Journal of Cellular Biochemistry 58:424–435 (1995)

ARTICLES

Possible Involvement of Focal Adhesion Kinase, p125^{FAK}, in Osteoclastic Bone Resorption

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Abstract Involvement of tyrosine phosphorylation in osteoclastic bone resorption was examined using osteoclast-like multinucleated cells prepared from co-cultures of mouse osteoblastic cells and bone marrow cells in the presence of 1α,25-dihydroxyvitamin D_3. When osteoclast-like cells were plated on culture dishes in the presence of 10% fetal bovine serum, they were sharply stained in their peripheral region by anti-phosphotyrosine antibody. Western blot analysis revealed that 115- to 130-kD proteins were tyrosine-phosphorylated in osteoclast-like cells. Using immunoprecipitation and immunoblotting, one of the proteins with 115–130 kD was identified as focal adhesion kinase (p125^{FAK}), a tyrosine kinase, which is localized in focal adhesions. Immunostaining with anti-p125^{FAK} antibody revealed that p125^{FAK} was mainly localized at the periphery of osteoclast-like cells. Herbimycin A, a tyrosine kinase inhibitor, not only suppressed tyrosine phosphorylation of p125^{FAK} but also changed the intracellular localization of p125^{FAK} and disrupted a ringed structure of F-actin-containing podosomes in osteoclast-like cells. Antisense oligodeoxynucleotides to p125^{FAK} inhibited dentine resorption by osteoclast-like cells, whereas sense oligodeoxynucleotides did not. These results suggest that p125^{FAK} is involved in osteoclastic bone resorption and that tyrosine phosphorylation of p125^{FAK} is critical for regulating osteoclast function.

Key words: osteoclast, focal adhesion kinase, tyrosine kinase, tyrosine phosphorylation, podosome

Osteoclasts are primary bone-resorbing cells, and play a critical role in bone remodeling [Suda et al., 1992]. However, progress in the biochemical and molecular analysis of osteoclasts has been slow because of the difficulty in obtaining a large number of mammalian osteoclasts. We previously reported that osteoclast-like multinucleated cells (OCLs) were formed in co-cultures of mouse osteoblastic cells and bone marrow cells in the presence of bone-resorbing agents such as 1α,25-dihydroxyvitamin D_3[1α,25$(OH)_2$-D_3], parathyroid hormone and prostaglandin E_2 [Takahashi et al., 1988; Akatsu et al., 1989]. More recently, we modified the co-culture system to prepare a large number of OCLs [Akatsu et al., 1992]. OCLs formed in this co-culture system were tartrate-resistant acid phosphatase (TRAP)-positive, possessed many calcitonin receptors, and formed resorption pits on dentine slices, satisfying major criteria of osteoclasts [Takahashi et al., 1988]. Therefore, OCLs are considered authentic mouse osteoclasts.

Several lines of evidence have proven that signaling mediated by protein tyrosine kinases is important in osteoclastic bone resorption. Targeted disruption of c-*src* in mice induced osteopetrosis, a disease characterized by reduced osteoclastic bone resorption [Soriano et al., 1991]. More recently, it was revealed that osteoclasts expressed high levels of p60$^{c\text{-}src}$ [Horne et al., 1992; Tanaka et al., 1992] and that osteoclasts of c-*src* gene-deficient mice had defects in their bone-resorbing activity [Boyce et al., 1992]. These results indicate that signaling mediated by p60$^{c\text{-}src}$ tyrosine kinase plays an important role in osteoclastic bone resorption.

Focal adhesion kinase (p125^{FAK}) was first identified as one of the tyrosine-phosphorylated proteins in chick embryo fibroblasts transformed by v-*src* or activated variants of c-*src* [Schaller et

Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; p125^{FAK}, focal adhesion kinase; OCLs, osteoclast-like multinucleated cells.

Received November 9, 1994; accepted November 29, 1994.

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al., 1992]. Almost simultaneously, the murine counterpart of $p125^{FAK}$ was cloned using a homology-based cDNA cloning approach to identify novel protein tyrosine kinases [Hanks et al., 1992]. The mRNA of $p125^{FAK}$ was expressed in various adult rat and mouse tissues and whole embryos [Hanks et al., 1992]. Immunostaining with anti-$p125^{FAK}$ antibody revealed that $p125^{FAK}$ was localized at focal adhesions of normal fibroblasts and KB carcinoma cells. This indicates that $p125^{FAK}$ is somehow involved in cytoskeletal organization [Schaller et al., 1992; Hanks et al., 1992; Burridge et al., 1992; Kornberg et al., 1992]. Various cytoskeletal proteins including talin [Pasquale et al., 1986], vinculin [Sefton et al., 1981], fibronectin receptors [Hirst et al., 1986], and p80/85 [Wu et al., 1991] are shown to be phosphorylated on tyrosine residues in RSV-transformed cells. A unique feature of $p125^{FAK}$ is that it is a novel type of tyrosine kinase [Schaller et al., 1992], and that tyrosine phosphorylation of $p125^{FAK}$ increases its tyrosine kinase activity [Guan and Shalloway, 1992]. It has also been shown that $p125^{FAK}$ is tyrosine-phosphorylated in response to cell attachment to extracellular matrices such as fibronectin and vitronectin, to stimulation of blood platelets by thrombin and collagen, to stimulation of Swiss 3T3 cells by several neuropeptides, and to the aggregation of high-affinity IgE receptors in rat basophilic leukemia cells [Burridge et al., 1992; Kornberg et al., 1992; Lipfert et al., 1992; Zachary et al., 1992; Hamawy et al., 1993]. These results suggest that $p125^{FAK}$ is involved in the signaling of various aspects of cell function.

We report here that $p125^{FAK}$ is phosphorylated on tyrosine residues in osteoclasts, and that antisense oligodeoxynucleotides to $p125^{FAK}$ inhibit bone-resorbing activity of osteoclasts. Herbimycin A not only inhibited tyrosine phosphorylation of $p125^{FAK}$ but also changed the intracellular localization of $p125^{FAK}$ in osteoclasts.

MATERIALS AND METHODS

Antibodies and Chemicals

Anti-phosphotyrosine monoclonal antibody (25.3G4) was kindly provided by Dr. T. Yamori (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research). Another anti-phosphotyrosine antibody (py20) was purchased from ICN Biomedicals (Tokyo, Japan). Anti-$p125^{FAK}$ monoclonal (2A7) and polyclonal (BC3) antibodies were kindly provided by Dr. J.T. Parsons (University of Virginia). Rhodamine-conjugated phalloidin was from Molecular Probes Inc. (Eugene, OR). Herbimycin A and methyl 2,5-dihydroxycinnamate were kindly provided by Kyowa Medex Co. (Tokyo). $1\alpha,25(OH)_2D_3$ was purchased from Wako Pure Chemical Co. (Osaka, Japan). Alpha-minimum essential medium (αMEM) was obtained from Flow Laboratories (McLean, VA). Other chemicals and reagents used in this study were of analytical grade.

Oligodeoxynucleotide Preparation

Synthetic phosphorothioate oligodeoxynucleotides that include ATG initiation codon of the cDNA for mouse $p125^{FAK}$ were used in the sense and antisense orientation:

Sense-1 ($p125^{FAK}$ sense-1)	5′-AAAAGAATG-GCAGCTGCTTA-TCTT-3′
Antisense-1 (antisense to sense-1)	3′-TTTTCTTACC-GTCGACGAATA-GAA-5′
Sense-2 ($p125^{FAK}$ sense-2)	5′-CTAGCATCT-AGCAAAAGAAT-GGCA-3′
Antisense-2 (antisense to sense-2)	3′-GATCGTAGA-TCGTTTTCTTA-CCGT-5′

These oligodeoxynucleotides were synthesized and purified by Sawadi Technology Co. (Tokyo) and added to pit formation assay on dentine slices. In some experiments, each antisense oligomer was mixed with its sense oligomer in an equal amount for 24 h. These preparations were also used for dentine-resorbing assay.

Cell Culture

Mouse osteoclast-like multinucleated cells (OCLs) were prepared according to the method previously reported using ddY mice [Akatsu et al., 1992]. Briefly, primary osteoblastic cells obtained from newborn mouse calvaria and bone marrow cells obtained from 7- to 9-week-old male mouse tibiae were co-cultured in αMEM containing 10% fetal bovine serum (FBS) (Gibco,

Grand Island, NY) in the presence of 10 nM $1\alpha,25(OH)_2D_3$. Culture dishes (ϕ 10 cm, Corning 25020, Corning, NY) were precoated with 4 ml of 0.2% collagen gel matrix (Nitta Gelatin Co., Osaka, Japan). OCLs were formed within 6 days of culture. They were released from dishes by treating with 4 ml of 0.2% collagenase (Wako, Osaka, Japan) and enriched by the 35% percoll density centrifugation technique. After percoll density centrifugation, the purity of OCLs was about 5% (enriched OCL fractions) [Akatsu et al., 1992]. These fractions were used for dentine resorption assay. For the purpose of protein analysis of OCLs, they were further purified according to the method previously reported for rabbit osteoclast preparations [Tezuka et al., 1992]. In short, enriched OCL fractions were replated on culture dishes (ϕ 10 cm, Corning) in the presence of 10% FBS. After cells were incubated for 4 h, dishes were treated with 0.001% pronase and 0.02% EDTA for 10 min in order to remove osteoblastic cells. After treatment with pronase and EDTA, more than 80% of the adherent cells on the dishes were multinucleated OCLs and TRAP-positive mononuclear cells, possible precursors of OCLs. In the final preparation, more than 90% of the total protein was estimated to be derived from OCLs (purified OCL fractions).

Dentine-Resorbing Assay

Dentine-resorbing activity of OCLs was assessed by the method previously reported by Tamura et al. [1993]. Enriched OCL fractions were suspended in αMEM containing 10% FBS (5×10^5 cells/ml) on dentine slices (ϕ 4 mm) placed in a 96-well culture plate (0.1 ml/slice). After incubation for 90 min at 37°C, the slices were put into 24-well plates (one slice/well), and incubated for 48 h with or without test compounds. At the end of the culture period, slices were placed in 1 M NH_4OH and adherent cells were removed by ultrasonication. Resorption pits were visualized by staining the slices with Mayer's hematoxylin solution. The resorbed surface area was measured using an image analysis system (LA-525, PIAS Co., Tokyo).

Western Blot Analysis

All extraction procedures were performed at 4°C. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), then lysed with RIPA buffer [10 mM Tris-HCl (pH 7.8), 1% (v/v) Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate, 1 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium fluoride, and aprotinin at 10 μg/ml]. RIPA extracts were prepared by centrifugation at 12,000 *g* for 15 min. Samples containing an equal amount of proteins were suspended in Laemmli sample buffer, and electrophoresed on SDS-polyacrylamide gels [Laemmli, 1970]. After electrophoresis, proteins were transferred to Immobilon-P (Millipore Co., Bedford, MA). Immunostaining with antibodies was performed using ECL Western blotting detection reagents (Amersham International plc., Amersham place, UK).

Immunoprecipitation

For immunoprecipitation of $p125^{FAK}$, lysates were incubated with anti-$p125^{FAK}$ polyclonal antibody BC3 (3 μl/mg of protein) for 1 h at 4°C. Immune complexes were recovered by adding protein G-agarose (PIERCE, Rockford, IL) to the mixture and incubated for 1 h at 4°C. Immune complexes were then collected by centrifugation, washed with RIPA buffer 3 times, and eluted by boiling for 3 min in Laemmli sample buffer.

Immunohistochemistry

Cultured cells were fixed with methanol/acetone (50:50, v/v) for 10 min at 4°C. Intrinsic peroxidase was blocked by incubating with 3% H_2O_2 for 10 min. Nonspecific staining was blocked with a blocking agent (Histofine, Nichirei Co., Tokyo). Cells were incubated with the first antibody for 1 h at room temperature, and the bound antibodies were visualized with biotinylated second antibody, avidin-biotin-conjugated peroxidase, and a DAB substrate kit (Histofine). For detecting actin localization, cells which had been precultured for 4 h on 24-well plates (Corning) were treated with or without 1 μg/ml of herbimycin A for 0, 0.5, 1, 4, and 16 h. Cells were then fixed with 4% formaldehyde for 10 min at 4°C, permeated with 0.1% Triton X-100 for 5 min, and incubated with rhodamine-conjugated phalloidin for 30 min [Kanehisa et al., 1990]. Cells were washed in PBS and examined under an Olympus microscope.

RESULTS

Distribution of Phosphotyrosine in OCLs

When mouse osteoblastic cells were co-cultured with mouse bone marrow cells on collagen-coated dishes in the presence of 10 nM $1\alpha,25(OH)_2D_3$, OCLs were formed within 6 days.

Enriched OCL fractions were prepared by collagenase digestion and percoll density centrifugation, suspended in αMEM containing 10% FBS, and plated on culture dishes (24-well plates). After the cells were incubated for 4 h, distribution of phosphotyrosine-containing proteins was examined by immunostaining with 25.3G4. OCLs were sharply stained by the antibody at their periphery, whereas osteoblastic cells were only stained weakly (Fig. 1A). Neither OCLs nor osteoblastic cells were stained by nonspecific IgG (Fig. 1B). A similar staining pattern was also observed using another anti-phosphotyrosine antibody (py20) (data not shown).

Tyrosine Kinase Inhibitors Inhibit Osteoclastic Bone Resorption

Herbimycin A, a benzoquinonoid ansamycin antibiotic, has been shown to induce inactivation of v-*src* tyrosine kinase and reduce cellular phosphotyrosine content in RSV-transformed cells [Uehara et al., 1986]. When graded concentrations of herbimycin A were added to enriched OCL preparations cultured on dentine slices in the presence of 10% FBS, dentine resorption by OCLs was inhibited dose-dependently. Herbimycin A at 1 μg/ml inhibited dentine resorption almost completely (Fig. 2). This concentration of herbimycin A was reported to cause no cytotoxicity on PT18 cells [Satoh et al., 1992]. In agreement with our findings, Yoneda et al. [1993] reported that herbimycin A inhibited osteoclastic bone resorption in vivo and in vitro. Another tyrosine kinase inhibitor, methyl 2,5-dihydroxycinnamate, also inhibited dentine resorption (Fig. 2). These results indicate that signaling mediated by tyrosine kinases is indeed critical for osteoclastic bone resorption.

Tyrosine Kinase Inhibitors Suppress Podosome Formation of OCLs

When enriched OCL preparations were plated on culture dishes in the presence of 10% FBS, OCLs spread out on the dishes and formed a ringed structure of F-actin-containing dots called podosomes (Fig. 3A) [Marchisio et al., 1984]. It has been reported that functionally active osteoclasts form podosomes [Lakkakorpi and Väänänen, 1991]. After the cells were incubated for 4 h, more than 90% of OCLs formed podosomes (Fig. 3C). When OCLs were treated with 1 μg/ml of herbimycin A, the intracellular distribution of actin-containing dots shifted from the periphery to the central region of OCLs (Fig. 3B). The redistribution was first observed in OCLs 4 h after the addition of herbimycin A. Most of the

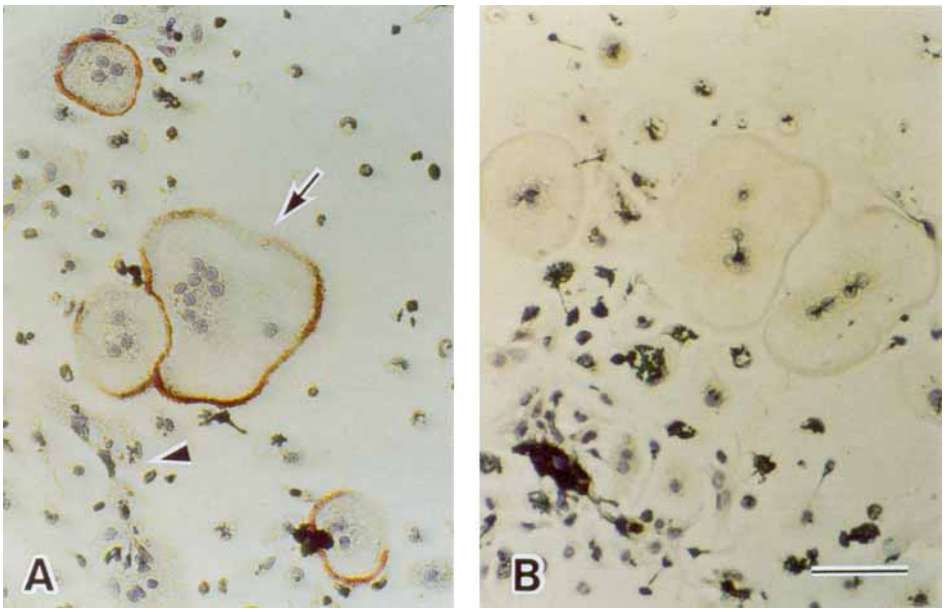

Fig. 1. Localization of phosphotyrosine in OCLs plated on culture plates. Enriched OCL fractions were plated on 24-well culture plates in the presence of 10% FBS for 4 h. Cells were then stained for phosphotyrosine (**A**) or nonimmune IgG (**B**). Note that OCLs were sharply stained with anti-phosphotyrosine antibody at the peripheral region (*arrow*). Osteoblastic cells showed only faint staining (*arrowhead*). Neither OCLs nor osteoblastic cells were stained with nonspecific IgG. Bar = 50 μm.

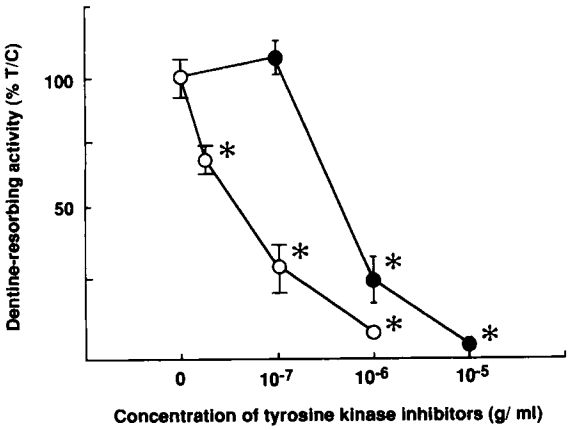

Fig. 2. Effects of tyrosine kinase inhibitors on dentine resorption by OCLs. Enriched OCL fractions were cultured on dentine slices with graded concentrations of tyrosine kinase inhibitors. After culturing for 2 days, cells were removed by ultrasonication and resorption pits formed on dentine slices were stained with Mayer's hematoxylin. Dentine-resorbing activity of OCLs was quantified by measuring the resorbed surface area using an image analysis system. Both herbimycin A (○) and methyl 2,5-dihydroxycinnamate (●) inhibited dentine resorption by OCLs dose-dependently. Surface area resorbed by OCLs in the control cultures without inhibitors was 48.0 ± 3.1% of the total surface area. *Significantly different from the group treated without inhibitors, $P < 0.01$.

OCLs with podosomes disappeared within 16 h (Fig. 3C). Another tyrosine kinase inhibitor (methyl 2,5-dihydroxycinnamate) had similar effects (data not shown). These results indicate that tyrosine kinases are important for cytoskeletal organization and activation of OCLs.

Western Blot Analysis of Tyrosine Phosphorylation in OCLs

OCLs were enriched and replated on plastic dishes in the presence of 10% FBS. After the cells were incubated for 4 h, contaminating osteoblastic cells were removed by treating the dishes with pronase and EDTA to yield purified OCL fractions. Western blotting with anti-phosphotyrosine antibody revealed that several proteins were phosphorylated on tyrosine residues in purified OCL fractions (Fig. 4A). Among them, proteins with molecular weight of 115–130 kD were preferentially phosphorylated. When purified OCL fractions were treated with 1 μg/ml of herbimycin A, tyrosine phosphorylation of the proteins decreased time-dependently (Fig. 4A). Protein contents appeared to be unaffected by the treatment of the cells with herbimycin A (Fig. 4B).

Tyrosine Phosphorylation of p125^{FAK} in OCLs

To examine tyrosine phosphorylation of p125^{FAK} in OCLs, cell lysates of the purified OCL fractions were immunoprecipitated with anti-p125^{FAK} polyclonal antibody (BC3). Tyrosine phosphorylation of the immunoprecipitates was analyzed by Western blotting with py20. p125^{FAK} was proved to be phosphorylated on tyrosine residues in OCLs, and the phosphorylation of p125^{FAK} was time-dependently inhibited by treating the cells with 1 μg/ml of herbimycin A (Fig. 5A). Parallel Western blots with anti-p125^{FAK} antibody BC3 indicated that the treatment with herbimycin A for 16 h induced no significant change in the amount of p125^{FAK} in the purified OCLs (Fig. 5B). This indicates that one of the tyrosine-phosphorylated proteins with 115–130 kD in OCLs is p125^{FAK}.

Herbimycin A Shifts the Intracellular Localization of p125^{FAK} in OCLs

To examine the distribution of p125^{FAK} in OCLs, enriched OCL fractions were immunostained with anti-p125^{FAK} antibody. Enriched OCL fractions were plated on culture dishes in the presence of 10% FBS. After the cells were incubated for 4 h, OCLs were stained with monoclonal antibody 2A7. As shown in Figure 6A,B, OCLs were clearly stained with antibody in the peripheral region. The staining pattern of OCLs with 2A7 was similar to that with anti-phosphotyrosine antibody (see Fig. 1A). When OCLs were treated with 1 μg/ml of herbimycin A for 4 h, distribution of p125^{FAK} shifted from the periphery to the central region of OCLs (Fig. 6C,D). Similar results were obtained using another anti-p125^{FAK} polyclonal antibody BC3 (data not shown).

Antisense Oligodeoxynucleotides to p125^{FAK} Inhibit Dentine Resorption by OCLs

To further confirm the importance of p125^{FAK} in osteoclastic bone resorption, the effects of antisense oligodeoxynucleotides to p125^{FAK} on dentine resorption by OCLs were examined (Fig. 7A,B). Treatment with antisense oligomers to p125^{FAK} at 75 μg/ml inhibited dentine resorption induced by OCLs to a level about 60% of the control (Fig. 7). In contrast, sense oligomers to p125^{FAK} had no inhibitory effect. The inhibitory effect of antisense oligomers was abolished, when antisense oligomers were preincubated with the respective sense oligomers (Fig. 7).

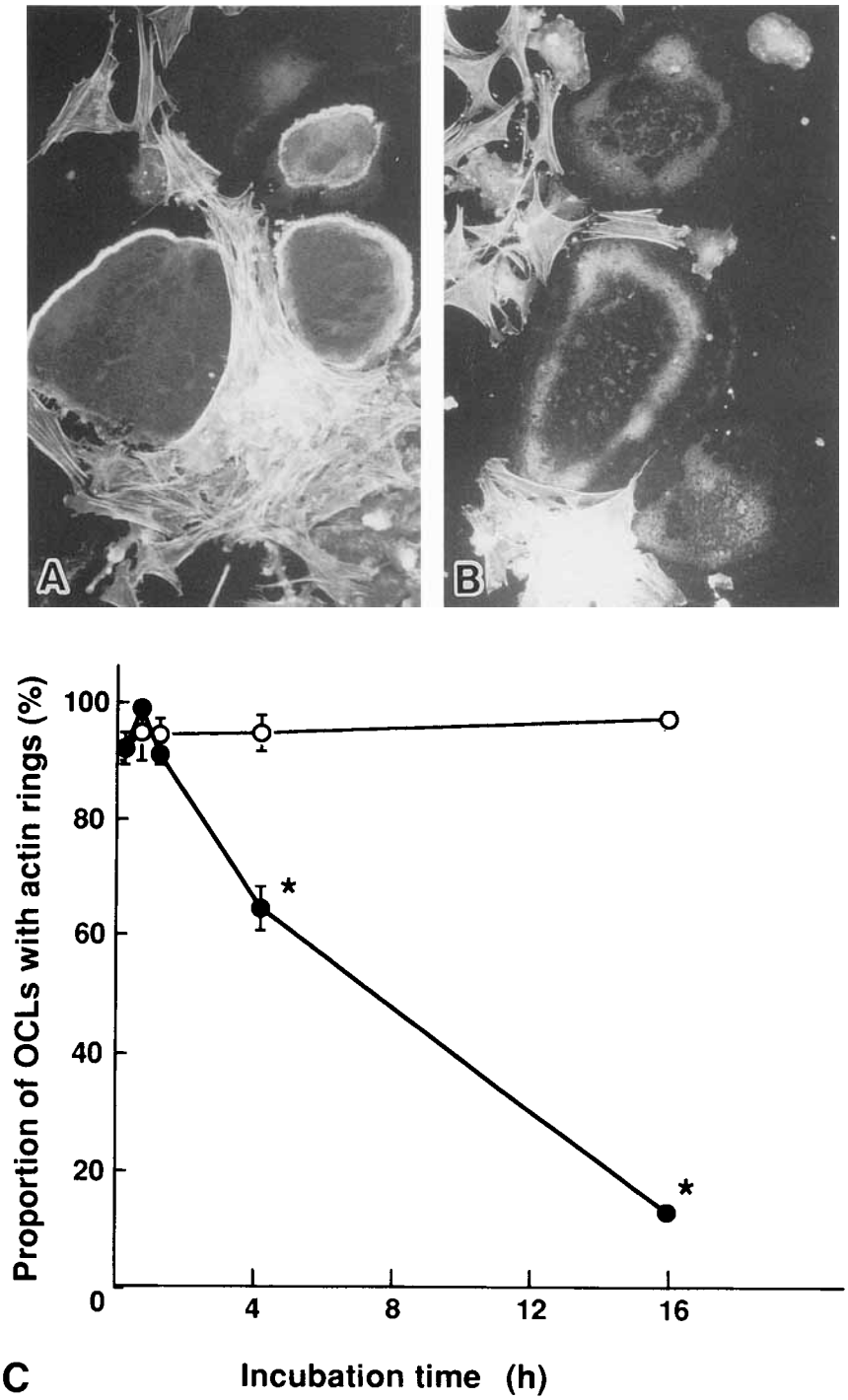

Fig. 3. Inhibition by herbimycin A of podosome formation by OCLs. Enriched OCL fractions were plated on 24-well plates in the presence of 10% FBS for 4 h. Cells were then cultured without (○) or with (●) 1 μg/ml of herbimycin A, and stained with rhodamine-conjugated with phalloidin. **A:** Distribution of F-actin in the control cultures. More than 90% of OCLs formed podosomes at the periphery region. **B:** Distribution of F-actin in cultures treated with 1 μg/ml of herbimycin A for 16 h. Adding herbimycin A disrupted the podosomes of OCLs and induced changes in the intracellular distribution of F-actin from the periphery to the central region. **C:** The OCLs that formed podosomes were counted. Bar = 50 μm. *Significantly different from the group before the treatment with herbimycin A, $p < 0.01$.

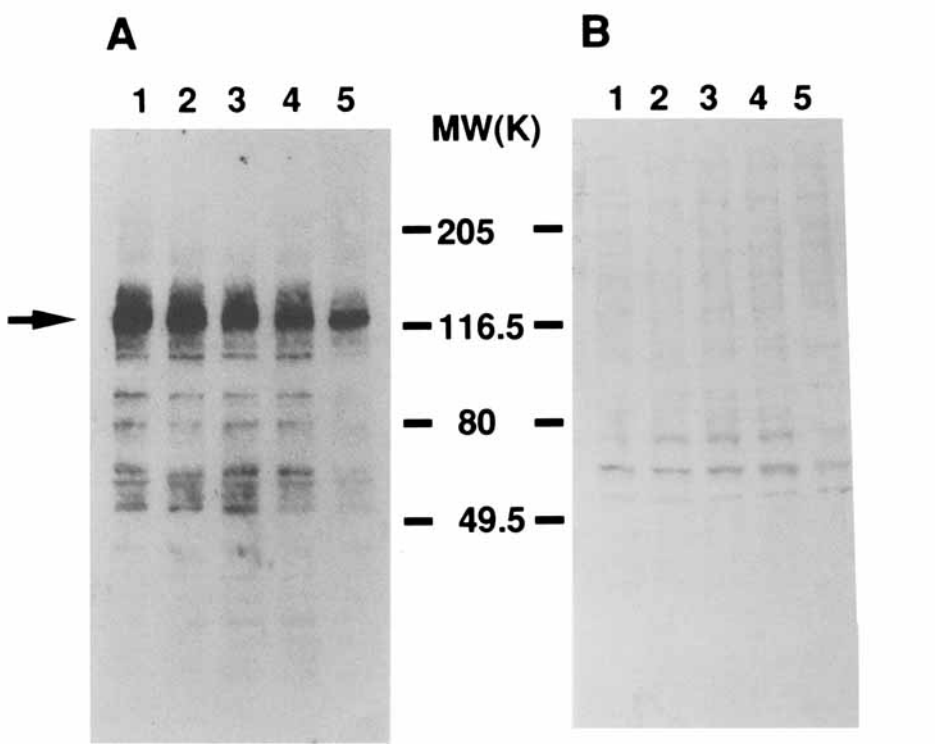

Fig. 4. Time course of change in the overall pattern of tyrosine-phosphorylated proteins in OCLs after herbimycin A was added. Enriched OCL fractions were replated on culture dishes, and OCLs were further purified according to the method described by Tezuka et al. (1992). Purified OCL fractions were treated with 1 μg/ml of herbimycin A for 0 h (*lane 1*), 0.5 h (*lane 2*), 1 h (*lane 3*), 4 h (*lane 4*), and 16 h (*lane 5*), respectively. Cells were lysed with RIPA buffer, and tyrosine-phosphorylated proteins in OCLs were detected by immunoblotting with py20 (**A**). The same membrane was stained with Coomassie brilliant blue (**B**). An intense broad band indicating tyrosine phosphorylation was detected at 115–130 kD (*arrow*), and herbimycin A decreased it time-dependently without any appreciable changes in the protein contents.

DISCUSSION

Signaling mediated by protein tyrosine kinases is involved in several aspects of normal cell function as well as transformation. Targeted disruption of c-*src* induced a functional disorder in the osteoclasts, indicating that the signaling mediated by $p60^{c\text{-}src}$ tyrosine kinase is important for osteoclastic bone resorption [Soriano et al., 1991; Boyce et al., 1992]. This hypothesis was further confirmed by the finding that osteoclasts express high levels of $p60^{c\text{-}src}$ [Horne et al., 1992; Tanaka et al., 1992]. Signaling of $p60^{c\text{-}src}$ tyrosine kinase is mediated by appropriate second messengers, and in this way, tyrosine-phosphorylated proteins in osteoclasts may play an important role in osteoclastic bone resorption. When OCLs were plated on culture dishes in the presence of 10% FBS, they were sharply stained at their peripheral region by anti-phosphotyrosine antibody. Distribution of tyrosine-phosphorylated proteins in OCLs was similar to that of F-actin-containing podosomes. The ringed structure of podosomes is reported to represent an active structure of the clear zone, a specialized area of adhesion of bone resorbing osteoclasts [Kanehisa et al., 1990; Lakkakorpi and Väänänen, 1991; Zambonin-Zallone et al., 1988]. Herbimycin A suppressed podosome formation of OCLs and inhibited dentine resorption by OCLs. In addition, herbimycin A has been shown to prevent hypercalcemia in vivo [Yoneda et al., 1993]. These results clearly indicate that the signaling mediated by tyrosine kinases is important for the cytoskeletal organization and activation of OCLs.

We showed that 115- to 130-kD proteins were mainly phosphorylated on tyrosine residues in OCLs. One of the 115- to 130-kD proteins was identified as $p125^{FAK}$ in OCLs. Using an immunofluorescent technique, Rathod et al. [1994] recently reported that chicken and human osteoclasts possess abundant levels of $p125^{FAK}$. Our immunohistochemical examination revealed that $p125^{FAK}$ was mainly localized at the peripheral

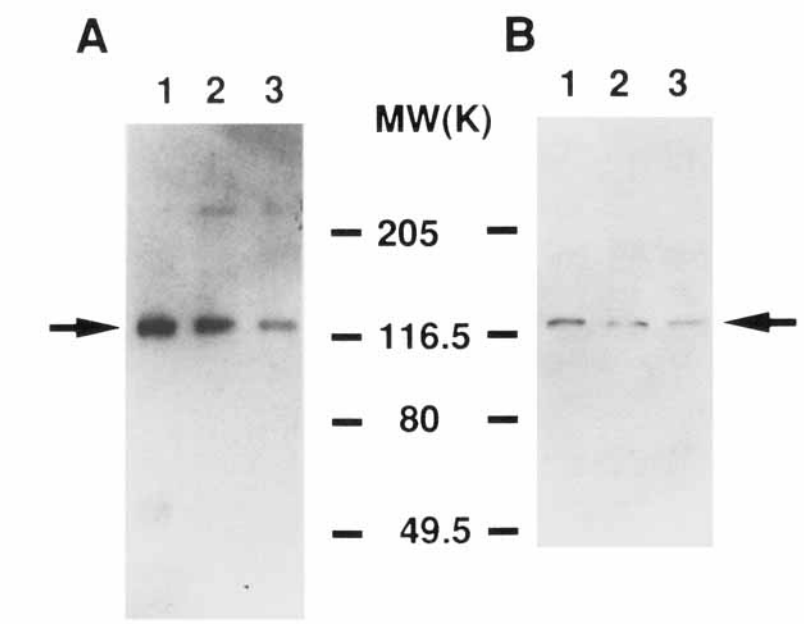

Fig. 5. Identification of p125^{FAK} as a tyrosine-phosphorylated protein in OCLs. **A:** Purified OCL fractions were treated with 1 μg/ml of herbimycin A for 0 h (*lane 1*), 4 h (*lane 2*), and 16 h (*lane 3*), and respective lysates were immunoprecipitated with anti-p125^{FAK} antibody. Tyrosine phosphorylation of p125^{FAK} was detected by immunoblotting with py20. Tyrosine phosphorylation of p125^{FAK} was reduced time-dependently by herbimycin A treatment. **B:** The cell lysates from purified OCL fractions treated with 1 μg/ml of herbimycin A (*lane 1*, 0 h; *lane 2*, 4 h; *lane 3*, 16 h) were subjected to parallel Western blot analysis with anti-p125^{FAK} antibody (BC3). Repeated experiments revealed that the amounts of p125^{FAK} in the purified OCL fractions were not significantly reduced by herbimycin A treatment.

region of OCLs. The localization of p125^{FAK} was similar to that of phosphotyrosine-containing proteins and podosomes. In immunoelectron microscopic examination, we also found that p125^{FAK} is preferentially localized at the clear zone of osteoclasts (data not shown). Herbimycin A not only inhibited phosphorylation of p125^{FAK}, but also shifted the intracellular localization of p125^{FAK} from the periphery to the central region in OCLs. These results suggest that the tyrosine phosphorylation of p125^{FAK} is important for the localization of p125^{FAK} and the actin ring formation in OCLs. Alternatively, collapse of the podosome structure by herbimycin A may have resulted in the decrease in tyrosine phosphorylation and the redistribution of p125^{FAK}. In fact, when actin polymerization was inhibited by cytochalasin D, tyrosine phosphorylation of p125^{FAK} and tensin was suppressed in fibroblasts [Burridge et al., 1992; Bockholt and Burridge, 1993]. Further experiments are needed to clarify the significance of tyrosine phosphorylation of p125^{FAK} and its intracellular localization in osteoclasts.

Experiments with antisense oligodeoxynucleotides provided further evidence of the importance of p125^{FAK} in osteoclastic bone resorption. Antisense oligomers to p125^{FAK} significantly inhibited dentine resorption by OCLs, while sense oligomers to p125^{FAK} did not. The inhibitory effects of antisense oligomers were neutralized by preincubating with their respective sense oligomers. Two different oligomers were used for these experiments, and similar results were obtained. Most of the inhibitors of bone resorption such as calcitonin, bafilomycin A_1 and prostaglandins are known to act directly on osteoclasts [Chambers and Magnus, 1982; Chambers and Ali, 1983; Sundquist et al., 1990]. Therefore, it is likely that the antisense oligomers to p125^{FAK} act directly on OCLs in the dentine resorption assay. However, osteoblastic cells contaminating in OCL preparations also expressed appreciable amounts of p125^{FAK} (data not shown). Thus, the possibility that the antisense oligomers act on cells other than OCLs to inhibit dentine resorption can not be excluded at present.

It is interesting to examine how p125^{FAK} is tyrosine-phosphorylated in OCLs. As has been shown previously, osteoclasts express high levels of p60$^{c\text{-}src}$, and the targeted disruption of c-*src* impairs bone-resorbing activity of osteoclasts [Soriano et al., 1991; Horne et al., 1992;

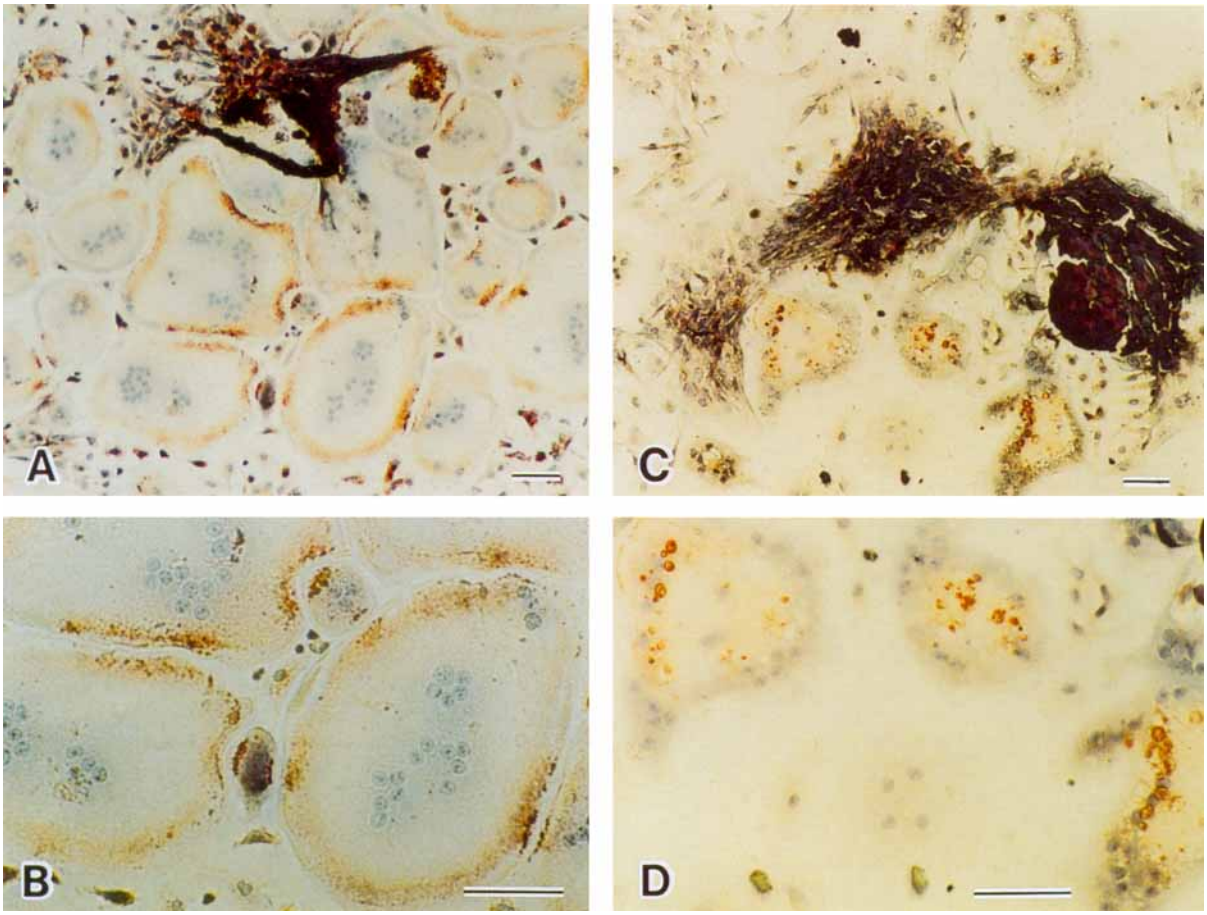

Fig. 6. Changes in the intracellular localization of p125^{FAK} in OCLs after herbimycin A treatment. Enriched OCL fractions were prepared from the co-cultures and plated on culture dishes in the presence of 10% FBS. Cells were further cultured for 4 h in the presence (**C**) or absence (**A**) of 1 μg/ml of herbimycin A. Cells were then fixed and stained with anti-p125^{FAK} antibody (2A7). **B,D:** High-power views of OCLs in **A** and **C**, respectively. Note that p125^{FAK} is localized in the peripheral region of OCLs in the absence of herbimycin A (**A,B**). Adding herbimycin A shifted the intracellular localization of p125^{FAK} from the periphery to the central region of OCLs (**C,D**). Bars = 50 μm.

Tanaka et al., 1992; Boyce et al., 1992]. Guan and Shalloway [1992] reported that overexpression of wild-type p60$^{c\text{-}src}$ increased p125^{FAK} phosphorylation, though the level of phosphorylation was much more modest than that induced by v-*src*. Therefore, it is possible to speculate that p60$^{c\text{-}src}$ is somehow involved in tyrosine phosphorylation of p125^{FAK}. Recently, Parson's group reported that the majority of tyrosine phosphorylation of p125^{FAK} is primarily the consequence of autophosphorylation but not of transphosphorylation by other cellular protein tyrosine kinases in chicken embryo fibroblasts [Hildebrand et al., 1993; Schaller et al., 1994]. Moreover, Cobb et al. [1994] reported that the tyrosine phosphorylated form of p125^{FAK} was tightly associated with pp60^{src} and pp59^{fyn}, and that the association protected the autophosphorylation site of p125^{FAK} from digestion with tyrosine phosphatases. These results provide additional evidence to the hypothesis that p60$^{c\text{-}src}$ is important for the stable tyrosine phosphorylation of p125^{FAK} in OCLs.

Several reports have demonstrated that p125^{FAK} phosphorylation is involved in the signaling of the adhesion receptors of the integrin family [Burridge et al., 1992; Guan and Shalloway 1992; Lipfert et al., 1992]. Vitronectin receptors, $\alpha_v\beta_3$ integrins, have been reported to be highly expressed in osteoclasts, and specific antibodies of the integrins and Arg-Gly-Asp (RGD)-containing peptides inhibited osteoclastic bone resorption [Davis et al., 1989; Horton et al., 1991]. Whether vitronectin receptors are involved in the phosphorylation of p125^{FAK} remains to be clarified and is now under investigation at our laboratory.

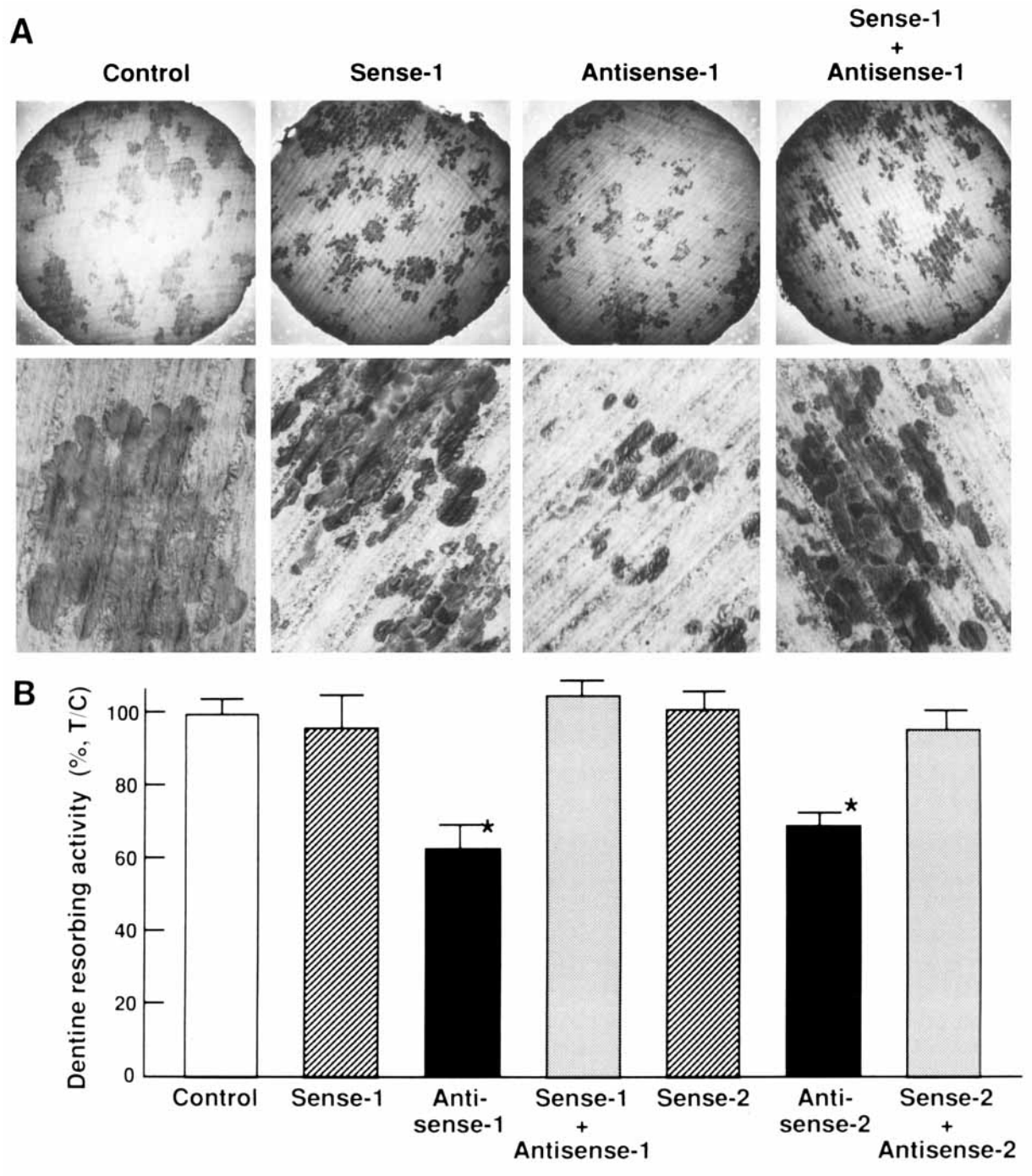

Fig. 7. Inhibition by antisense oligodeoxynucleotides to $p125^{FAK}$ of dentine resorption by OCLs. Enriched OCL fractions were cultured for 48 h on dentine slices (ϕ 4 mm) with sense (sense-1 and sense-2) or antisense (antisense-1 and antisense-2) oligodeoxynucleotides to $p125^{FAK}$ at 75 μg/ml, or a mixture of the sense (75 μg/ml) and antisense oligomers (75 μg/ml). Resorption pits formed by OCLs were stained with Mayer's hematoxylin. **A:** *Upper panels,* typical patterns of resorption pits formed by OCLs on dentine slices in the presence and absence of sense-1, antisense-1, or sense-1 plus antisense-1 oligodeoxynucleotides to $p125^{FAK}$. *Lower panels,* a portion of respective slices shown in the upper panels. **B:** The surface area resorbed by OCLs was measured with an image analysis system. Antisense oligomers (antisense-1 and antisense-2) significantly inhibited dentine resorption by OCLs, while sense oligomers did not. No inhibitory effect of antisense oligomers was observed by adding a mixture of the sense and the antisense oligomers. In the control cultures, 32.1 ± 2.0% of the total surface area was resorbed by OCLs. Experiments were repeated 3 times with 2 different antisense oligomers (antisense-1 and antisense-2). Similar results were obtained. *Significantly different from the control group treated without oligodeoxynucleotides, $p < 0.01$.

ACKNOWLEDGMENTS

We express our thanks to Drs. T. Yamori (Japanese Foundation for Cancer Research) and J.T. Parsons (University of Virginia) for their generous gifts of antibodies. We are also grateful to Dr. Y. Fukui (University of Tokyo) for helpful suggestions in performing Western blot analysis. Also, we are grateful to Drs. A. Bouton (University of Virginia) and J.T. Parsons for their constructive comments and discussion for preparing the manuscript.

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