial development (1). Skeletal turnover stems from a complex remodeling process of microscopic dimensions in which osteoclasts resorb bone mineral and organic matrix and osteoblasts proceed to repair the resorption cavity (2, 3). Resorptive and formative events at each remodeling locus are congruent in space, sequentially coupled in time, and balanced in quantity, so that the mass of bone does not change, at least not in the short term. This spatial, temporal, and quantitative coordination points to the participation of local signals in the control of remodeling, and it has prompted a search for locally elaborated regulatory factors. Cells of the monocyte macrophage series may well generate such factors. Not only are they thought to be the precursors of mature osteoclasts (4–12), they are known to release agents that promote bone resorption both directly and indirectly (13). Furthermore, macrophage-like cells occupy resorption cavities during the time interval between resorption and formation, so that they are appropriately positioned to influence formative events (14, 15). We have examined the possibility that macrophages elaborate a factor (or factors) that promote(s) the proliferation of osteoblasts, thereby acting to couple formation and resorption. We describe herein a heat-stable peptide activity in macrophage-conditioned medium (MCM), which promotes the growth of osteoblasts and chondroblasts *in vitro*.

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MATERIALS AND METHODS

Isolation and Culture of Rat Resident Peritoneal Macrophages. Resident peritoneal macrophages were isolated from fluid obtained by peritoneal lavage of male Sprague–Dawley rats (150–200 g). The rats were killed by cervical dislocation and the peritoneum was lavaged with a previously described chemically defined medium (CDM) (16, 17) supplemented with 5 units of heparin sodium per ml. The peritoneal cells were collected from the lavage solution by centrifugation at 200 × *g* for 10 min at 4°C and subsequently washed 3 times with 40 vol of CDM. To prepare MCM, 3×10^4 to 6×10^5 cells/cm^2 in CDM were placed in 60-mm-diameter plastic tissue culture dishes. After incubation at 37°C for 1 hr, the cultures were washed twice with CDM to remove nonadherent cells and then incubated with 6 ml of either CDM containing 10 μg of transferrin per ml (modified CDM) or 6 ml of CDM containing 2% fetal bovine platelet-poor plasma-derived serum (PDS) at 37°C in humidified 4% CO_2/96% air. [PDS was obtained from Sterile Systems (Logan, UT) and prepared as described; see ref. 18.] In some instances, macrophages were cultured in the presence of 5 μg of indomethacin per ml and control and treated cultures were assayed for prostaglandin E_2 by the method of Reingold *et al.* (19). The lower limit of detection in this assay was 200 pg/ml. The conditioned medium was collected after 19 hr of incubation, centrifuged at 1,000 × *g* for 20 min, and frozen until assayed.

Greater than 90% of the cells exhibited a positive reaction for nonspecific esterase activity (α-naphthyl acetate substrate) (20) and a similarly high proportion of the cells were capable of phagocytizing opsonized zymosan particles.

Preparation of Fetal Rat Calvarial Osteoblast-like Cells and Chondrocytes, and Fetal Rat Skin Fibroblasts. Primary cultures enriched in osteoblast-like cells or chondrocytes were prepared from the calvaria of rat fetuses (day 20–21) as described (16, 17) with some modifications. Osteoblast-like cells were released from the frontal bones and chondrocytes were released from the chondrocyte-enriched portions of the interparietal bones by incubation for 60 min in modified CDM containing 2 mg of crude collagenase per ml (70% type I, 30% type II; Sigma) (140–160 units/mg) at pH 7.5. The released cells were grown in 24-well tissue culture plates (Costar, Cambridge, MA) (5×10^4 cells per well) in CDM containing 5% fetal bovine serum (Sterile Systems, Logan, UT) at 37°C, in humidified 4% CO_2/96% air.

Osteoblast-like cells were also incubated at 2×10^5 cells per 35-mm plate in CDM unsupplemented or in a mixture (1:3) of serum-free MCM and CDM. On days 1, 5, and 12, the cells were stained histochemically to detect alkaline

Abbreviations: CDM, chemically defined medium; PDS, platelet-poor plasma-derived serum; IL-1, interleukin 1 (lymphocyte-activating factor); MDGF, macrophage-derived growth factor; MCM, macrophage-conditioned medium.

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phosphatase activity (Sigma kit 85-L). Sister cultures were scraped into distilled water, sonicated, and chemically analyzed for alkaline phosphatase according to the method of Lowry *et al.* (21).

Fetal rat skin fibroblasts were isolated and grown in the same manner as described for the calvarial cells. Fibroblasts were isolated by collagenase digestion from the skin of the same fetuses used to obtain the other cell types, and they were cultured under the same conditions.

Assay for DNA Synthesis and Proliferation. MCM was assayed for DNA synthesis-promoting activity in primary osteoblast-like cells, chondrocytes, and skin fibroblast cultures. The cells, seeded into 24-well tissue culture plates as described above, were maintained in culture for 5 days without a medium change, by which time they had grown to confluence. The cultures were washed twice with modified CDM and incubated for an additional 24 hr in 1 ml of modified CDM. After this serum-free period, 20–200 μl of test material was added to each culture well or the medium was removed from each well and replaced with test material that had been serially diluted in the same medium. After 19–20 hr, the cells were pulsed for 2 hr with 1 μCi of [^{3}H]thymidine (specific activity, 20 Ci/mmol; 1 Ci = 37 GBq), then washed twice with ice-cold 10% (wt/vol) trichloroacetic acid and once with ethanol/ether (3:1) (vol/vol). After the cell layer had dried, the trichloroacetic acid-insoluble material was dissolved in 0.2 M NaOH, and its total radioactivity was estimated by liquid scintillation spectrometry.

To determine the effects of MCM on proliferation, bone and cartilage cells were cultured for 24 hr in 35-mm plastic tissue culture dishes (2×10^5 cells per dish) in CDM supplemented with 0.6% PDS, which was found in separate experiments not to affect the seeding efficiency. Eighteen hours after culture initiation, the medium was removed and replaced with one of the following: control medium (CDM supplemented with 0.6% PDS), test medium (a 1:3 dilution of conditioned medium derived from macrophages cultured in 2% PDS-enriched CDM), or CDM supplemented with 5% fetal bovine serum (positive control). The number of cells per replicate culture was estimated every other day by enzymatic dispersion (0.05% trypsin and 0.02% EDTA) and counting in a hemocytometer.

Interleukin 1 (IL-1; Lymphocyte-Activating Factor) Assay. IL-1 activity of serum-free conditioned medium and column fractions was assayed as described by Lachman (22). IL-1 standards included that derived from the J774A.1 cell line, as described by Lachman *et al.* (23), and purified IL-1 (Genzyme, Boston, MA).

Partial Purification of Macrophage-Derived Growth Factor (MDGF). Macrophages were cultured at 6×10^6 cells per 60-mm tissue culture dish in 6 ml of modified CDM. Supernatants were collected after a 19-hr incubation period and centrifuged at $1000 \times g$ for 20 min to remove any cellular debris. The conditioned medium (250 ml) was then concentrated 25-fold using an amicon YM5 ultrafiltration membrane, dialyzed against deionized distilled water, and lyophilized. The lyophilized material was reconstituted to 0.5 ml with phosphate-buffered saline (pH 7.2) and applied to either a 0.9 × 30 cm Sephadex G-75 or Sephacryl S-200 column equilibrated with phosphate-buffered saline (pH 7.2) containing 50 μg of gentamycin per ml. Elution was carried out with the same buffer at a flow rate of 3.2–5.2 ml/hr and fractions of 0.4–0.5 ml were collected. Aliquots of each fraction were assayed for osteoblast mitogenic activity (20 μl) and thymic lymphocyte proliferation activity (10 μl) (IL-1).

RESULTS

Mitogenic Activity of MCM. Incubation medium exposed to resident peritoneal macrophages (MCM) stimulated DNA synthesis markedly in cultured osteoblast-like cells and chondrocytes when compared to control medium, but failed to modify DNA synthesis in cultured skin fibroblasts (Table 1). Additionally, epidermal growth factor (50 ng/ml) and fibroblast growth factor (20 ng/ml) stimulated osteoblast, chondrocyte, and fibroblast DNA synthesis to a similar degree above background (data not shown). Macrophages incubated in serum-free or PDS-containing medium elaborated an equivalent amount of stimulatory activity. The conditioning process was complete within 20 hr; medium exposed to macrophages for an additional 24–48 hr exhibited no further mitogenic activity (data not shown). MCM was effective at a dilution of 1:6, which represents <200 ng of total macrophage-derived protein, and that dilution was used to estimate the relationship between macrophage density per culture and growth factor activity (Fig. 1). The mitogenic activity of MCM was nondialyzable, was markedly decreased by prior trypsinization but not by simultaneous treatment with trypsin and soybean trypsin inhibitor, and was not altered by heating at 56°C for 30 min.

Osteoblast-like Cell and Chondrocyte Growth and Differentiation in Response to MCM. MCM was assayed for its ability to enhance the actual multiplication of osteoblast-like cells and chondrocytes. Exposure of each cell type to MCM caused a substantial increase in cell number within 2 days. Whereas the use of control medium was associated with a 32% increase in the number of osteoblast-like cells and an 18% increase in chondrocytes, MCM supported increases of 106% and 69%, respectively. By the seventh day in culture, the number of osteoblast-like cells had increased by 165% and chondrocytes by 68% in control media; the increments in cell number were 356% and 314%, respectively, in the presence of MCM (Fig. 2).

To determine whether MCM promotes the emergence of differentiated cells, we tested its effect on alkaline phosphatase activity in the osteoblast-like culture system. For this purpose, alkaline phosphatase activity was used as an osteoblast marker. Histochemical studies disclosed intense alkaline phosphatase activity in the vast majority of cells after 12 days in the presence or absence of MCM. MCM increased the absolute number of alkaline phosphatase-containing cells. Enzymatic analysis disclosed that incubation in MCM increased the total amount of alkaline phosphatase activity per culture (control = 102 nmol/hr per culture; MCM-treated = 181 nmol/hr per culture), with little change in specific activity (control = 59 nmol/hr per 10^5 cells; treated = 48 nmol/hr per 10^5 cells). Hence, as judged by enrichment in alkaline phosphatase, the cells stimulated to proliferate were osteoblast-like in nature.

Evidence That MDGF Is Not a Prostaglandin and That Prostaglandin Production Does Not Mediate Its Action on Osteoblast-like Cells. Since macrophages produce prostaglandins (24), we examined the possibility that these substances might be responsible for the growth-promoting activity of

Table 1. Enhancement of [^{3}H]thymidine incorporation in bone and cartilage cells with MCM

Cell type	[^{3}H]Thymidine incorporation, % of control		
	Exp. 1	Exp. 2	Exp. 3
Osteoblast	569 ± 19	528 ± 25	428 ± 16
Chondrocyte	413 ± 24	350 ± 28	251 ± 21
Fibroblast	108 ± 38	69 ± 9	ND

Aliquots of MCM diluted 1:3 were tested under serum-free conditions in triplicate on the cell types shown above, grown in 24-well tissue culture plates; 5% fetal calf serum was used as a positive control (5×10^5 cpm per well); background was ≈10% of the serum stimulation. Data are expressed as the mean ± SEM. ND, not determined.

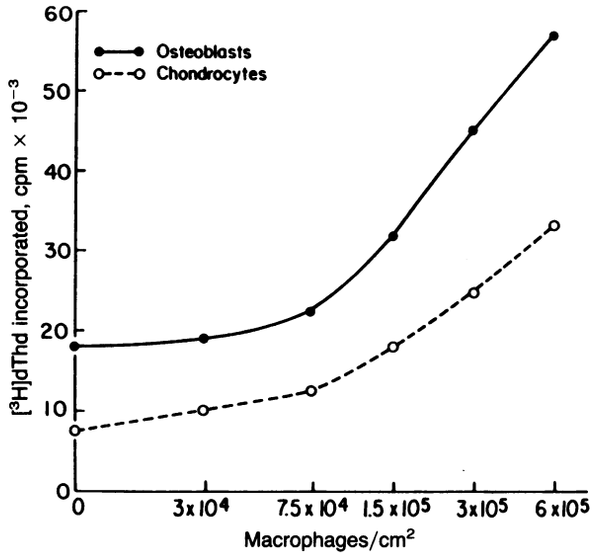

FIG. 1. Effect of macrophage density on the DNA synthesis-promoting activity of MCM. Macrophages were cultured at the indicated densities in 60-mm tissue culture plates (6 ml of medium) for 19 hr. The medium was then collected from each culture and assayed at a 1:6 dilution for DNA synthesis-promoting activity on osteoblasts (●) and chondrocytes (○). Osteoblasts and chondrocytes were seeded at 5×10^4 cells per 16-mm-diameter well.

MCM. Conditioned medium was collected from macrophages cultured in the presence of indomethacin, a potent cyclooxygenase inhibitor, and assayed as described. Indomethacin, at a concentration sufficient to exhibit prostaglandin release by the cultured macrophages (control, 280 pg/ml; indomethacin-treated, undetectable), did not modify the growth-promoting activity of MCM.

The identification of prostaglandins in cultures of osteoblasts during a logarithmic phase of growth (25) suggested the possibility that the MDGF might act indirectly by stimulating the release of mitogenic prostanoids. To test this hypothesis, we treated bone cells with indomethacin 24 hr prior to their exposure to MCM and supplemented the MCM with fresh indomethacin prior to assay. Indomethacin did not alter the mitogenic response of the bone cells to MCM (data not shown). Additionally, bone cells produced no detectable prostaglandin E_2 either under serum-free conditions alone or when supplemented with serum-free MCM (data not shown).

Partial Purification of MDGF. When the concentrated serum-free MCM was gel filtered on Sephadex G-75, two major and two minor peaks of activity were observed. The major peaks eluted at apparent M_rs of 43,000 and 10,000, while the two minor peaks eluted at apparent M_rs of ≥68,000 and 18,000, respectively (Figs. 3 and 4). The M_r 43,000 and the M_r 10,000 peaks markedly stimulated DNA synthesis in osteoblast-like cells (Figs. 3 and 4).

Analysis of MCM for IL-1 Activity. Macrophages from many sources have been reported to elaborate a factor(s) that stimulates the proliferation of thymic lymphocytes (IL-1). IL-1, or a closely related macrophage-derived factor, has also been shown to promote the growth of fibroblasts. We therefore explored the possibility that IL-1 activity might be present in the fractions that stimulated the growth of osteoblast-like cells. MCM and fractions from the gel-filtration columns were assayed for thymocyte proliferation activity (IL-1) according to the method of Lachman *et al.* (22). The crude MCM was found to contain IL-1 activity. Analysis of the major gel filtration fractions that stimulated osteoblast growth indicated that IL-1 activity coeluted with the M_r 43,000 activity but was not detectable in the M_r 10,000 activity (Fig. 4).

DISCUSSION

Our experimental results indicate that macrophages elaborate a factor (or factors) that stimulates the growth of chondroblasts and osteoblast-like cells cultured from rat cranial tissues. This factor, which was released from attached mac-

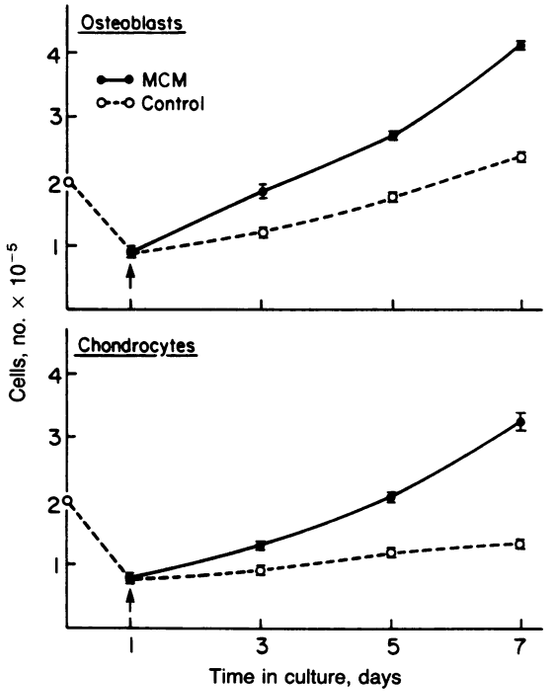

FIG. 2. Stimulation of osteoblast (*Upper*) and chondrocyte (*Lower*) growth by MCM. Cells were seeded on day 0, and MCM (●) or control medium (○) was added on day 1. On each day, replicate cultures were counted.

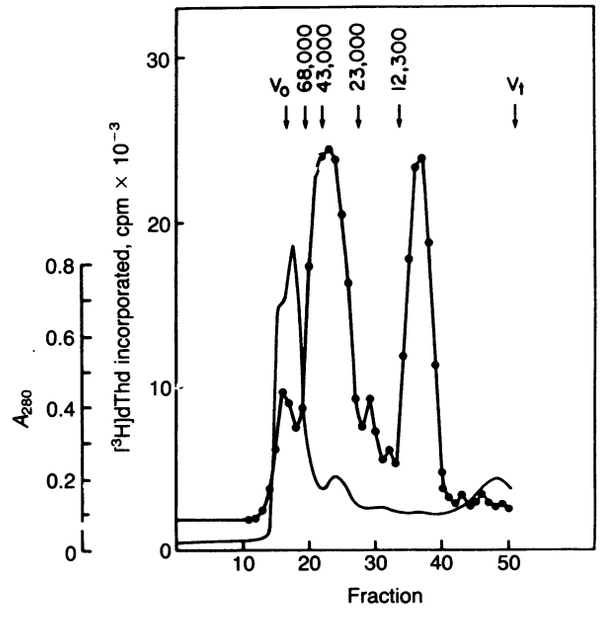

FIG. 3. Sephadex G-75 gel filtration of the macrophage-derived growth activities. Concentrated serum-free MCM was applied to the column and eluted with phosphate-buffered saline (pH 7.2) at a flow rate of 3.2 ml/hr. Fractions (0.4 ml) were collected and 25-μl aliquots of each were assayed for osteoblast DNA synthesis-promoting activity. Osteoblasts were seeded at 5×10^4 cells per 16-mm-diameter well. ●, [^{3}H]dThd incorporated; ——, A_{280}.

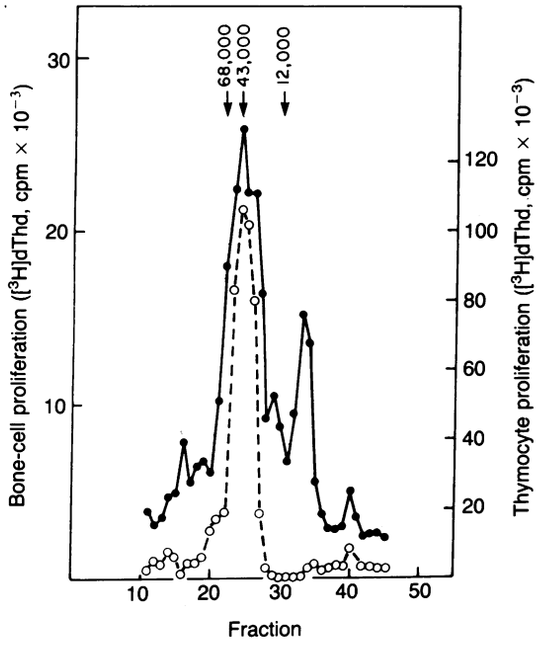

FIG. 4. Sephacryl S-200 gel filtration of the macrophage-derived factors. Concentrated MCM was applied to the column and eluted with phosphate-buffered saline (pH 7.2) at a flow rate of 5.2 ml/hr. Fractions (0.5 ml) were collected, 20-μl aliquots were assayed for osteoblast DNA synthesis-promoting activity (●), and 10-μl aliquots were assayed for thymocyte-proliferation activity (○).

rophages within the first 20 hr of culture, is a true mitogen in that it enhanced cell proliferation as well as DNA synthesis. Furthermore, the factor is a macrophage product rather than a modified macromolecular constituent of serum, because it appeared in a chemically defined medium that was devoid of exogenous protein, except for transferrin. In separate experiments (data not shown), we have determined that transferrin does not stimulate growth of isolated cultured osteoblast-like cells and chondrocytes. We considered the possibility that the observed growth-promoting activity might be a prostaglandin, because macrophages are known to elaborate prostanoids (24), and prostaglandins may be capable of stimulating bone cell growth (25). Virtual blockade of macrophage prostaglandin synthesis with indomethacin, as judged by the absence of measurable prostaglandins in the conditioned medium, did not decrease the mitogenic activity of that medium. Furthermore, activity was nondialyzable, trypsin-inhibitable, and eluted from gel columns in association with several macromolecular fractions. Gel filtration studies revealed multiple peaks of growth factor activity; however, the bulk of biological activity appeared in two peaks, corresponding to M_rs of ≈43,000 and 10,000. Although the significance of these peaks is unknown, the higher-molecular-weight peak may represent an aggregate of smaller peptides with biological activity. Separation by gel filtration permitted us to explore the relationship between MDGF and other macrophage products of biological interest, including IL-1 (22, 23, 26–30), which stimulates the production of the lymphocyte mitogen interleukin-2 by thymus lymphocytes (31–34), and fibroblast growth-promoting activity (35–37).

We cannot exclude the possibility that bone cell growth-stimulating activity is, in part, identical or closely similar to IL-1. IL-1 activity was consistently found in MCM and in the larger of the principal fractions obtained from MCM by gel filtration. By contrast, assays carried out on the dominant low-molecular-weight peak indicate that macrophages are in fact capable of elaborating a bone cell mitogen devoid of IL-1 activity. Low as well as high-molecular-weight forms of IL-1 have been identified, but only the latter appears during the incubation of certain macrophage cell lines in the absence of serum (38). Hence, the serum-free conditions used in the present experiments may have prevented the release of low-molecular-weight IL-1 but not of the low-molecular-weight bone-cell growth stimulator.

Recently, cells of the monocyte–macrophage series have been shown to release peptides that are mitogenic for fibroblasts, smooth muscle cells, and endothelial cells (18, 34, 39–43) and that may or may not have IL-1 activity (33). Although high- and low-molecular-weight fibroblast growth stimulators have been identified (35), both of these species are larger than the respective high- and low-molecular-weight fractions that promote osteoblast growth. Furthermore, MCM failed to stimulate the proliferation of skin fibroblasts in our studies. There may be bone fibroblasts that differ from skin fibroblasts in their responses to growth factors but that are not osteoblast precursors.

Although the bone-cell population used in our studies has been termed osteoblast-like, because of the possibility that it contains nonosteoblast elements, a large body of evidence indicates that osteoblasts or their immediate precursors are the predominant if not the exclusive cell type in this system. Moreover, we employed a widely used osteoblast marker, alkaline phosphatase, in determining whether the osteoblast was the MDGF-responsive cell. The finding that MDGF-mediated stimulation of growth was accompanied by a substantial increase in alkaline phosphatase activity per culture and by a sharp increase in the number of alkaline phosphatase-containing cells suggests that the osteoblast (or its precursor) is the responsive element in the culture system. A high growth rate in the absence of external stimuli has thus far prevented the demonstration of MDGF activity in osteoblast-like clones of osteogenic sarcoma cells (44).

Macrophage-derived peptides that are mitogenic for osteoblasts and chondrocytes may serve as regulators of skeletal repair, bone growth, and bone formation during remodeling. Macrophages accumulating in response to injury may sustain fracture healing by releasing such factors. Recently, Gowen *et al.* have shown that macrophage-derived peptides stimulate bone resorption *in vitro* (45, 46). Hence, the purposive participation of macrophages in the remodeling cycle (1–15) may include the coordinated stimulation of formation and resorption.

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