EXPERIMENTAL STUDY

The role of phosphoinositide 3-kinase in spreading osteoclasts induced by colony-stimulating factor-1

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Abstract

Background: Colony-stimulating factor-1 (CSF-1), a growth and survival factor for osteoclasts, stimulates these cells to spread and migrate towards a gradient of CSF-1. This may support the translocation of osteoclasts to new sites on the bone surface to be resorbed. Phosphoinositide 3-kinase (PI 3-K) is a lipid kinase participating in various signal transduction pathways.

Objective: To investigate the role of PI 3-K in the CSF-1-induced spreading of osteoclasts.

Methods: In isolated rat osteoclasts treated with or without CSF-1, the distribution of PI 3-K and proteins phosphorylated on tyrosine were investigated using immunofluorescence. In murine osteoclast-like cells grown from bone marrow cells co-cultured with osteoblasts, the activation of the PI 3-K by CSF-1 was determined both *in vivo* and *in vitro*. *In vivo*, the enzyme product in the cell was determined after extraction and separation with thin layer chromatography; *in vitro*, PI 3-K activity was measured in the pellet immunoprecipitated from the cell lysate.

Results: Inhibition of PI 3-K blocked the CSF-1-induced spreading of osteoclasts. In spreading osteoclasts, a portion of PI 3-K was translocated to the periphery where proteins phosphorylated on tyrosine appeared simultaneously. In osteoclast-like cells, CSF-1 stimulated PI 3-K activity. This activity could be immunoprecipitated with antibody against phosphotyrosine residues.

Conclusion: PI 3-K participates in the CSF-1-induced spreading of osteoclasts. The activated PI 3-K may induce the reorganization of the cytoskeleton resulting in spreading and migration.

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Introduction

Colony-stimulating factor-1 (CSF-1), also known as macrophage colony-stimulating factor, is one of the hemopoietic growth factors required for proliferation, differentiation, activation and survival of the cells of the mononuclear phagocytic system (1). Its cellular effects are mediated via a high affinity cell surface receptor that belongs to the tyrosine kinase receptor family and is encoded by the proto-oncogene *c-fms* (1).

CSF-1 is essential in supporting the development of osteoclasts and, consequently, bone resorption (2). The osteopetrotic mouse strain of the type *op* proved to be a useful tool to study the function of CSF-1 in this process. Homozygous *op/op* mice are deficient in the synthesis of biologically active cytokines (3). The *op* phenotype is characterized by a low number of macrophages and osteoclasts (4), the latter causing the impaired bone resorption that leads to osteopetrosis. Daily injection of CSF-1 into *op/op* mice induced the formation of osteoclasts and reversed the osteopetrotic phenotype, providing conclusive evidence that the development of osteoclasts depends on this cytokine (5).

Osteoclast precursor cells and mature osteoclasts contain transcripts encoding *c-fms* and express binding sites for CSF-1 (6–8), suggesting that the cytokine not only supports osteoclastogenesis by a direct action on cells of the osteoclast lineage but that it also acts on mature osteoclasts. Indeed, CSF-1 was shown to be necessary for the survival of osteoclasts *in vitro*. Furthermore, it stimulates spreading and migration against a gradient of CSF-1 (9) and fusion of osteoclasts (10). The effect of CSF-1 on bone resorption is not clear. Experiments with osteoclasts incubated on bone slices demonstrate contradictory results. One group reported inhibition (9), another stimulation (11). Inhibition could be explained by the osteoclast not resorbing bone during migration. By contrast, the osteoclast has to move over the bone surface to initiate new sites of bone resorption; therefore, migration may promote bone resorption. The effects of CSF-1 on bone resorption *in vitro* might depend on the experimental conditions.

It has been shown that phosphoinositide 3-kinase (PI 3-K) is involved in membrane ruffling and chemotaxis induced by platelet-derived growth factor (12). Therefore, we investigated the role of PI 3-K in the spreading

of osteoclasts induced by CSF-1. Several isoenzymes belong to the PI 3-K family (13). They are lipid kinases that phosphorylate phosphatidylinositols (PtdIns) and its phosphate esters at the D-3 position of the inositol ring. The products of PI 3-Ks act as second messengers in signal transduction, inducing various cellular processes including mitogenesis, transformation, membrane ruffling, regulation of the actin cytoskeleton and vesicle movement (12, 14–16).

The most extensively investigated isoform of PI 3-K is a heterodimer consisting of the regulatory subunit p85 and the catalytic subunit p110. *In vivo*, the preferred substrate of this heterodimer is PtdIns-4,5-diphosphate (PtdIns-4,5-P₂) (13). The regulatory subunit p85 contains two SH2 domains by which it binds to phosphorylated tyrosine residues. This interaction modulates the regulatory subunit and, as a consequence, leads to an activation of the catalytic subunit (17, 18). Thus, the heterodimer p85/p110 is indirectly activated by receptor and non-receptor tyrosine kinases phosphorylating tyrosine residues. Furthermore, the small GTP-binding protein Ras has been found to interact directly with the catalytic subunit of PI 3-K in a GTP-dependent manner through the Ras effector site and to stimulate the enzyme activity (19).

Inhibitors of PI 3-K block bone resorption *in vitro* and *in vivo* (20–22). The mechanism by which PI 3-K participates in this process is not fully clear, but some functions have been demonstrated. The enzyme is involved in the formation of ruffled borders (23). When osteoclasts attach to mineralized bone surfaces, they are activated and undergo drastic morphological and functional changes. The cells polarize and form specialized membrane areas along with the reorganization of cytoskeletal structures (24). During this cellular attachment, PI 3-K translocates from the cytosol to the cytoskeleton (25). Inhibition of PI 3-K inhibits the attachment of osteoclasts, suggesting that the enzyme is required for this process.

In the present study, the effect of CSF-1 on PI 3-K of osteoclasts has been investigated. As mentioned above, CSF-1 stimulates the spreading and migration of isolated osteoclasts *in vitro* (9). We demonstrate that wortmannin, an inhibitor of PI 3-K (26), impairs the CSF-1-induced spreading of osteoclasts, suggesting a role of PI 3-K in this cellular process. Treatment of osteoclasts with CSF-1 leads to a translocation of a portion of the enzyme to the periphery of the cells, where it is localized with proteins phosphorylated on tyrosine. Furthermore, treatment of osteoclast-like cells with CSF-1 results in an activation of PI 3-K.

Materials and methods

Materials

Media were obtained from Gibco BRL, Life Technologies, Basel, Switzerland; leupeptin, aprotinin and

pepstatin were purchased from Bachem, Bubendorf, Switzerland; phenylmethylsulfonyl-fluoride (PMSF), dithioerythritol (DTT), phosphatidylinositol, phosphatidylserine, phosphatidylinositol-4-phosphate, phosphatidylinositol-4,5-bisphosphate and phosphoinositides purified from brain extract were obtained from Sigma, Buchs, Switzerland and [³²P]H₃PO₄ (carrier-free, concentration 10 mCi/ml) and [γ -³²P]ATP (specific activity 3000 Ci/mmol, concentration 10 mCi/ml) was purchased from Amersham Pharmacia Biotech, Bucks, UK). Inorganic salts and organic compounds with small molecular weight were obtained from Merck, Grogg AG, Bern, CH and Fluka AG, Buchs, Switzerland. Recombinant human CSF-1 was generously donated by Cetus Oncology, Chiron Corporation, Emeryville, CA, USA, 1,25-dihydroxycholecalciferol from Hoffmann-LaRoche, Basel, Switzerland and wortmannin was a generous gift from T Payne, Novartis Pharma AG, Basel, Switzerland.

Cell culture

In vitro formation of mouse osteoclast-like cells

Osteoclast-like cells were grown in 8-cm diameter tissue culture dishes (27) and plated directly onto the plastic of the culture dish instead of onto a collagen gel. Thus, 4.5×10^6 bone marrow cells from femurs and tibias of 6–8-week-old male ddy mice were co-cultured with 1.2×10^6 osteoblasts in α -MEM containing Earle's salts, 10% fetal bovine serum (FBS), 50 mg/l ascorbate, 10^{-8} M 1,25-dihydroxycholecalciferol, 10^{-6} M prostaglandin E₂ (PGE₂; Alexis, Läufelfingen, Switzerland) and antibiotics. When the medium was changed on day 3, PGE₂ was not added further. Osteoblasts were removed on day 5 by treatment with 0.02% EDTA (3 min) or with 0.02% collagenase/dispase (Roche Molecular Biochemicals, Basel, Switzerland) dissolved in PBS (20–30 min). The remaining adherent cells were tartrate-resistant acid phosphatase-positive multinuclear cells with less than 10% mononuclear cells.

The osteoblasts required for the co-culture were prepared as described previously (28). After incubation in 4 mM EDTA–PBS for 30 min, calvaria of 1–3-day-old ddy mice were minced. The bone fragments were incubated in Petri dishes (8 cm diameter) in MEM–Earle's containing 50 mg/l ascorbate, 15% FBS and 2% collagen (Cellon SA, Strassen, Luxembourg). After 6 days, the osteoblasts that had grown out from the bone fragments, were harvested by digesting the collagen gel and kept frozen in liquid nitrogen. When required for co-culture, the osteoblasts ($10^4/\text{cm}^2$) were grown for 5 days in α -MEM–Earle's containing 10% heat inactivated FBS, 5×10^{-5} M mercaptoethanol, antibiotics and 50 mg/l ascorbate and collected by trypsin digestion.

Preparation of disaggregated rat osteoclasts Osteoclasts were isolated from tibiae and femurs of 1–2-day-old rats as described previously (29). The bones

were curreted in Medium 199–Hank's. The cells were collected by centrifugation and resuspended in medium containing 30% FBS. Aliquots of 50 μ l were pipetted onto 13-mm diameter glass coverslips (four coverslips per rat). The coverslips had been treated overnight with NaCl solution containing 30% FBS. After incubation for 1 h at 37 °C, non-adherent cells were washed off and the osteoclasts adherent to the coverslips treated as described below.

Cell spreading

The osteoclasts on the coverslips were incubated with or without CSF-1 in Medium 199–Hanks containing 0.1% bovine serum albumin (BSA)-RIA (Sigma) for 20 min. To test the effect of wortmannin, the cells were pre-incubated for 10 min in PBS containing the drug or the vehicle (DMSO). After fixation with 4% para-formaldehyde in PBS, the cells were analyzed under the microscope using phase contrast. Spread osteoclasts were distinguished from non-spread cells according to their form (see Fig. 1). They were counted blinded and expressed as a percentage of the total number of osteoclasts. The area of the cells could not be used for comparison because of the size of individual cells varied too much.

Immunofluorescence

PI 3-K Osteoclasts on glass cover slips treated $\pm$ CSF-1 were washed with ice-cold PBS containing 1 mM Na_3VO_4 , fixed with ice-cold 1:1 acetone:methanol (v/v) and dried at room temperature. Unspecific binding sites were blocked by incubating the cells for 3 h in PBS containing 0.02% Tween 20 and 1.5% donkey serum. They were then incubated overnight at 4 °C with 2 μ g/ml of rabbit antibody against p85 of PI 3-K (Santa Cruz Biotechnology, Dr Glaser AG, Basel, Switzerland) or rabbit IgG (negative control), then for 1 h at room temperature with biotinylated donkey anti-rabbit IgG antibody (Amersham Pharmacia AG, Zurich, Switzerland), diluted 1:2000, and finally with Streptavidin–FluoroLink Cy2 (Amersham Pharmacia), diluted 1:1000. The antibodies and the streptavidin were diluted in PBS containing 0.02% Tween 20 and 1.5% donkey serum. After embedding, the preparations were observed with a Nikon fluorescence microscope equipped with a DC 100 W high pressure mercury lamp and filter HQ470/40 for exiting light (455–485 nm), filter HQ525/50 for the fluorescence light (505–545 nm) and a beam splitter reflecting light < 495 nm.

Phosphotyrosine residues The staining was carried out as described above, except for the following variation. The cells were washed once with ice-cold PBS containing 1 mM Na_3VO_4 , fixed with 4% formaldehyde dissolved in PHEM (60 mM PIPES, 10 mM HEPES, 10 mM EGTA, 2 mM MgCl_2 , pH 6.9), (30) for

Figure 1 Spreading of disaggregated osteoclasts induced by CSF-1. Osteoclasts were isolated from long bones of 1–2-day-old rats and allowed to adhere to glass coverslips for 1 h. After washing off the non-adhering cells, the osteoclasts were incubated in the absence or presence of 1 nM CSF-1 for 20 min in Medium 199–Hank's, containing 0.1% RIA–BSA. Additionally, they were treated with 100 nM wortmannin or the vehicle DMSO. In the absence of CSF-1 (a), the osteoclasts were not spread. In the presence of CSF-1 (b), the osteoclasts, including the granulated area, were spread, and all the nuclei could be identified. The central area was surrounded by an area with low contrast, which was probably thin and contained little cellular material. The granulated area was contracted around the nuclei. In the presence of wortmannin without (c) and with (d) CSF-1, the osteoclasts were not spread in a similar pattern to (a). They had filopodia and small lamellipodia. The nuclei were partially

15 min at 37 °C and washed three times with PBS. For permeabilization, the osteoclasts were treated for 2.5 min at 37 °C with 0.5% Triton X-100 dissolved in PHEM. Unspecific binding was blocked by incubating the cells with PBS containing 1% BSA and 10% sheep serum over night at 4 °C. The cells were then incubated for 1 h at 37 °C with 20 μ g/ml murine monoclonal antibody against phosphotyrosine residues (clone 4G10; Upstate Biotechnology, Lucerna Chem AG, Luzern, Switzerland), then at room temperature with a sheep biotinylated antibody against murine IgG diluted 1:2000 (Amersham Pharmacia), and finally with Streptavidin–FluoroLink Cy2, diluted 1:1000. The antibodies and streptavidin were diluted with PBS containing 1% RIA–BSA. The fluorescence microscopy was carried out as described above.

Determination of PI 3-K activity in vivo

For the determination of the PI 3-K activity in the intact osteoclast-like cells, they were grown in dishes of

5 cm diameter, labeled with [^{32}P] PO_4^{3-} , and after stimulation with ± 3 nM CSF-1, the product of the enzyme reaction was extracted. In detail, the osteoblasts were removed, the osteoclast-like cells washed with phosphate-free Hanks solution and subsequently air-incubated in PO_4^{3-} -free MEM containing Hanks salts, 5 mM HEPES, pH 7.3, and 10% FBS at 37 °C for 1 h. The FBS had been dialyzed against isotonic NaCl and once against phosphate-free medium. For labeling, the cells were air-incubated in 1.5 ml of the same medium containing 75 μCi [^{32}P] PO_4^{3-} for 1 h. After incubation in PBS with wortmannin or DMSO (DMSO had no effect) for 10 min, the cells were stimulated for various time periods in medium containing $\pm$ CSF-1 and 10% heat inactivated FBS. The incubation was terminated by aspirating the medium and adding 1 ml of ice-cold extraction buffer (11 parts 2 mM DTT in 4.8 M HCl, 25 parts 1 mM CaCl_2 , 4.9 mM KCl, 136 mM NaCl and 10 mM HEPES, pH 7.4, and 64 parts methanol) as described. After addition of 50 μg of PIs purified from brain extract (used as carrier and standard), the cellular material of each dish was collected by scraping with a rubber spatula. After sonication for 30 s and addition of 1.25 ml chloroform, the phospholipids were extracted into the chloroform phase (31). After evaporation under nitrogen they were separated on oxalate-impregnated Silica gel 60 thin layer plates (Merck, Darmstadt, Germany), that were activated at 110 °C for 40 min immediately before use. The plates were developed for 4 h in chloroform:acetone:methanol:acetic acid:water (80:30:26:24:14 v/v). The spots originating from phosphatidylinositol phosphate (PtdInsP), PtdInsP_2 and $\text{PtdIns}(3,4,5)\text{P}_3$ were identified after exposure to iodide vapor. The radioactivity of the product $\text{PtdIns}(3,4,5)\text{P}_3$, which is specific for PI 3-K, was quantified with a PhosphorImager (Storm, Molecular Dynamics). The background on the lane corresponding to each spot was subtracted.

Determination of PI 3-K activity in vitro

After removal of the osteoblasts, osteoclast-like cells were air-incubated in α -MEM–Hanks containing 1% FBS for 2 h to up-regulate the CSF-1 receptor. After stimulation with or without CSF-1 for various times, the medium was removed, the cells were washed with ice-cold PBS containing 1 mM Na_3VO_4 and lysed for 20 min with 0.4 ml of ice-cold buffer containing 137 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4 , 1% Triton X-100, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin and 10 mM Tris-HCl, pH 7.4. The lysate of two dishes was pooled and centrifuged at 12 000 *g* for 20 min at 4 °C. The supernatant (0.5 ml containing 0.5 mg protein) was cleared for 30 min with 10 μl control serum and 10 μl of Gamma Bind G Sepharose Media (Pharmacia Biotech, Dübendorf, Switzerland) and then incubated with 5 μg of antibody against phosphotyrosine (Transduction

Laboratories, Mächler AG, Basel, Switzerland) or with IgG (negative control) overnight at 4 °C under rotation. After incubation with G Sepharose for 2 h at 4 °C, the beads with the bound immunocomplex were washed three times. For immunoprecipitation of the CSF-1 receptor, an antiserum against the whole protein of this receptor was used (generously donated by E R Stanley, Albert Einstein College of Medicine, Bronx, NY, USA).

The PI 3-K-activity was determined by incubating the immunoprecipitate at 37 °C for 15 min in 50 μl of the following reaction buffer: 0.2 mM phosphatidylserine, 0.2 mM $\text{PtdIns}(4,5)\text{P}_2$, 0.1% Na-cholate, 50 mM NaCl, 10 mM glycerol-3-phosphate, 0.1 mM Na_3VO_4 , 10 mM NaF, 5 mM Na-PPi, 20 mM MgCl_2 , 1 mM DTT, 10 mM [γ - ^{32}P]ATP (2 μCi per tube), and 50 mM Tris-HCl, pH 7.5. The phospholipids were dissolved as described (32). The reaction was stopped by the addition of 0.1 ml 1 N HCl, and 0.2 ml of a mixture of 1:1 methanol chloroform. The phospholipids were extracted, separated on thin layer plates, and the radioactivity of $\text{PtdIns}(3,4,5)\text{P}_3$ determined as described above. The protein was determined by the Bradford method (kit obtained from BIO-RAD, Glattbrugg, Switzerland).

Statistics

The results are presented as means $\pm$ S.E.M. and analyzed by analysis of variance (Student's Newman–Keuls multiple comparison test).

Results

Inhibition of cell spreading by wortmannin

When osteoclasts isolated from long bones of young rats were incubated with CSF-1, they spread and formed circular cells (Fig. 1b). The nuclei were distributed in the granulated area and easy to identify. An area of little contrast, probably very thin, surrounded this granulated area. Wortmannin, an inhibitor of PI 3-K, inhibited spreading (Fig. 1d). In the absence of CSF-1 (Fig. 1a), as well as in the presence of wortmannin (Fig. 1c, d), the cells were not spread, and the largest part of the cell was granulated. The nuclei were partially hidden in the granules. The inhibition of the CSF-1-induced spreading by various concentrations of wortmannin is presented in Fig. 2; half maximal inhibition occurred at a wortmannin concentration of approximately 20 nM.

Localization of PI 3-K and phosphotyrosine residues

The inhibition of the spreading of osteoclasts by wortmannin suggested the involvement of PI 3-K in this process. Immunostaining of the regulatory subunit

Figure 2 Wortmannin inhibits the CSF-1-induced spreading of disaggregated osteoclasts. Osteoclasts isolated from rat long bones were allowed to adhere to glass coverslips as described in Fig. 1. After pre-incubation with various concentrations of wortmannin or DMSO in PBS, the osteoclasts were treated for 20 min with 1 nM CSF-1 (●) or without CSF-1 (○) as described in Fig. 1. Spread and unspread osteoclasts were counted blinded under the microscope. A total of 170–350 osteoclasts was counted for each treatment. The results present the means $\pm$ S.E.M. of four experiments.

p85 was applied to demonstrate an effect of CSF-1 on this lipid kinase. Figure 3 demonstrates the distribution of PI 3-K in osteoclasts treated with $\pm$ 3 nM CSF-1. Osteoclasts spread within 2 min of addition of CSF-1 (Fig. 3c), and membrane ruffling appeared (black arrow). A fraction of PI 3-K was translocated to the periphery of the cell, more strongly to sites where membrane ruffling was observed (Fig. 3d and h). The maximal effect was reached 5 min after addition of CSF-1 and remained for at least for 10 min (data not shown). In the absence of CSF-1 (Fig. 3b and f) and in the presence of wortmannin (Fig. 3j), PI 3-K remained in the center, in the granulated area of the cell.

Figure 4 demonstrates the distribution of proteins phosphorylated on tyrosine residues in osteoclasts treated with or without CSF-1. In the absence of CSF-1, staining for phosphotyrosine residues appeared inside and at the periphery of the osteoclasts (Fig. 4b, f). Most osteoclasts possessed lamellipodia (Fig. 4b and f), some filopodia (Fig. 4f) staining for phosphotyrosine residues. In a few osteoclasts, rings staining for phosphotyrosine residues were observed inside the cells (Fig. 4b). When CSF-1 was added, the osteoclasts spread and the staining for phosphotyrosine residues at the periphery appeared as a continuous line (Fig. 4d and h). Wortmannin inhibited spreading and the immunofluorescent signal was distributed similarly to osteoclasts not treated with CSF-1 (Fig. 4j). The similar distribution of PI3-K and tyrosine phosphorylated proteins along the periphery of the spreading osteoclast suggests an interaction between the two.

Activation of PI 3-K activity by CSF-1

In order to demonstrate the activation of PI 3-K activity by CSF-1, murine osteoclast-like cells were generated *in vitro*. The enzyme activity was determined by quantitating the product of the enzyme reaction in the cells treated $\pm$ 3 nM CSF-1. For this purpose, the cells were pre-labeled with [32 P]PO $_4^{3-}$. After stimulation with CSF-1, the phospholipids were extracted, separated by thin layer chromatography and the radioactivity of the enzyme product, PtdIns(3,4,5)P $_3$, was determined. Figure 5 (top) shows the autoradiograph of the thin layer chromatography, and the bottom of the figure shows the quantitation of the autoradiograph. The highest quantity of the product was formed within 2 min of addition of CSF-1. Afterwards PtdIns(3,4,5)P $_3$ decreased slowly and reached control levels after 20 min. Wortmannin (100 nM) inhibited the enzyme reaction to below control levels (about 50% of control).

The activity of the PI 3-K was also determined in the pellet after immunoprecipitation from the cell lysate with an antibody against phosphotyrosine. As seen in Fig. 6, in osteoclast-like cells treated with CSF-1, the activity of PI 3-K reached a maximum within 2 min and decreased rapidly thereafter. Pre-incubation of the cells with 100 nM wortmannin inhibited the reaction to nearly 0% of the control. When immunoprecipitated with an antibody against the receptor of CSF-1, the stimulation of PI 3-K activity by CSF-1 was not significantly different to controls (data not shown).

Discussion

The present results demonstrate the involvement of PI 3-K in the spreading of osteoclasts induced by CSF-1. It has also been demonstrated by others that wortmannin inhibits spreading and chemotaxis of rat osteoclasts (33). Using immunostaining we demonstrated that PI 3-K and proteins phosphorylated on tyrosine residues were localized at the periphery of spreading osteoclasts treated with CSF-1. Furthermore, PI 3-K activated by CSF-1 could be immunoprecipitated with antibody against phosphorylated tyrosine residues. These results suggest an association of PI 3-K with tyrosine phosphorylated proteins at the periphery of spread osteoclasts. Our results are in agreement with those of a recent publication (34).

The effect of CSF-1 has also been investigated in the macrophage cell line BAC1.2F5 (35). A large complex consisting of many proteins phosphorylated and not phosphorylated on tyrosine residues has been isolated from these cells. The results suggest that tyrosine phosphorylation induced by CSF-1 induces the organization of a complex consisting of cytoskeletal and other proteins that mediate the CSF-1-regulated mobility of

Figure 3 Effect of CSF-1 on the localization of PI 3-K in disaggregated osteoclasts. Osteoclasts isolated from long bones of 1–2-day-old rats were seeded to glass coverslips and after adherence treated ± 3 nM CSF-1 for various time periods: 2 min (line 1); 5 min (lines 2 and 3). After fixation, the cells were immunostained with an antibody against the regulatory subunit p85 of PI 3-K and photographed illuminated with light exciting the fluorochrome Cy2 (columns 2 and 4), or with white light using phase contrast (columns 1 and 3). In the absence of CSF-1 (a, b, e and f), the osteoclasts remained contracted, and PI 3-K was observed in the center in the granulated area of the cell. On addition of CSF-1 (columns 3 and 4), the osteoclasts spread and membrane ruffling appeared at the periphery of the cell (black arrows in c, g and k). A portion of the PI 3-K appeared at the periphery of the osteoclast, at sites where ruffling was observed (white arrows in d and h). No immunostaining was observed when the antibody against p85 was replaced by control IgG (k and l). Wortmannin inhibited CSF-1-induced spreading (i and j). Scale bar, 100 μ m. The findings described here were observed in three experiments.

macrophages. CSF-1 may have similar effects in osteoclasts.

PI 3-K is activated in that the two SH2 domains of the regulatory subunit p85 interact with tyrosine phosphorylated proteins, which results in the activation of the catalytic subunit p110 (17, 18). Thus, it has been demonstrated that PI 3-K interacts with phosphorylated tyrosine residues of the intracellular domain of the activated CSF-1 receptor (36, 37); however, we did not observe a significant stimulation of the PI 3-K activity in the pellet immunoprecipitated with an antibody against the CSF-1 receptor (not shown). The reason could be that the activated PI 3-K is not directly associated with the CSF-1-receptor but with proteins phosphorylated on tyrosine that are bound to the receptor. During lysis and immunoprecipitation, the receptor may dissociate from this complex, with PI 3-K

remaining bound to proteins phosphorylated on tyrosine. This could explain that PI 3-K activity stimulated by CSF-1 was observed in the pellet immunoprecipitated with antibodies against phosphotyrosine residues but not against the receptor.

An important non-receptor tyrosine kinase in osteoclasts is c-Src. Mice, in which the *c-src* gene has been disrupted, show normal osteoclast development, but bone resorption is impaired (38). c-Src participates in the formation of the ruffled border (39), but it is also required for spreading of osteoclasts induced by CSF-1 (40, 41). In avian osteoclasts, c-Src is involved in the activation of PI 3-K induced by osteopontin, a matrix protein that binds to the integrin $\alpha_v\beta_3$ (42). Grey *et al.* reported in their recent publication that in osteoclast-like cells stimulated with CSF-1 c-Src associates with its SH3 domain with the proline-rich sequence of the

Figure 4 Effect of CSF-1 on the localization of phosphorylated tyrosine residues in disaggregated osteoclasts. Osteoclasts isolated as in Fig. 3 were treated without CSF-1 (columns 1 and 2, except i and j), and with 3 nM CSF-1 (columns 3 and 4 and i and j) for various time periods: 2 min (line 1); 5 min (line 2 and 3). After fixation, the cells were immunostained with an antibody against phosphotyrosine and illuminated with light exciting the fluorochrome Cy2 (columns 2 and 4) or with white light using phase contrast (columns 1 and 3). In the negative control (k and l), IgG was added instead of the first antibody. In the absence of CSF-1, the osteoclasts remained contracted (a and e). In the presence of CSF-1, the osteoclasts spread and ruffling appeared at the periphery of the cell (black arrow in c, g and k), being maximal at 5 min. In the absence of CSF-1, immunostaining for phosphorylated tyrosine residues was observed in the granulated area and at the periphery of the cell. The staining was relatively strong in the center, varied from cell to cell, and was time independent. Two examples are demonstrated in b and f. Most osteoclasts possessed lamellipodia (b and f; white arrow head) and some also filopodia (f, small white arrow) staining for phosphotyrosine residues. In the presence of CSF-1, the immunostaining in the center was weaker. At the periphery, it appeared as a continuous line (white arrow in d and h), similar to that observed for PI 3-K in Fig. 3. When the antibody against phosphotyrosine was replaced with control IgG (k and l), the signal was much weaker. Wortmannin inhibited spreading (i and j), and the immunostaining appeared in the center and in lamellipodia of the cell. Scale bar, 100 μ m. The findings described here were observed in two experiments.

regulatory subunit p85 of PI 3-K and thus might support the activation of the catalytic subunit p110 (34). Our data do not contradict these results. The antibody against phosphotyrosine residues may immunoprecipitate c-Src associated with PI 3-K.

We observed that the PI 3-K activity of osteoclast-like cells not stimulated with CSF-1 was relatively high, about half of that of cells stimulated with the cytokine. Both activities were inhibited by wortmannin. These results were obtained whether the PI 3-K activity was determined either by quantitating the product of the enzyme reaction *in vivo* directly in the cultured cells or by measuring the enzyme activity in the pellet

immunoprecipitated from cell lysate. The observation could be explained by PI 3-K being not only activated by CSF-1 that induces cell spreading and migration but also by integrins binding to matrix proteins supporting attachment of osteoclasts and finally stimulating the bone resorbing activity. In the culture dish, osteoclast-like cells interact with matrix proteins, which may activate PI 3-K. It has been demonstrated that attachment of osteoclasts to plastic or bone induces a translocation of PI 3-K from the cytosol to the cytoskeleton (25). Furthermore, treatment of chicken osteoclasts with osteopontin activates PI 3-K (42). However, PI 3-K does not seem to be associated with

Figure 5 Effect of CSF-1 on PI 3-K activity in osteoclast-like cells *in vivo*. After removal of the osteoblasts by collagenase/dispase treatment from the osteoclast-like cells grown in co-culture in 5 cm-diameter culture dishes, osteoclast-like cells were labeled with $[^{32}\text{P}]\text{PO}_4^{3-}$. The cells were treated ± 3 nM CSF-1 for different time periods. The reaction was stopped with an ice-cold mixture of methanol, water and HCl. For the determination of the product of the enzyme reaction, the lipids were extracted and separated by thin layer chromatography. The radioactive spots on the thin layer plate were visualized and determined with a PhosphorImager. The lower half of the thin layer plate is shown in (A). W, wortmannin; T, incubation time in min; 1, PtdInsP; 2, PtdInsP₂; 3, PtdIns(3,4,5)P₃. (B) The radioactivity incorporated into PtdIns(3,4,5)P₃ is presented as a percentage of the control (no CSF-1) as means $\pm$ S.E.M. of 9–20 culture dishes collected from two independent experiments. At 20 min, the experiment was performed only once, assaying three culture dishes. The inhibition by 100 nM wortmannin was tested in 3–5 dishes. (■), In the presence of CSF-1; (□), in the absence of CSF-1; (●) in the presence of CSF-1 + wortmannin; (○) absence of CSF-1 + wortmannin. *Significantly different from control (no CSF-1), $P < 0.01$; # significantly different from CSF-1 + wortmannin, $P < 0.01$.

p130^{Cas} (Crk-associated substrate) and the focal kinase related PYK2 (proline-rich kinase 2), which both localize with cytoskeletal proteins in the actin ring when osteoclasts attach to surfaces coated with $\alpha_v\beta_3$ integrins (43). The function of PI 3-K in the attachment of the osteoclast to the substrate is not yet clear.

PI 3-K is involved in the reorganization of the cytoskeleton, which results in cell spreading and migration of various cell types (12, 15, 16). The product of the enzyme reaction, PtdIns(3,4,5)P₃, is a phospholipid integrated in the cell membrane. Acting as a second messenger, it binds proteins with a pleckstrin (PH) domain and translocates them to the cell membrane, which results in their activation and signal transduction (14). The signaling proteins inducing migration have not yet been identified fully. Some data suggest that the small GTP-binding proteins Rac and Cdc42, downstream of PI 3-K, activate PAK (p21-activated kinase), which finally results in the reorganization of the cytoskeleton (44).

In summary, we suggest that CSF-1 induces the tyrosine phosphorylation of proteins and stimulates PI 3-K, which is partially translocated to the periphery of the osteoclast. There, the enzyme may take part in the reorganization of the cytoskeleton required for cell spreading and migration.

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Figure 6 Effect of CSF-1 on PI 3-K-activity in osteoclast-like cells measured in the anti-phosphotyrosine-immunoprecipitate. After removal of the osteoblasts, osteoclast-like cells grown in co-culture in 8 cm-diameter culture dishes were preincubated for 2 h in α -MEM, 10% FBS and then treated with ± 3 nM CSF-1 for different time periods. After lysis of the cells and immunoprecipitation with antibody against phosphotyrosine, the activity of PI 3-K was measured in the immunoprecipitate. The results are expressed as a percentage of the control (no CSF-1) as means $\pm$ S.E.M. of 5–10 culture dishes of three independent experiments. The inhibition by 100 nM wortmannin was determined with two cultures. (■), With CSF-1; (□), without CSF-1.

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