Isolation of a murine osteoclast colony-stimulating factor
(growth factor/bone resorption/colony assay/mammary tumor)

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ABSTRACT

Cultures of a cell line derived from a murine mammary carcinoma that induces hypercalcemia were examined for soluble products that could induce osteoclasts to differentiate from murine bone marrow cells. The serum-free culture supernatant of this cell line stimulated growth of colonies from bone marrow cells that exhibited tartrate-resistant acid phosphatase activity. These TRAPase-positive colonies demonstrated essential features of osteoclasts when cocultured with mineralized bone or dentin. The culture period required for colony development and the frequency of colony-forming cells indicated that relatively primitive marrow progenitors were stimulated by a tumor-derived factor(s) to form immature osteoclasts. Other colony-stimulating factors (CSFs), including granulocyte CSF, macrophage CSF, granulocyte-macrophage CSF and interleukin 3, were ruled out as the source of the activity produced by the tumor cells. The biological activity was successfully purified by gel filtration chromatography and reverse-phase HPLC. By SDS/PAGE, the activity was traced to a protein of $\approx 17$ kDa. Functional and biochemical studies of the purified factor suggest that it is distinct from any known CSF of myeloid cells. This protein appears to be a CSF for the osteoclast lineage, osteoclast CSF (O-CSF).

Although it has been clearly established that osteoclasts are derived from a hemopoietic stem-cell population (1–3), the identification of osteoclasts in their early stages of development has been difficult, and little is known about their precise numbers or growth requirements. In contrast to colony-stimulating factors (CSFs) of hemopoietic cells, there is no known specific growth factor that stimulates the proliferation and differentiation of progenitors of the osteoclast lineage. To define the regulation of osteoclast generation, we examined the cell-culture supernatant of a well-characterized murine mammary tumor that causes excessive bone resorption in mice (4–7). A protein factor was isolated that stimulates the clonal growth of osteoclast progenitors in semisolid cultures of murine bone marrow cells in vitro.

MATERIALS AND METHODS

Tumor Cell Cultures. Cloned cell lines of a hypercalcemia-and granulocytosis-inducing murine mammary adenocarcinoma designated CESJ (6, 7) were used. Each CESJ clone produces bone-modulating activity in addition to granulocyte CSF (G-CSF) and macrophage CSF (M-CSF) (6, 7). Two other murine tumors were used as controls: (i) a mammary carcinoma clone (Bc66), which does not induce neutrophilia or hypercalcemia in mice (8) and produces M-CSF but not G-CSF (7), and (ii) a murine fibrosarcoma clone (NFSA-c9; provided by M. Shikita, National Institute of Radiological Sciences, Chiba, Japan), which causes neutrophilia (9) but not hypercalcemia (M.Y.L., unpublished data) and is a known source of murine G-CSF and M-CSF (7). All tumor cells were cultured and maintained in serum-free, protein-defined HL-1 medium (Ventrex, Bio Ventures Groups, Portland, ME), supplemented with 2 mM L-glutamine and 50 units of penicillin, 50 $\mu$g of streptomycin, and 0.125 $\mu$g of amphotericin B (GIBCO) per ml.

Tumor Cell-Conditioned Medium. The supernatant of culture medium in which tumor cells had been cultured for 7–8 days was concentrated $\approx 500$-fold by ultrafiltration (Amicon YM10 membranes, Amicon) and filtered (Milllex GV, 0.2 $\mu$m, Millipore). For the purification of the growth factor, CESJ cells were cultured in 5 liters of HL-1 medium in a stirred flask. Cells in the active growth phase were collected; washed twice; suspended in 3 liters of serum-free medium 199 (Whittaker Bioproducts) supplemented with 40 $\mu$g of L-asparagine per ml, 10 $\mu$M sodium pyruvate (GIBCO), and 50 units of penicillin, 50 $\mu$g of streptomycin, and 0.125 $\mu$g of amphotericin B per ml; and cultured for 3 days to obtain tumor cell-conditioned medium free of exogenous protein.

Bone Marrow Progenitor Analysis. Bone marrow cells of young adult (BALB/c × C3H)F1 mice were cultured in 35 × 10 mm Petri dishes or in 15 × 10 mm Linbro wells (Flow Laboratories) at 10$^5$ cells per ml in supplemented medium 199 containing 20% (vol/vol) fetal calf serum (HyClone), 0.3% Bacto agar (Difco), and various concentrations of added tumor cell-conditioned medium or other test samples (10). Cultures were incubated at 37°C in a humidified atmosphere with 5% CO$_2$ for 7–21 days. Culture of spleen cells was similar to that of bone marrow cells but with 5 × 10$^5$ cells per ml. Colonies derived from putative osteoclast progenitors were identified by staining for tartrate-resistant acid phosphatase (TRAPase) activity. After the agar gel was transferred from the culture dish onto a glass slide and fixed (10), slides were stained for TRAPase activity by using hexazotized pararosaniline as a coupling dye and then counter-stained (11). Mononuclear cells containing TRAPase activity were distinctively stained bright red. The colonies were examined under an inverted microscope and assigned to three categories based on their percentage of red (TRAPase-positive) cells: positive (>90%), mixed (10–90%), and negative (<10%). Colonies were defined as groups of 50 or more cells, and clusters were defined as groups of >8 but <50 cells. All colonies and clusters in the agar plate were scored, and the results were expressed as colony or cluster numbers per 10$^3$ bone marrow or per 5 × 10$^5$ spleen cells.

Cocultivation of Bone Marrow Cells with Devitalized Bone Pieces. Bone marrow colonies were grown in the presence of an optimal concentration of tumor cell-conditioned medium as described above but with 0.8% methylcellulose instead of agar. On day 14 of the culture, colonies were collected and gently dispersed, and the cells were cocultured with devitalized:

Abbreviations: CSF, colony-stimulating factor; O-CSF, osteoclast CSF; G-CSF, granulocyte CSF; M-CSF, macrophage CSF; G-M-CSF, granulocyte–macrophage CSF; IL-3 and IL-1, interleukins 3 and 1; TGF-$\beta$, transforming growth factor $\beta$; TRAPase, tartrate-resistant acid phosphatase.

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ized mouse calvaria at $2 \times 10^5$ cells per calvarium for 48 hr in 1.0 ml of medium 199 containing 20% fetal calf serum and the optimal concentration of tumor cell-conditioned medium. Calvaria were fixed in formalin and decalcified, and 4-μm thick sections were stained for TRAPase activity.

**Demonstration of Resorption Lacunae.** Sterilized dentin slices ($8 \times 8 \times 0.1$ mm) were prepared from cow teeth and placed at the bottom of Linbro wells. Bone marrow cells ($5 \times 10^4$) were cultured over the dentin slices in 0.5 ml of medium containing 20% fetal calf serum, the optimal concentrations of tumor cell-conditioned medium, and 0.3% agar. In some instances, $10^4$ marrow cells were cultured in 0.5 ml of liquid culture medium lacking agar but containing the other ingredients as above. After 14–28 days of incubation at $37^\circ$C in 5% CO$_2$/95% air, colonies that developed in the agar or on the dentin were stained for TRAPase, and the location of positive and negative colonies in relation to the dentin slice was recorded. The dentin pieces were subsequently immersed in distilled water or in 50% (vol/vol) sodium hypochlorite for 30 min, washed, and dehydrated in ethanol (12). The specimen was sputter-coated with gold/palladium and viewed in an ETEC scanning electron microscope.

**Growth Factors and Antibodies.** Recombinant growth factors were from the following sources: murine G-CSF (S. Nagata, Osaka Bioscience Institute, Osaka); murine granulocyte-macrophage CSF (GM-CSF; Genzyme); murine interleukin 3 (IL-3; Bingen); human interleukin 1 (IL-1; Hausserman-LaRoche); murine IL-1 (Genzyme); and transforming growth factor β1 (TGF-β1) (Oncogen). Purified murine M-CSF (R. Shadduck, Montefiore Hospital, Pittsburgh), or L cell-conditioned medium was used as a source for murine M-CSF. Biological activities of these CSFs were tested by standard colony assays of bone marrow cells (10). G-CSF activity was also assessed by proliferation of factor-dependent NFS-60 cells (7). Rabbit anti-serum raised against murine M-CSF was a kind gift from R. Shadduck. Anti-murine GM-CSF serum (13) and rat anti-mouse IL-3 monoclonal antibody (14) were gifts from D. Mochizuki (Immunex, Seattle) and J. Abrams (DNAX), respectively.

**Gel Filtration Column Chromatography.** A column (2.6 x 100 cm) of Sephacryl HR S-200 (Pharmacia Fine Chemicals) was equilibrated in phosphate buffer (0.05 M NaH$_2$PO$_4$/0.05 M NaCl, pH 7.2) containing 0.02% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS) detergent (Calbiochem). Two milliliters of 500 x-concentrated CESJ tumor cell-conditioned medium was dialyzed against the above elution buffer, and the sample, containing 4–5 mg of protein per ml, was eluted at room temperature at a flow rate of 20 ml/hr; 5-ml fractions were collected. The column effluent was monitored for protein absorbance at 280 nm. Fractions were sterilized by filtration (0.22 μm) and stored at 4°C for biological assays.

**Reverse-Phase HPLC.** Fractions containing TRAPase-positive colony-stimulating activity were pooled from several gel filtration runs, concentrated by Amicon YM10 ultrafiltration, dialyzed against 0.05 M NaH$_2$PO$_4$ buffer (pH 7.2), and then against 0.1% CF$_3$COOH. A 2-ml sample was applied to a C$_{18}$ reverse-phase radial pressure column (Waters). Elution solvents were: A, 0.1% CF$_3$COOH/H$_2$O, and B, 0.1% CH$_3$COOH/60% acetonitrile. The sample was eluted with a complex gradient from 0 to 40% B (0–5 min), 40–72% B (5–40 min), then isocratically at 72% B (40–70 min), and finally 72–95% B (70–120 min) at a flow rate of 1 ml/min; 3-ml fractions were collected in polypropylene tubes. Individual HPLC fractions were dialyzed against 0.05 M NaH$_2$PO$_4$ buffer containing 0.02% Tween 20, concentrated to 0.5 ml by using Centricon 10 filtration units (Amicon), and sterilized by passage through 0.22-μm filters before CSF bioassay.

**Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis (SDS/PAGE).** The method of Laemmli (15) was used. Samples of individual fractions spanning the peak of TRAPase-positive colony-stimulating activity from the reverse-phase HPLC column were run on 15% polyacrylamide slab gels and were stained for protein with Coomassie blue.

**RESULTS**

**Demonstration of TRAPase-Positive Colony-Stimulating Activity from the CESJ Culture Medium.** When culture medium exposed to CESJ cells was concentrated and added to bone marrow cell cultures, distinct colonies composed predominantly of TRAPase-positive cells consistently developed after 14 days of incubation (Fig. 1). As the concentration of CESJ-conditioned medium increased in culture, the number of TRAPase-positive colonies and the intensity of TRAPase expression increased. At optimal concentrations (7.5–10%, vol/vol) of the conditioned medium in culture, 75–100 colonies were stimulated per 10$^5$ bone marrow cells, and >90% of them were strongly TRAPase positive. At a higher concentration (20%, vol/vol) of the conditioned medium, colony numbers sharply decreased, while all colonies were strongly positive for the TRAPase activity. In contrast, similarly prepared culture medium of Bc66 or NFS-A-c9 tumor cells stimulated many colonies, but virtually all of them were composed of macrophages negative for TRAPase activity (data not shown). In the presence of an optimal concentration of CESJ medium, the number of TRAPase-positive colonies increased linearly with increasing numbers of cells plated, and the regression line intercept zero, indicating each

**Fig. 1.** Photomicrographs of bone marrow colonies stimulated by CESJ-conditioned medium. A red TRAPase-positive colony (A) is contrasted with a blue TRAPase-negative colony (B). (×250.)
TRAPase-positive colony was clonally derived. There was no apparent strain specificity.

Elimination of Known Myeloid CSFs as TRAPase-Positive Colony-Stimulating Activity of CESJ-Conditioned Medium. The effect of specific antisera to known CSFs was assessed against the TRAPase-positive colony-stimulating activity. Various dilutions of antisera or purified antibodies were tested in the colony assays by using a constant optimal dose of CESJ-conditioned medium. Anti-M-CSF, anti-GM-CSF, and anti-IL-3 had no effect on the TRAPase colony-stimulating activity of the conditioned medium, whereas the same antibodies completely neutralized the respective colony-stimulating activities of M-CSF, GM-CSF, and IL-3. Since conditioned media of NFSA-c9 cells, which constitutively produce G-CSF and M-CSF, and of B566 cells, which produce M-CSF, do not stimulate TRAPase-positive colonies, the ability of CESJ-conditioned medium to stimulate TRAPase-positive colonies must involve factor(s) other than M-CSF or G-CSF. The lack of neutralization of TRAPase-positive colony-stimulating activity by anti-sera to GM-CSF or IL-3 also supports our previous findings that neither GM-CSF nor IL-3 is expressed in CESJ cells (7). Thus, G-CSF, M-CSF, GM-CSF, and IL-3 were excluded as factors responsible for the TRAPase-positive colony-stimulating activity.

Elimination of Other Factors. Since TGF-β-like activity was demonstrated in the crude conditioned medium of the tumor (5), we examined the effect of recombinant TGF-β1 on TRAPase-positive colony formation at 0–500 pM in the presence or absence of G-CSF (25–100 units/ml) and/or M-CSF (25–100 units/ml). None of these factor combinations stimulated TRAPase-positive colonies. In fact, TGF-β1 inhibited colony formation. Production of prostaglandins or vitamin D metabolites from the CE tumor has been excluded (5). Parathyroid hormone and parathyroid hormone-related protein were ruled out by the failure to stimulate cAMP production from isolated kidney cells in culture (5). The CESJ tumor did not express mRNA for IL-1, and the tumor-conditioned medium contained no IL-1 activity (7). Furthermore, human or murine IL-1 (102 to 108 units/ml of culture), with or without G-CSF (25–100 units/ml) or M-CSF (25–100 units/ml), did not stimulate TRAPase-positive colonies (data not shown). Tumor necrosis factor and platelet-derived growth factor were absent from the CESJ tumor products by L929 cell assay and radioreceptor assay, respectively (data not shown).

Osteoclastic Function of TRAPase-Positive Colony Cells. Dentin slices were placed at the bottom of Linbro wells and were overlaid with bone marrow colony assays. On days 14, 21, and 28 of culture, colonies in the agar or on the dentin were evaluated, and the dentin pieces were processed for scanning electron microscopy. Corresponding to the site of TRAPase-positive colonies, clusters of what appeared to be rudimentary resorption lacunae were observed on dentin pieces that were cocultured for 14 days (Fig. 2 A and B). Such resorption lacunae were not found in the areas where TRAP-
ase-negative colonies were present. Histological sections of calvaria cocultured with cells grown in the presence of CESJ-conditioned medium showed that 95–98% of the cells attached to the concave surface of the calvaria were TRAPase-positive, some of which demonstrated multinuclearity and a ruffled border, both characteristic features of the functional osteoclast (Fig. 3). In contrast, calvaria cocultured with cells grown in the presence of L-cell-conditioned medium revealed that >95% of cells attached were TRAPase-negative (data not shown). These studies provided strong evidence that TRAPase-positive colony-stimulating activity of the CESJ-conditioned medium indeed stimulates the growth of osteoclast progenitors.

Biochemical Isolation of TRAPase-Positive Colony-Stimulating Activity. To isolate the osteoclast-stimulating factor, medium exposed to CESJ tumor cells was concentrated and fractionated by gel filtration chromatography, followed by reverse-phase HPLC. Initial experiments with HL-1 culture medium, which contained supplementary exogenous proteins, were not successful because these proteins tended to coelute with the protein of interest. This problem was avoided by using protein-free medium 199 to prepare tumor cell-conditioned medium that exhibited the same biological activities as that prepared in HL-1 medium.

Gel Filtration Chromatography. Concentrated CESJ-conditioned medium containing 8–9 mg of protein was applied to the gel filtration column (Fig. 4). TRAPase-positive colony-stimulating activity appeared as two peaks clearly separated from the major protein peak. The first peak was associated with macrophage colonies that were TRAPase-negative at fractions corresponding to a molecular mass of about 150 kDa. A second distinct peak of the activity was observed in fractions corresponding to an apparent molecular mass of about 30 kDa. In these fractions, almost all colonies and clusters were TRAPase-positive, and only a few TRAPase-negative macrophage colonies were present. A peak of G-CSF activity also was eluted in this region (Fig. 4). Repeated gel filtration separations with different batches of conditioned medium consistently demonstrated similar protein and activity profiles. From these replicate separations, fractions 55–70 were pooled, concentrated, and applied to a C18 reverse-phase column.

Reverse-Phase HPLC. Two milliliters of the pooled fractions from the gel filtration procedure containing 2–5 mg of protein were chromatographed. A single peak of TRAPase-positive colony- and cluster-stimulating activity was repeatedly found in fractions 30–33 (Fig. 5). In contrast to the gel filtration chromatography, G-CSF activity was completely separated from this peak (Fig. 5). The HPLC fractions were tested individually for their ability to form TRAPase-positive cells capable of excavating dentin. We found groups of resorption lacunae corresponding to the location of colonies containing TRAPase-positive cells when dentin pieces were cocultured with marrow cells in the presence of material from fractions 30–33 (Fig. 2C). Such excavations were not found when other fractions were tested or in areas on the dentin where TRAPase-negative cells were present.

SDS/PAGE Gel Analysis. Reverse-phase HPLC fractions containing TRAPase-positive colony- and cluster-stimulating activity (fractions 28–37) were analyzed by SDS/PAGE without disulfide cleavage. A band running with an apparent molecular mass of 17 kDa coincided in intensity with the peak of biological activity on HPLC. When an SDS/PAGE gel was sectioned and eluted, the biological activity was in slices corresponding with a molecular mass of 15–19 kDa (data not shown).

DISCUSSION

By a combination of gel filtration and reverse-phase chromatography, we have isolated an osteoclast colony-stimulating factor from the proteins secreted by a hypercalcemia-inducing murine mammary tumor cell line. Colonies and clusters stimulated from bone marrow cultures by the initial conditioned medium or by the purified factor were strongly positive for TRAPase, a cytochemical marker used to detect osteoclasts in bone (16). Upon cocultivation with
devitalized bone or dentin pieces, these TRAPase-positive colony cells demonstrated some of the typical morphological features of osteoclasts and formed rudimentary resorption lacunae. These data suggest that the factor present in the tumor-conditioned medium stimulated clonal growth of osteoclast progenitors. In addition, the TRAPase colony-stimulating activity was only elaborated from a hypercalcemia-inducing mammary tumor and not from control tumors, emphasizing the significant biological role for this factor in bone resorption in vivo. We propose the term osteoclast CSF (O-CSF), for this tumor-derived factor. Clear biochemical separation of O-CSF from other CSFs also produced from our tumor cells, as well as comparative functional and immunological studies of O-CSF with other known CSFs, suggests that it is a distinct factor from other known CSFs for myeloid cells.

Little is known about the factors that regulate osteoclasts in their early stages of development. Although IL-3, GM-CSF, and M-CSF are reported to be involved in the formation of osteoclast-like cells in the presence of 1,25-dihydroxy vitamin D$_3$, it is unresolved as to which CSF has definitive effects on osteoclast generation (17–20). IL-3 and GM-CSF are absent in our tumor culture medium, and the factor we have purified is capable of stimulating proliferation and differentiation of TRAPase-positive osteoclast progenitors in the absence of added vitamin D metabolites in the culture.

Recently, Yoshida et al. (21) reported a mutation in the coding region of the M-CSF gene in an osteopetrotic (op) mouse that presumably caused the osteopetrosis. In our studies, M-CSF-producing tumors did not induce hypercalcemia, and treatment of CE mammary tumor-bearing mice with antiserum to M-CSF did not affect the tumor-induced hypercalcemia (7). Therefore, elevated levels of M-CSF in the circulation did not seem to affect osteoclast function. However, it is possible that M-CSF is needed for the local activation of osteoclasts so that a complete lack of M-CSF in op mice may lead to osteopetrosis (22). Further characterization of O-CSF would elucidate the relationship of O-CSF to M-CSF as well as to a recently identified stem-cell factor (23).

In summary, we have isolated a specific CSF for the osteoclast lineage from conditioned medium of a hypercalcemia-inducing murine mammary tumor. This osteoclast CSF appears to be a distinct molecule from other known CSFs of myeloid cells. The availability of purified O-CSF should provide a new tool to investigate the physiology of osteoclast generation.

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