Requirement of pp60$^{c\text{-src}}$ Expression for Osteoclasts to Form Ruffled Borders and Resorb Bone in Mice

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Abstract

Targeted disruption of the c-src proto-oncogene in mice has shown that src expression is required for normal bone resorption, since the src-deficient mutants develop osteopetrosis. To evaluate the mechanisms by which src-deficiency affects osteoclast function, we treated src-deficient mice with the stimulants of bone resorption, IL-1, parathyroid hormone, and parathyroid hormone-related protein, and analyzed the effects by quantitative bone histomorphometry and electron microscopy. Increased numbers of multinucleated cells with the morphological characteristics of osteoclasts appeared on bone surfaces, but these cells did not form ruffled borders or normal resorption lacunae. To confirm these in vivo findings, we cultured src-mutant bone marrow cells on dentine slices in the presence of 1,25 dihydroxyvitamin D_3. Increased numbers of multinucleated cells were formed, but unlike normal murine bone marrow cells, they did not form resorption pits. These results indicate that osteoclasts appear in the absence of pp60$^{c\text{-src}}$, but that pp60$^{c\text{-src}}$ expression is required for mature osteoclasts to form ruffled borders and resorb bone. (*J. Clin. Invest.* 1992. 90:1622-1627.) Key words: osteopetrosis • proto-oncogene • histomorphometry • electron microscopy • calcium

Introduction

Targeted mutations introduced into the germ line of mice after homologous recombination in embryonic stem cells allow new insights into the functions of individual gene products in the animal. Using this technology, it was shown recently that several independent mutations in the c-src gene cause osteopetrosis in mice (1), the disease characterized by impairment of the formation of normal bone marrow cavities (2). The best char-

acterized animal model of the human disease is the op variant in which there is a point mutation in the coding region of macrophage-colony stimulating factor (M-CSF)[1] (3). In this strain of mice, there is a failure in formation of osteoclasts (4). In contrast, examination of the behavior of osteoclasts in src-deficient mice both in vitro and in vivo revealed that multinucleated cells formed on bone surfaces, but they did not form Howship's lacunae. These results show that expression of pp60$^{c\text{-src}}$ is not required for the formation of osteoclasts, but rather for mature osteoclasts to resorb bone.

Methods

Initially, we performed detailed histomorphometric examination of humeri and vertebrae of untreated src-deficient (src-) mutant and normal wild type (WT) mice aged 6-8 wk. The src-mutant mice (8-12 g) were smaller than the wild type mice (16-20 g). All bone samples were fixed in 10% buffered formalin, decalcified in 14% EDTA, and embedded in paraffin. The following variables were measured in representative 3-μm thick sagittal sections cut through the vertebrae using a Bioquant Image Analysis System (R & M Biometrics, Nashville, TN) and a digitizing tablet along with a light microscope and a drawing tube attachment: cartilaginous end plate thickness, primary (1°) spongiosa thickness; vertebral length, cancellous bone volume (expressed as a percentage of the total cancellous space), and osteoclast numbers in the cancellous space (expressed per millimeter of the cancellous bone surface). The following variables were measured in sagittal sections cut through the humeri: epiphyseal plate thickness, 1° spongiosa thickness; 1° and secondary (2°) spongiosa bone volumes (expressed as a percentage of the space occupied by the 1° and 2° spongiosae); and osteoclast numbers in the 1° spongiosa (expressed per millimeter of the 1° spongiosa bone surface).

To determine whether increased osteoclast numbers and activity could occur in vivo in src-deficient mutants, we treated groups of src-deficient mutants and wild type normal mice with the maximal stimulators of bone resorption, human recombinant IL-1α, synthetic human parathyroid hormone (1-34) (PTH), and synthetic human parathyroid hormone-related protein (1-34) (PTH-rP). Using previously described methods (5-8), we injected small volumes (10 μl) of each agent into the loose subcutaneous tissue overlying the calvaria of mice four times daily for 3 d. We tested the effects of each agent (PTH and PTH-rP, 3 μg, four times daily and IL-1, 0.1 μg, four times daily for 3 d) in src- and WT mice in separate experiments using three or four mice in each group. The weights of the src-mutant mice (6-12 g) in each group were approximately half of those of the WT mice (12-20 g). Thus, in

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1. *Abbreviations used in this paper:* 1,25D_3, 1,25 dihydroxyvitamin D_3; M-CSF, macrophage-colony stimulating factor; MNC, multinucleated cells; PTH, parathyroid hormone; PTH-rP, parathyroid hormone-related peptide; src-, src-deficient; TRAP, tartrate-resistant acid phosphatase; TRAP(+)MNC, TRAP-positive MNC.

	Vertebrae			Humeri	
Measurements	src-	WT	Measurements	src-	WT
End plate thickness (mm)	0.08±0.006	0.08±0.003	Epiphyseal plate thickness (mm)	0.13±0.01	0.15±0.01
Vertebral length (mm)	1.8±0.02*	2.6±0.04	1° Spongiosa thickness (mm)	0.58±0.06*	0.15±0.01
Bone volume (%)	50±6*	13±2	1° & 2° Spongiosa bone volume (%)	56±1.3*	22.8±3.8
Osteoclast number (per mm cancellous bone surface)	2.8±0.4*	0.6±0.1	Osteoclast number (per mm 1° spongiosa bone surface)	2.0±0.4	1.2±0.3

* Mean values are significantly different from values in WT mice ($P < 0.005$; by Student's t test).

most experiments, although the src-mutant mice were given the same dose locally over the calvaria, they received approximately twice the dose given to the WT mice per gram body weight. In these circumstances, more marked systemic effects on, for example, blood calcium concentrations would be anticipated than in normal mice. To determine whether hypercalcemia could occur in the src-deficient mutants in response to IL-1, PTH, and PTH-rP, we measured whole blood ionized calcium in retro-orbital venous samples (30–40 μl) in the mutants and wild type mice 2 h after the 11th injection in the above experiments using the 634 ISE Ca^{++} pH Analyzer from Ciba Corning (Medfield, MA).

The posterior halves of the calvariae were fixed, decalcified, and processed in the same manner as the vertebrae and the following variables were measured on representative 3-μm thick sections: osteoclast numbers within the bone marrow cavities (expressed per square millimeter of the total bone area, i.e., the bone and bone marrow between the outer and inner periosteal surfaces); the osteoclast surface, i.e., the extent of osteoclasts along the interface surface between bone and bone marrow (expressed as a percentage of the interface, and includes resorbing and nonresorbing osteoclasts; the inactive eroded surface, i.e., the extent of the bone surface that has been eroded by osteoclasts but is no longer covered by them; and the mean osteoclast resorption depth, i.e., the average depth that each osteoclasts erodes into the bone matrix from the preexisting bone surface. The position of the preexisting bone surface overlying each osteoclast is estimated by drawing a line between the edges of the unresorbed bone surface on either side of each resorption lacuna, such that the line maintains the typical concavity of bone marrow spaces in the calvaria. The distance between this line and the bone surface beneath each osteoclast is measured and the sum of these distances in each section is divided by the number of osteoclasts. We chose to assess the resorption capacity of the osteoclasts directly using this method, rather than by measuring resorption-related changes in the bone matrix area, because there is a wide range in the porosity of the

normal calvaria, and the period of observation to observe a significant effect on bone volume (72 h) was short.

To examine the ultrastructural appearance of the osteoclasts, the anterior halves of the calvariae of the mice treated with PTH-rP and IL-1 were fixed in 4% formaldehyde and 1% glutaraldehyde in phosphate buffer. The specimens from the PTH-rP–treated mice were decalcified in 14% EDTA before embedding in Poly-Bed 812 (Polysciences Inc., Warrington, PA) but those from the IL-1-treated mice were embedded undecalcified and ultrathin sections from all specimens were examined in a JEOL JEM 100 CS transmission electron microscope.

We studied osteoclast formation in culture by using a modification of mouse bone marrow long-term cultures described by Takahashi et al. (9). Bone cells were isolated from 4–6-wk-old male ICR Swiss mice (Harlan Industries, Indianapolis, IN). Nonadherent marrow cells were suspended in α-MEM supplemented with 10% FCS (Hyclone Laboratories Inc., Logan, UT) and no antibiotics at a final density of 4×10^6 cells/ml. Half a milliliter of cell suspension (2×10^6 cells/well) was inoculated onto sperm whale dentine in 24-well plates (Corning Glass Inc., Corning, NY), and 1,25 dihydroxyvitamin D_3 (1,25D_3) (Hoffman–La Roche, Nutley, NJ) was added to each well at a final concentration of 10^{-8} M. The cells were fed every 2 d with 0.3 ml of fresh α-MEM with 10% FCS and 10^{-8} M 1,25D_3. After 6 d, the cells on the dentine slices were stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (Sigma Chemical Co., St. Louis, MO). TRAP-positive (red-stained) multinucleated (three or more nuclei) cells (TRAP(+)MNC) were counted under light microscopy at a magnification of 200. For examination of resorption lacunae, the slices were sonicated in 0.1 M NaOH to remove the cells and were stained with 0.1% (vol/vol) toluidine blue. Lacunae were examined using light microscopy and the plane area of matrix resorbed was quantitated using a computer-assisted morphometric program on an Olympus image and process analysis system. Data were analyzed by pattern

	No. of osteoclasts per mm² TBA		Osteoclast surface (%)		Inactive eroded surface (%)		Resorption depth (μm)		Blood Ca^{++} (mmol/liter)	
	src-	WT	src-	WT	src-	WT	src-	WT	src-	WT
PTH	43±2*	41±9*	13±1*	19±1	1±1	11±1	0.5±0.1	7±1.5*	1.67±0.1*	1.61±0.03*
PTH-rP	27±7*	45±8*	12±3*	16±4*	1±1	10±3*	1±0.3	6±1.3*	1.58±0.06*	1.55±.01*
IL-1	37±10	53±10*	11±4*	28±2*	3±1	49±2*	1±0.2	14±0.2*	1.28±0.02	1.44±0.03*
Untreated	5±1	3±1	1.5±4	1±0.3	0.4±0.3	0.5±0.3	3±1	2±1	1.29±0.02	1.28±0.01

TBA, total bone area. * Indicates values which are significantly different from the values in the untreated mice using Dunnett's one-tailed t test for comparison of treatment groups against a control. Mice (three or four per group) were treated with each agent PTH and PTH-rP, 3 μg, four times daily and IL-1α, 0.1 μg, four times daily for three days) in separate experiments. Whole blood ionized Ca^{++} was measured 2 h after the 11th injection, and the mice were killed 14 h after the last injection.
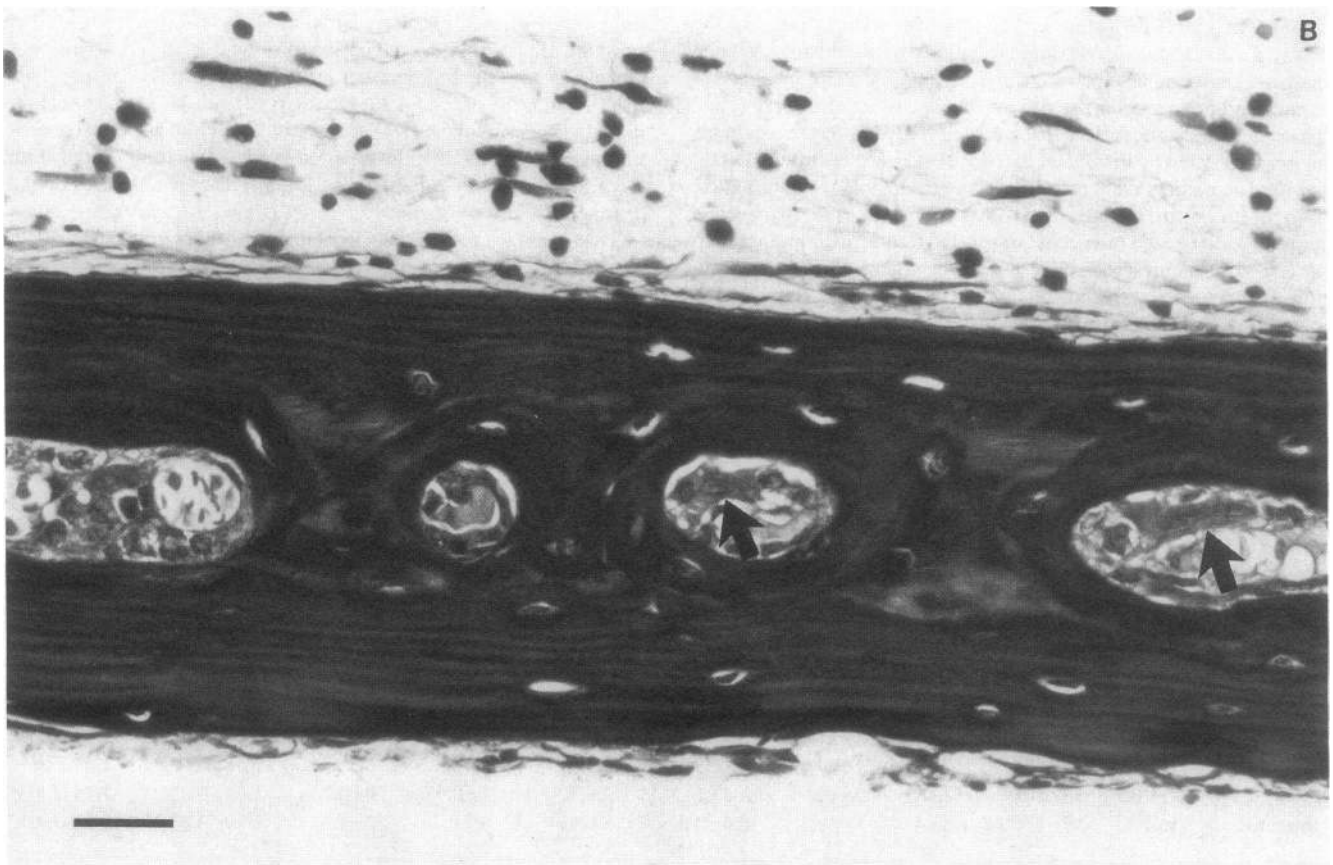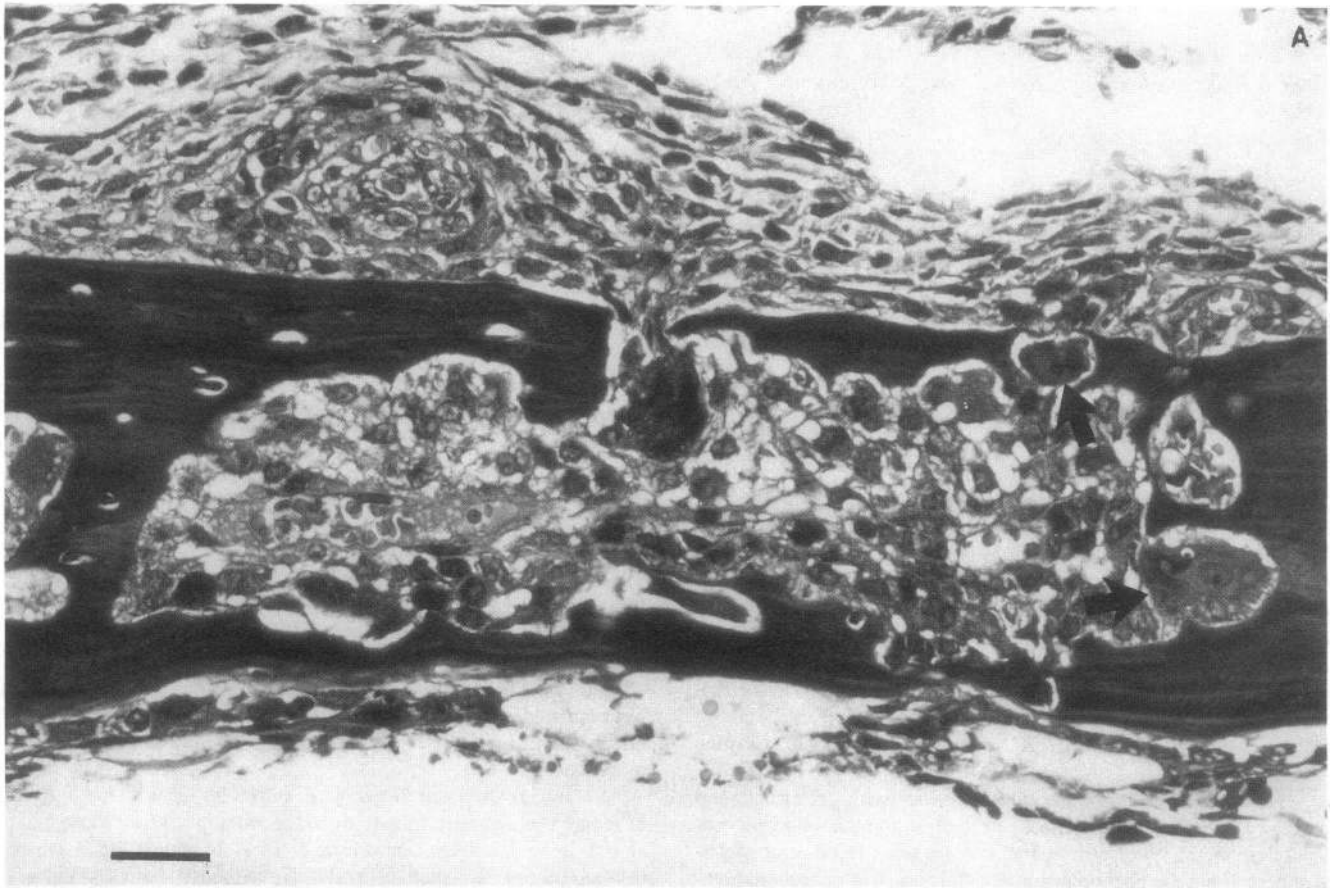
Figure 1. (*A*) Histologic section through the posterior half of the calvaria of a wild type mouse treated with four daily local injections of 0.1 μg IL-1α for three days to stimulate bone resorption. Multinucleate osteoclasts (*arrows*) are seen within deep Howship's lacunae resorbing the bone matrix. A normal ruffled border is extending from the osteoclast to the bone surface (*bottom right arrow*). The bar represents 25 μm. (*B*) Histologic section through the posterior half of the calvaria of a src- mutant mouse treated with four daily local injections of 0.1 μg IL-1α for three days. Multinucleate osteoclasts (*arrows*) have appeared on the bone surface but have failed to form Howship's lacunae or ruffled borders. The bar represents 25 μm.
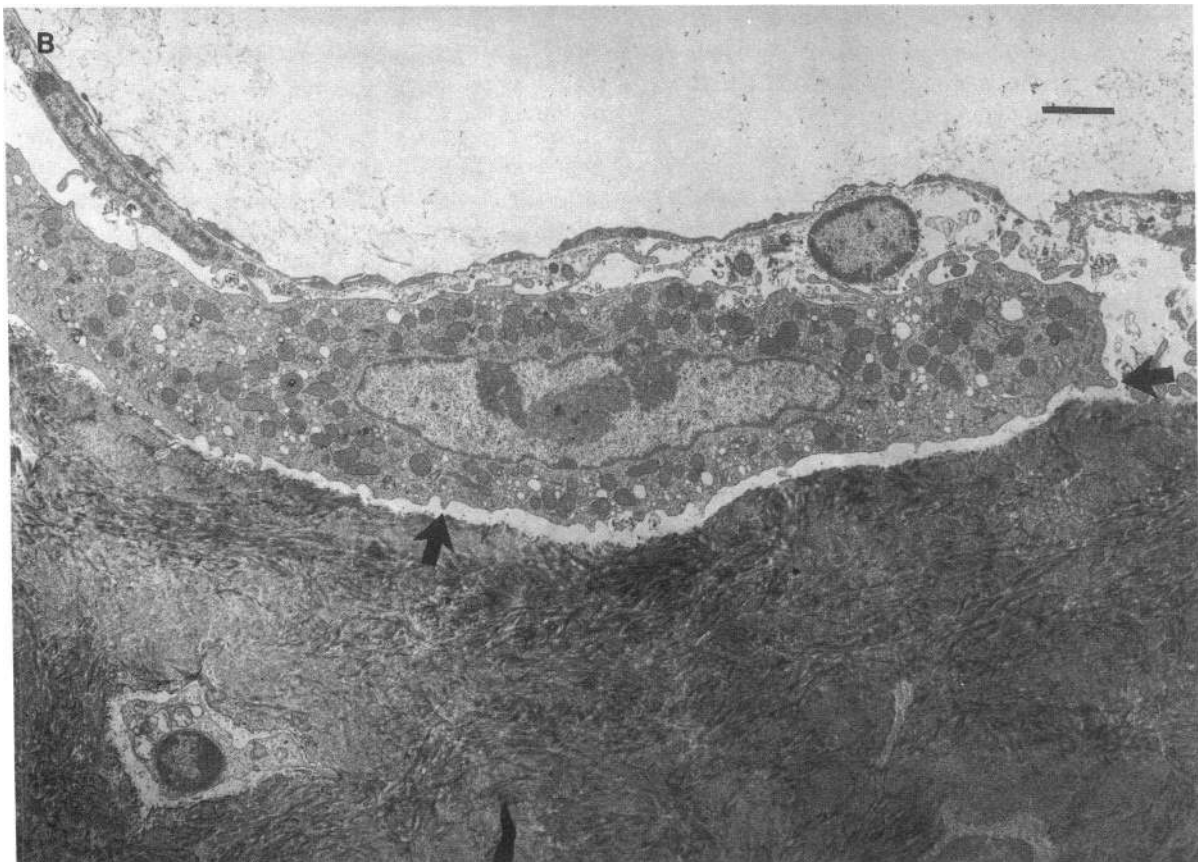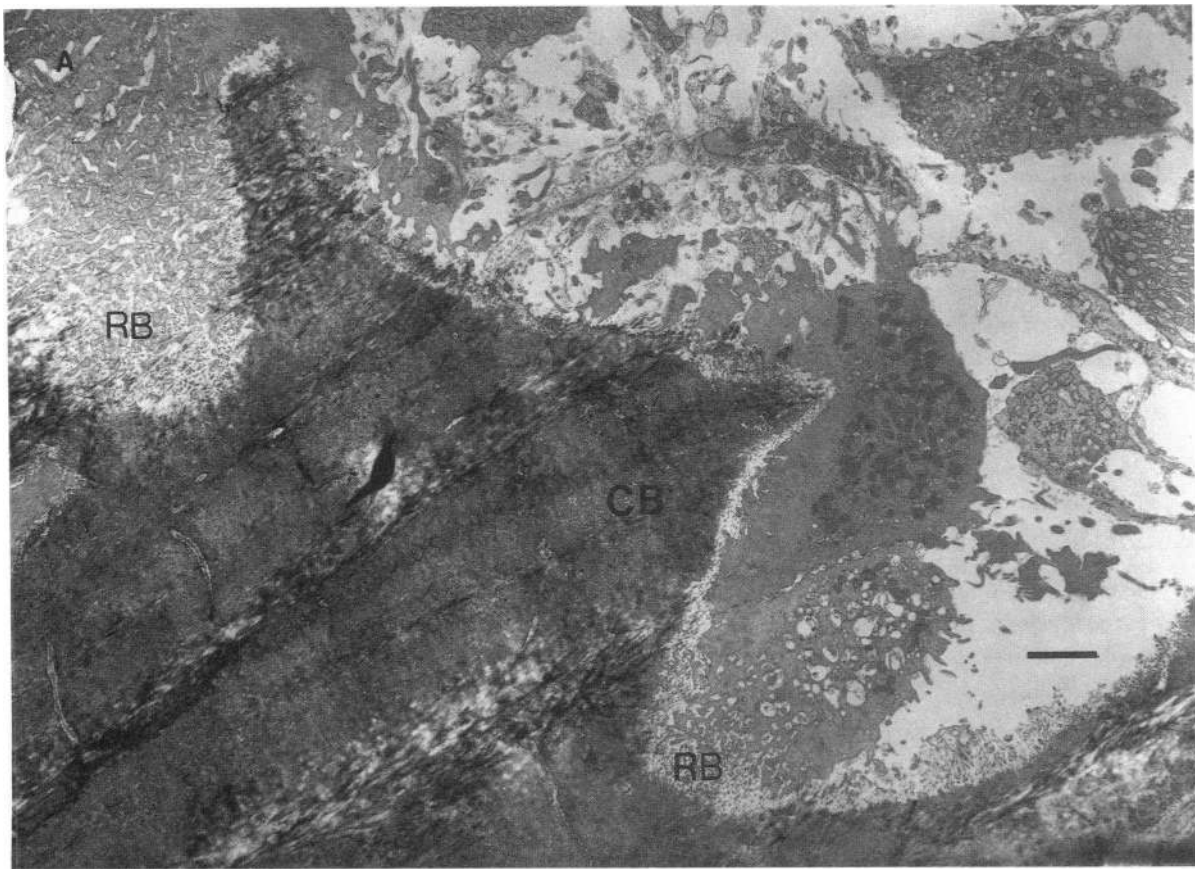
Figure 2. (*A*) Electron micrograph of a section of calvarial bone from a wild type mouse treated with multiple daily local injections of PTH-rP for three days. Osteoclasts are resorbing the calcified bone within Howship's lacunae, where their ruffled borders are closely applied to the bone surface. The bar represents 2 μm. (*B*) Electron micrograph of a section of calvarial bone from a src-mutant mouse treated with multiple daily local injections of PTH-rP for three days. An elongated osteoclast with abundant intracytoplasmic vacuoles and mitochondria is present on the bone surface. Although occasional cytoplasmic extensions (*arrows*) extend from the cytoplasmic membrane to the bone surface, the cell has not formed a ruffled border. The bar represents 2 μm.

analysis of variance. The total resorption area was divided by the number of TRAP(+) multinucleated cells to give an estimate of the mean area resorbed by each osteoclast.

Results

The primary spongiosa thickness and bone volumes in the humeri and vertebrae of the src-deficient mutants were greater than those in the wild type mice (Table I). However, there was no difference in the thickness of the epiphyseal plates of the humeri or of the cartilaginous end plates of the vertebrae, indicating that there was no major defect in the formation of cartilage. Osteoclast numbers, expressed per millimeter of the cancellous bone surface in the vertebral bodies, were significantly higher in the mutants than in the wild type mice. However, there was no significant difference in osteoclast numbers expressed per millimeter of the 1° spongiosa bone surface of the humeri in the mutant or wild type mice. These data suggest that the increased amount of bone in the mutant mice may be caused by a defect in the function rather than in the formation of osteoclasts.

In normal wild type mice, PTH, PTH-rP, and IL-1 led to increased osteoclast numbers in the calvariae of the treated animals (Table II). There was also an increase in response to each of these factors as assessed by the percentage of bone surface eroded by osteoclasts and of the bone surface covered by osteoclasts. In addition, there was an increase in the depth of resorption lacunae under the osteoclasts. IL-1 increased the resorption depth under the osteoclasts even more than PTH or PTH-rP. In src-deficient mice, all three factors caused an increase in osteoclast number and osteoclast surface, comparable with the increase seen in wild type mice (Fig. 1). However, there was no significant resorption under the osteoclasts or increase in the extent of the inactive eroded surface without osteoclasts in the src-deficient animals (Table II). Furthermore, the appearance of the osteoclasts in the src- mutant mice differed from that in the wild type mice. Most of these cells were flat and elongated with smooth surfaces and lacked the cytoplasmic ruffling seen in the activated and more rounded osteoclasts in the wild type mice (Fig. 1). Thus, although increased numbers of osteoclasts appeared on the bone surfaces, they did not resorb the bone matrix.

In the IL-1–treated mice, blood calcium levels in the wild type mice were significantly greater than in the untreated mice, but there was no increase in blood calcium in the src-deficient mice (Table II). In the PTH– and PTH-rP–treated src-deficient mice, there was a similar increase in blood calcium to that seen in wild type mice treated with these factors. This increase in blood calcium in the src-deficient mice was presumably attributable to the well-known effects of PTH and PTH-rP on the kidney to increase the reabsorption of calcium from the renal tubules.

Ultrastructural examination of osteoclasts in each of the three specimens from the IL-1– and PTH-rP–treated groups confirmed the absence of ruffled borders, the complex cytoplasmic folds typical of actively resorbing osteoclasts, in the src-mutants but not in the wild type mice (Fig. 2).

After 6-d culture of bone marrow cells from WT mice on the dentine slices in the presence of 1,25D$_3$, many TRAP(+)MNCs were formed (Fig. 3), and these cells formed resorption pits. Comparable numbers of TRAP(+)MNCs were also seen in src-mutant marrow cell cultures. However,
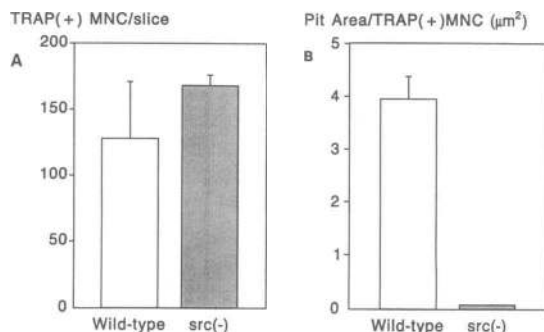

Figure 3. (*A*) Formation of TRAP(+)MNC in WT and src-mutant mice. Bone marrow cells (1 × 10^6/well, 24-well plate) from WT and src-mutant mice were cultured on the dentine slices in the presence of 10^{-8} M 1,25D$_3$ for 6 d. The number of TRAP(+)MNC with more than three nuclei was counted under light microscopy. Values are mean±SEM of four wells. The experiment was performed twice and produced similar results. (*B*) Area of resorption pits formed under TRAP(+)MNC in wild type and src-mutant mice. After cells were removed, the dentine slices were stained with toluidine blue and pit area was measured as described in Methods. Despite the large number of TRAP(+)MNC present, no resorption pits were seen on the dentine slices on which the src-mutant cells had been cultured.

they failed to form resorption pits (Fig. 3). These in vitro results are consistent with the in vivo findings described above and further support the notion that pp60^{c-src} expression is crucial for osteoclast function, but not formation.

Discussion

Our findings indicate that the expression of pp60^{c-src} is necessary for the formation of normal ruffled borders in activated osteoclasts. Although it is not certain from our studies whether the effect is direct or indirect, the absence of src is clearly associated with a failure of ruffled border formation and impaired osteoclastic bone resorption. Other, as yet undiscovered, factors may also be necessary. The absence of these or pp60^{c-src} could be responsible for the failure of ruffled border formation that has been observed in some other forms of osteopetrosis in humans and animals (2). Little is known about the regulation of the formation of ruffled borders in osteoclasts. However, it is likely that reorganization of cytoskeletal proteins, such as actin, α-actinin, talin, and tubulin, takes place in activated osteoclasts (10), and tubulin has been shown to be a substrate for pp60^{c-src} in normal brain cells (11, 12). Tubulin, vinculin, talin, and the transmembrane receptor for fibronectin are substrates for pp60^{v-src} in Rous sarcoma virus transformed cells, and some of these are localized to sites of cytoplasmic ruffling (13). Thus, the absence of ruffled border formation in the osteoclasts of src-mutant mice may be related to defective tyrosine phosphorylation of cytoskeletal proteins. Together with a recent preliminary report of the detection of pp60^{c-src} expression in chicken osteoclasts (14), this suggests that the major defect in the src-deficient mutants may be in the osteoclast. However, osteoblasts have a pivotal role in the control of bone resorption (15), and it remains possible that normal pp60^{c-src} expression is required in these cells for osteoclastic bone resorption to occur.

Recent observations in a naturally occurring murine model of osteopetrosis indicate that other tyrosine kinases may also be

essential for normal osteoclast function. In the op mouse mutation, there is a decrease in the formation of osteoclasts (2). Thus, this variant of murine osteopetrosis appears to be distinct from the osteopetrosis in src-mutant mice where osteoclast formation is not altered. These mice have impaired expression of M-CSF (3), the colony stimulating factor for cells of the monocyte-macrophage lineage, and the defect can be cured by treatment with M-CSF (4, 16), which is produced in bone marrow by accessory cells (9), but not by bone marrow transplantation. The receptor for M-CSF is c-fms, a receptor tyrosine kinase (17). Since this variant of osteopetrosis is caused by decreased osteoclast formation, and in the src-deficient mice the defect is not in osteoclast formation, but rather in the capacity of mature osteoclasts to form ruffled borders and resorb bone, these data together show that separate and distinct tyrosine kinases are required for both normal osteoclast generation from hematopoietic progenitor cells and bone resorption by mature osteoclasts.

Acknowledgments

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