J. Biochem. **139**, 583–590 (2006)
doi:10.1093/jb/mvj066

Pepstatin A, an Aspartic Proteinase Inhibitor, Suppresses RANKL-Induced Osteoclast Differentiation

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Received November 27, 2005; accepted January 24, 2006

Pepstatin A is well known to be an inhibitor of aspartic proteinases such as pepsin, cathepsins D and E. Except for its role as a proteinase inhibitor, however, the pharmacological action of pepstatin A upon cells remain unclear. In this study, we found that pepstatin A suppressed receptor activator of NF-κB ligand (RANKL)–induced osteoclast differentiation. Pepstatin A suppressed the formation of multinuclear osteoclasts dose-dependently. This inhibition of the formation only affected osteoclast cells, *i.e.*, not osteoblast-like cells. Furthermore, pepstatin A also suppressed differentiation from pre-osteoclast cells to mononuclear osteoclast cells dose-dependently. This inhibition seems to be independent of the activities of proteinases such as cathepsin D, because the formation of osteoclasts was not suppressed with the concentration that inhibited the activity of cathepsin D. Cell signaling analysis indicated that the phosphorylation of ERK was inhibited in pepstatin A-treated cells, while the phosphorylation of IκB and Akt showed almost no change. Furthermore, pepstatin A decreased the expression of nuclear factor of activated T cells c1 (NFATc1). These results suggest that pepstatin A suppresses the differentiation of osteoclasts through the blockade of ERK signaling and the inhibition of NFATc1 expression.

Key words: aspartic proteinase, cathepsin, osteoclast, pepstatin A.

Abbreviations: ERK, extracellular signal-regulated kinase; IκB, inhibitor of NF-κB; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony–stimulating factor; NFATc1, nuclear factor of activated T cells c1; OPG, osteoprotegerin; PI3K, phosphatidylinositol-3 kinase; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; RT-PCR, reverse transcription–polymerase chain reaction; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; TRAP, tartrate-resistant; acid phosphatase.

Bone undergoes continuous remodeling through bone formation and resorption. The balance between them is tightly regulated to maintain the homeostasis of our skeleton. When the balance is disturbed, osteopenia or osteopetrosis ensues. This is a natural consequence in the process of aging, with increasing age bones becoming more prone to fracture because of diminished bone mineral density. Numerous reports have documented the effects of cytokines on the differentiation and/or activation of osteoclasts. Among these cytokines, receptor activator of nuclear factor-κB (RANK) ligand (RANKL), expressed by osteoblastic cells, plays a pivotal role in osteoclastogenesis as well as in osteoclast activation and survival (*1–8*). After RANKL binds to its receptor, RANK (*5–10*), several tumor necrosis factor (TNF) receptor-associated factors (TRAFs) bind directly to the cytoplasmic domain of RANK (*11–17*). The interaction between TRAF and RANK appears to require an oligomerized receptor cytoplasmic domain and is thought to be responsible for initiation of the activation of most of the RANK-mediated signaling pathways such as NF-κB, Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38MAPK), extracellular signal regulated kinase (ERK), phosphatidylinositol-3 kinase (PI3K), and calcium signaling pathways (*18–21*). RANK occupancy mobilizes intracellular calcium, a requisite for calcineurin-mediated nuclear factor of activated T cell (NFAT) activation (*22*). NFAT binds to its DNA response element via a ternary complex with AP-1 proteins, including Fos/Jun, to transactivate target genes including tartrate-resistant acid phosphatase (TRAP) (*23*, *24*). As a result, NF-κB, AP-1 and NFAT play essential roles in osteoclast differentiation, fusion and activation (*25*, *26*). On the other hand, osteoprotegerin (OPG), which is ubiquitously expressed, acts as a secreted decoy receptor of RANK to negatively regulate osteoclastogenesis (*27–29*). In bone remodeling, osteoblastic cells and stromal cells regulate osteoclastogenesis by controlling the ratio of OPG to RANKL.

Numerous researchers have indicated that bone resorption is regulated by several types of proteinases, including cathepsin K (CK), an osteoclast-specific cysteine proteinase, and matrix metalloproteinase-9 (MMP-9). These proteinases are secreted from the ruffled border of osteoclasts and degrade type I collagen, the most abundant protein in bone. The degraded collagen is endocytosed, sorted to

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lysosomes of osteoclasts and then digested into smaller fragments. In these compartments, it is likely that cathepsins B, L and D play a role in these events (*30*, *31*). Two separate lines of evidence have suggested the importance of CK for osteoclast activity. First, CK–/– mice exhibit an osteopetrotic phenotype in their long bones (*32*, *33*). Further support came from a genetic study in which nonsense, missense and stop codon mutations in the gene encoding CK were found in patients with pycnodysostosis, a skeletal disease with an osteopetrotic phenotype (*34*). It has recently been reported that PTH-induced osteoclastogenesis was suppressed by cystatin C and other cysteine proteinase inhibitors (*35*). Their enzymatic activity does not seem to be involved in osteoclastogenesis because non-reactive derivatives of the inhibitors did not affect the PTH-stimulated formation of TRAP-positive osteoclasts.

Aspartic proteinases such as cathepsins D (CD) and E (CE) are also expressed in osteoclasts. It is thought that these proteinases contribute to bone resorption in the endosome/lysosome system because they are predominantly active at acidic pH levels. In addition, the localization of CE in the ruffled border of mature osteoclasts has been reported (*36*). In this study, we investigated the effect of an aspartic proteinase-specific inhibitor, pepstatin A (Iva-Val-Val-Sta-Ala-Sta), on osteoclastogenesis. Non-toxic concentrations of pepstatin A suppressed the osteoclastogenesis in a dose-dependent manner. The concentration of pepstatin A required for the suppression of osteoclastogenesis was higher than that for complete inhibition of the aspartic proteinase activity in intact cells. In addition, pepstatin A was a potent inhibitor of both ERK phosphorylation and NFATc1 accumulation in bone marrow macrophages stimulated by RANKL.

EXPERIMENTAL PROCEDURES

Reagents—Pepstatin A was purchased from Peptide Inc. (Osaka, Japan). Recombinant M-CSF was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). The His6-tagged soluble human RANKL plasmid was a kind gift from Hitoshi Amano, Showa University, Tokyo, Japan. Protein was expressed in Codon-Plus BL21 (DE3) RL competent cells (Stratagene, La Jolla, CA), and purified from a cell extract using the culture medium and nickel-nitrilotriacetic acid column chromatography (Qiagen).

Cell Culture—Primary osteoblastic cells were isolated from 2-day-old mice calvariae after routine sequential digestion five times with 0.1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and 0.2% dispase (Godo Shusei, Tokyo, Japan), as previously described (*37*). For the co-culture experiments, murine bone marrow cells flushed out from the tibias and femurs of 4–6-week-old male ddY mice, and murine calvaria–derived osteoblastic cells were co-cultured in the presence of 1,25-$(OH)_2$ D_3 (1×10^{-8} M) and prostaglandin E_2 (1×10^{-6} M). In several experiments, bone marrow cells were cultured at 1×10^6 cells/ml in 48-well plates for 3 or 5 days, under osteoclastogenic conditions, *i.e.*, in α-minimal essential medium supplemented with 10% FBS at 37°C in 5% CO_2 in the presence of 10 ng/ml of M-CSF and 50 ng/ml of RANKL. The medium was changed on day 2 of culture, and supplemented with M-CSF and RANKL. Pepstatin A was added at 15–120 μM with M-CSF and RANKL to the growth medium.

Tartrate-Resistant Acid Phosphatase (TRAP) Staining—Osteoclasts were identified as those cells staining positively for TRAP as described previously (*38*). TRAP-positive mono- or multinuclear cells were counted. Any cells containing three or more nuclei were counted as multinuclear cells.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted using TRIsol reagent (Invitrogen, CA, US), and first strand cDNA was synthesized using RevaTra Ace (TOYOBO, Osaka, Japan). For PCR analysis, cDNA was amplified with *Taq* DNA polymerase (TAKARA Biomedicals, Tokyo, Japan). A thermal cycle of 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s was performed 30 times for RANKL, M-CSF and OPG. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal control for PCR, the cycle was repeated for 25 times. The amplified products were separated by electrophoresis on a 2% agarose gel. The primer sequences used for mouse RANK: 5′-CGCCATGGCCCCGCGCG-CCCGG-3′ and 5′-GGCAGGTAAGCCTGGGCCTCC-3′; M-CSF: 5′-AGACTGATGGTACATCCACG-3′ and 5′-CCC-CACAGAAGAACCAATG-3′; RANKL: 5′-CCACGGTCT-AGATCTCAGTCTATGTCCTGAACTTTG-3′ and 5′-GTA-TATCCAGTGTGGTGGCACATTGTGGGGCCACAGCGC-TTC-3′; OPG: 5′-ATGAACAAGTGGCTGTGCTG-3′ and 5′-ACGGATTGAACCTGATTCCC-3′; c-fms: 5′-TGTGCAA-GACCATGGTGAAT-3′ and 5′-GTCACCCACAGACACC-TCCT-3′; F4/80: 5′-CTGTAACCGGATGGCAAACT-3′ and 5′-CATCACTGCCTCCACTAGCA-3′; and GAPDH: 5′-ATGTCGTGGAGTCTACTGGC-3′ and 5′-TGACCTTGCC-CACAGCCTTG-3′.

Real-Time RT-PCR—Using a Mx3005P™ Real-Time PCR System (Stratagene, CA, US), the reverse-transcribed cDNA was amplified with specific primers according to the manufacturer's instructions. The primer sequences used for amplification were as follows: mouse GAPDH: 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-CACATTGGGGGTAGGAACAC-3′; NFATc1: 5′-TCATC-CTGTCCAACACCAAA-3′ and 5′-TCACCCTGGTGTTCTT-CCTC-3′. The thermal cycle was the same as for RT-PCR. The relative amount of each mRNA was normalized as to GAPDH expression.

Aspartic Proteinase Assay—The activity was determined with acid-denatured bovine hemoglobin as a substrate at pH 3.5 as previously described (*39*), except that acid-soluble degradation products were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co.).

Western Immunoblotting—Bone marrow cells flushed out from the tibias and femurs of 4–6-week-old male ddY mice were cultured with M-CSF (100 ng/ml) at 1×10^7 cells per 10 ml in a 10 cm culture dish. After 3-day culture, the cells were washed with culture medium to remove any nonadherent cells. The remaining adherent cells were harvested by vigorous pipetting, replated at 1×10^6 cells per 10 ml in a 10 cm culture dish, and then cultured for 3-days. We used these cells as purified bone marrow-derived macrophages (BMMs) in this study. The cells were washed and incubated with serum-free medium for 3 h. The cells were then incubated with 50 ng/ml of RANKL or 10 ng/ml of M-CSF for the indicated times in

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the individual experiments at 37°C. The cells were washed three times with phosphate-buffered saline containing 1 mM sodium vanadate, and then were solubilized in 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 units/ml aprotinin). A supernatant was obtained by centrifugation at 12,000 rpm for 10 min in a microcentrifuge. The protein concentration of each sample was measured with BCA protein assay reagent. Twenty micrograms of protein were separated by 10% SDS–polyacrylamide gel electrophoresis and then electroblotted onto a polyvinylidine difluoride membrane. The blots were blocked with 5% milk protein for 1 h at room temperature, probed with primary antibodies (Akt and phospho Ser473 Akt, ERK and phospho ERK, and IκB and phospho IκB; Cell Signaling Technology, Inc.) overnight at 4°C, washed, incubated with HRP conjugated secondary antibodies, and finally detected with ECL-plus (Amersham Pharmacia Biotech).

RESULTS

Pepstatin A Inhibits Osteoclastogenesis—To determine whether pepstatin A can inhibit osteoclastogenesis, we checked the effect of pepstatin A on osteoclastogenesis in a co-culture system. Pepstatin A suppressed the formation of TRAP-positive multinuclear cells in a dose-dependent manner at 15–120 μM (Fig. 1A). A similar inhibitory effect of pepstatin A on osteoclastogenesis was observed with another osteoclastogenesis system, induced with RANKL/M-CSF from bone marrow cells (Fig. 1B).

In a co-culture system, osteoblastic cells and stromal cells regulate osteoclastogenesis by controlling the ratio of OPG to RANKL. We next examined whether pepstatin A affects the OPG/RANKL ratio in osteoblast-like calvarial cells. While the expression of RANKL mRNA increased during 5-day culture in the presence of 1,25-$(OH)_2D_3$ and prostaglandin E_2, pepstatin A failed to inhibit the increase in RANKL mRNA. We could not find any differences between the control, and prostaglandin E_2 and 1,25-$(OH)_2D_3$ stimulated cultures with or without pepstatin A in the OPG and M-CSF expression levels (Fig. 1C). In addition, no significant effect of pepstatin A was found on the growth of either calvarial cells or MC3T3-E1 osteoblastic cells. Although we also examined the expression of these genes after 3-day stimulation, no effect of pepstatin A was found (not shown). Furthermore, in order to determine whether pepstatin A can inhibit osteoclast formation in the absence of stromal cells, we used bone marrow cells that were separated from stromal cells on a Sephadex G-10 column. As shown in Fig. 1D, pepstatin A dose-dependently suppressed the RANKL-induced osteoclastogenesis from stromal cell–deprived bone marrow cells in a similar manner to as seen in the co-culture system (Fig. 1A) and bone marrow culture (Fig. 1B).

Pepstatin A Strongly Inhibits an Early Stage of Osteoclast Formation—We next examined at which stage pepstatin A inhibits osteoclast formation. Osteoclasts were generated from bone marrow cells stimulated with RANKL and M-CSF for 5 days. Pepstatin A was added at 0–3 days or 3–5 days. Incubation in the presence of pepstatin A during incubation from day 0 through day 3 was as effective as treatment with pepstatin A throughout the entire incubation period. In comparison, when pepstatin A was only included during incubation from day 3 through day 5, it inhibited osteoclastogenesis less effectively, and ~60% of the control number of TRAP-positive multinuclear cells were formed (Fig. 2). We next examined the effect of pepstatin A on the RANKL-induced formation of TRAP-positive mononuclear preosteoclasts from a bone

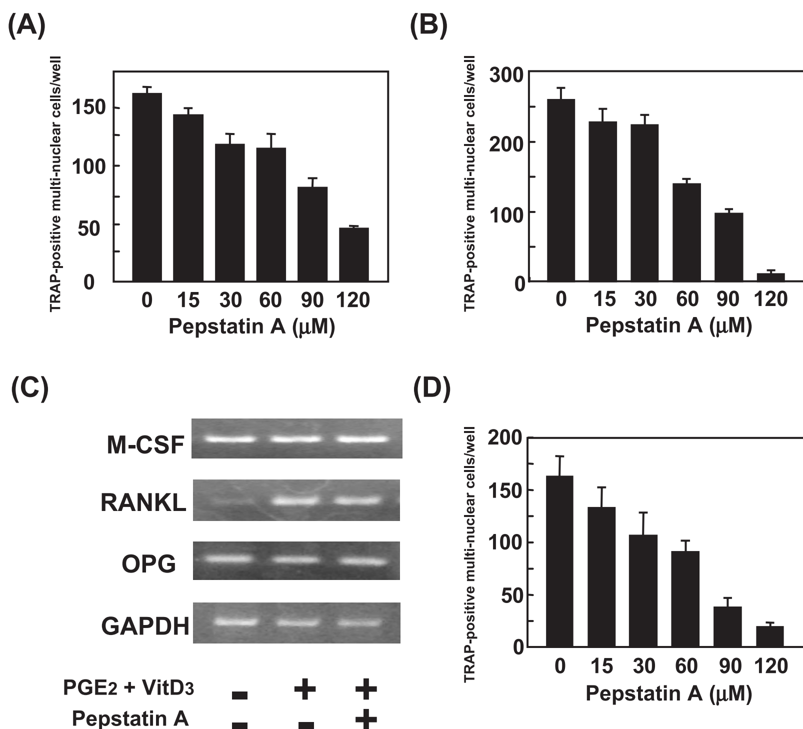

Fig. 1. **Pepstatin A inhibits osteoclastogenesis.** Osteoclasts were generated from a co-culture stimulated with prostaglandin E_2 and 1,25-$(OH)_2D_3$ (A), or bone marrow cells stimulated with RANKL and M-CSF (B) with the concentration range of 0–120 μM pepstatin A. TRAP-positive multinuclear cells were counted. (C) Cells were treated with prostaglandin E_2, 1,25-$(OH)_2D_3$, or 120 μM pepstatin A. Control cells were treated with the vehicle DMSO. Total RNA was extracted and RT-PCR was performed using specific primers as described under Experimental Procedures. (D) A bone marrow cell suspension was applied to a Sephadex G-10 column to remove stromal cells. The collected cells were cultured in the presence of RANKL and M-CSF with the concentration range of 0–120 μM pepstatin A.

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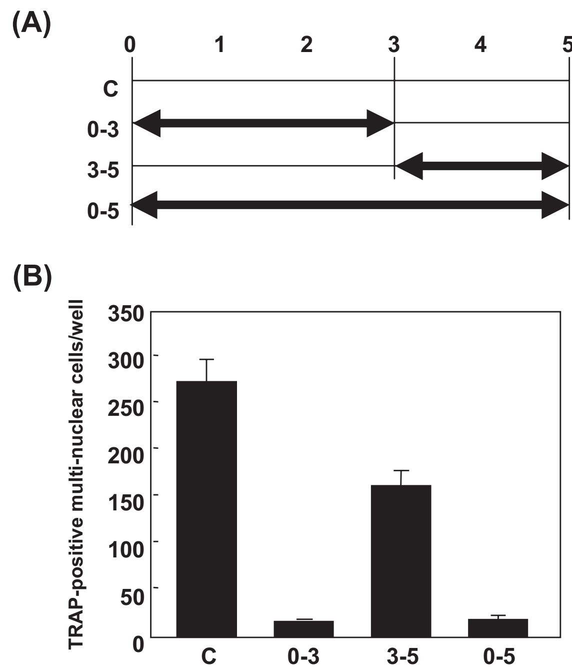

Fig. 2. **Pepstatin A strongly inhibits the early stage of osteoclast formation.** (A) The schematic features of the period when pepstatin A was added. (B) The number of TRAP-positive multi-nuclear cells in a bone marrow culture after 3 or 5 days with 120 μM pepstatin A. C, control (no pepstatin A).

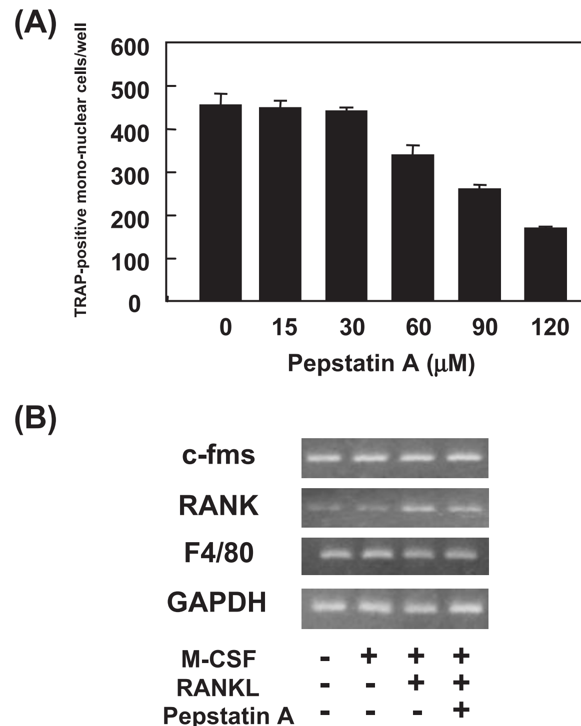

Fig. 3. **Pepstatin A inhibits RANKL-induced mono-nuclear osteoclast formation.** (A) The number of TRAP-positive mono-nuclear cells in a bone marrow culture after 3 days with the concentration range of 0–120 μM pepstatin A. C, control (no pepstatin A). (B) RT-PCR analysis of RANK, c-fms and F4/80 in bone marrow cells on a Sephadex G-10 column after 3 days. M-CSF, 10 ng/ml; RANKL, 50 ng/ml; pepstatin A, 120 μM.

marrow culture. Pepstatin A dose-dependently inhibited the TRAP-positive mononuclear osteoclasts during 3-day stimulation of RANKL (Fig. 3A). These results suggest that pepstatin A preferentially acted on the osteoclastic precursor cells at an early stage of cell differentiation. We then examined the expression of mRNAs encoding osteoclast lineage-specific cell surface receptors RANK and c-fms using RT-PCR. While treatment of bone marrow cells with M-CSF for 3 days caused no significant changes in the expression levels of these genes, treatment with RANKL simultaneously with M-CSF increased the RANK expression. The expression levels of these genes were independent of pepstatin A treatment. It has been well documented that cytokine IL-3 does not affect RANK or c-fms expression but irreversibly inhibits osteoclast differentiation by diverting the cells to the macrophage lineage (*40*). To examine the possibility that pepstatin A diverts osteoclast precursors to different cell lineages, the expression of F4/80, a monocyte/macrophage marker, was evaluated by RT-PCR. The expression of F4/80 was not affected by pepstatin A treatment (Fig. 3B). This indicates that the mode of inhibition of pepstatin A is different from that of IL-3. Another piece of evidence supporting these findings is that when pepstatin A was withdrawn on day 3, RANKL-mediated osteoclastogenesis was strongly suppressed on day 5, however, the osteoclasts were fully developed on day 9 or later (not shown).

Suppression of Intracellular Aspartic Proteinase Activity by Pepstatin A Is Not Sufficient for the Inhibition of Osteoclastogenesis—As pepstatin A is an aspartic proteinase inhibitor, we examined whether the proteinase activity participated in osteoclast formation. Bone marrow cells were cultured with 0–120 μM pepstatin A for 3 days. The cells were extensively washed with PBS and lysed, and then the aspartic proteinase activity was measured. A pepstatin A concentration of 15 μM was sufficient for substantial inhibition of the aspartic proteinase activity in the cells, while complete inhibition was seen at 90 μM (Fig. 4). In contrast to the inhibition of the proteinase activity, the suppressive effect of pepstatin A on osteoclastogenesis was less effective. At the concentration of 15 μM, pepstatin A was almost totally ineffective against the osteoclastogenesis, as shown in Figs. 1 and 3. In addition, at the concentration of 90 μM, pepstatin A did not completely suppress the differentiation of osteoclasts. Together, these results suggest that the inhibitory effect of pepstatin A on osteoclast formation is not due to the inhibition of proteinase activity.

Pepstatin A Blocks RANKL-Induced ERK Kinase Phosphorylation and Decreases the Expression of NFATc1—In order to elucidate the effect of pepstatin A on the known major RANKL-induced signal transduction, osteoclasts were pretreated with pepstatin A for 3h and then stimulated with RANKL. The stimulation of osteoclasts with RANKL-induced phosphorylation of ERK observed at 5 min (Fig. 5A). Pepstatin A pretreatment for 3 h strongly suppressed both the baseline level (time 0) and RANKL-induced phosphorylation of ERK at 5 min. Although Akt phosphorylation induced by RANKL was weak in our experimental design, pepstatin A pretreatment did not generally affect Akt phosphorylation (not shown). As IκBα is a regulatory protein that binds to and inhibits

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NF-κB, we evaluated the kinetics of IκBα degradation. After treating BMMs with RANKL, IκB was phosphorylated for 5 min and then degraded for 15 min. The pretreatment with pepstatin A caused almost no inhibition of either IκBα phosphorylation or degradation (Fig. 5A). These results are consistent with our observation that the RANKL-induced nuclear translocation of NF-κB p65 was not prevented by pepstatin A (not shown). RANKL triggers the sustained NFATc1-dependent transcriptional program, through autoamplification of NFATc1 gene expression, during osteoclast differentiation (*41*). To examine the expression of NFATc1 mRNA and protein, BMMs were cultured in the presence of M-CSF and/or RANKL and/or pepstatin A. The cells were collected, and then real-time RT-PCR and immunoblot analysis were performed. As shown in Fig. 5B and C, NFATc1 was up-regulated on days 2 and 3 during 3-day cultivation with M-CSF and RANKL. On the other hand, pepstatin A strongly suppressed the induction of NFATc1.

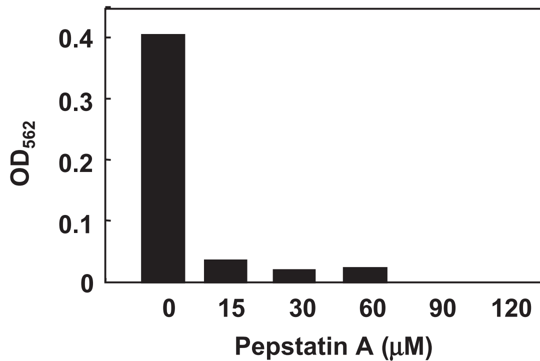

Fig. 4. **Proteolytic activity in pepstatin A–induced osteoclasts after 3 days.** CD activity was determined with acid-denatured hemoglobin as the substrate at pH 3.5. The data are expressed as the absorbance at 562 nm equivalent to 1 μg of tyrosine in 1 min.

DISCUSSION

CD, one of the most typical aspartic proteinases, is also expressed in osteoclasts, as observed on immuno- light and -electron microscopy (*30*, *31*). In the present paper, we report that pepstatin A, an aspartic proteinase-specific inhibitor, suppresses osteoclast formation. Although pepstatin A is mainly an inhibitor of CD and CE intracellularly, osteoclast formation was not suppressed even with concentrations with which the activities of these proteinases were completely inhibited. This may suggest that suppression of osteoclastogenesis by pepstatin

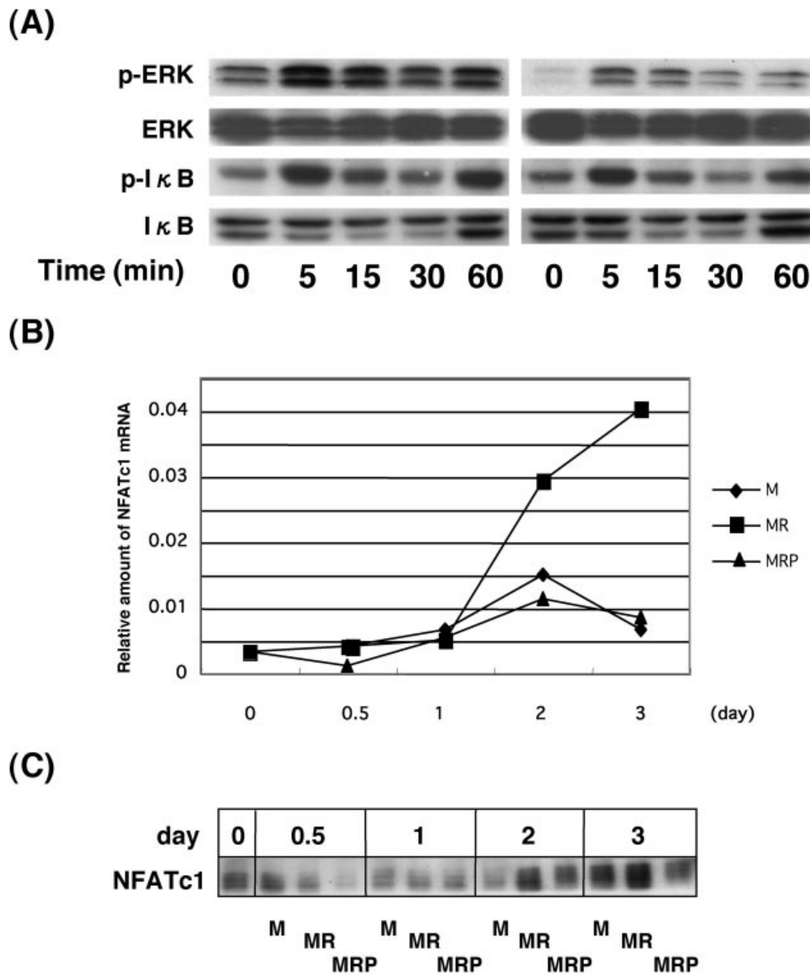

Fig. 5. **Pepstatin A inhibits RANKL-induced ERK activation and NFATc1 expression in osteoclasts.** (A) Serum-starved osteoclasts were pretreated with or without pepstatin A for 3 h and then stimulated with 50 ng/ml of RANKL for the indicated times. Cell lysates were immunoblotted with anti–phospho-ERK and -IκB antibodies. Purified bone marrow cells treated with or without pepstatin A were collected at the indicated times, and then real-time RT-PCR (B) and Western blot analysis (C) were performed. M, M-CSF only; MR, M-CSF and RANKL; MRP, M-CSF, RANKL and pepstatin A.

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A is independent of the activities of CD and CE. Previously, Chen et al. reported a method that comprises the localization of CD with BODIPY FL-pepstatin A by fluorescence microscopy (*42*). In their study, BODIPY FL-pepstatin A inhibited the CD activity, with an IC_{50} of 10 nM *in vitro*, and stained lysosomes, where it was co-localized with CD, in a murine macrophage-like cell line (J774A.1) within 30 min. From this, it is thought that 120 μM pepstatin A completely inhibits the CD activity in osteoclasts. These results indicate that the proteolytic activity of CD is not related with the inhibition of osteoclastogenesis by pepstatin A. In agreement with this, bone marrow from the CD-deficient mice was confirmed to include multinuclear osteoclasts similar to those observed in wild-type mice, even though we used CD-deficient 3-week-old mice, because CD-deficient mice tend to die at about 25 days (not shown). As CE is an intracellular but a non-lysosomal proteinase, the idea that suppression of osteoclastogenesis by pepstatin A might be due to inhibition of the activity of CE could not be completely ruled out. To clarify this point, more experiments are needed to examine this issue.

To determine whether pepstatin A can inhibit osteoclast formation in the absence of stromal cells, we used bone marrow cells that had been passed through a Sephadex G-10 column, and had been stimulated with M-CSF and RANKL. As shown in Fig. 1D, TRAP-positive multinuclear cells decreased on treatment with pepstatin A in a dose-dependent manner, which is similar to the observation for a bone marrow culture. These results suggest that pepstatin A acts directly on osteoclasts. We next examined the expression of cell surface receptors including RANK and c-fms using RT-PCR. However, pepstatin A did not affect their expression (Fig. 3B), thus suggesting that pepstatin A acts downstream of RANK and/or c-fms. After RANKL has bound RANK, a set of TRAFs is recruited to near RANK and transferred its signals to downstream kinases, such as ERK, p38MAPK, JNK and PI3K. To examine the signal transduction downstream of RANK, the phosphorylation of ERK, IκB and Akt through RANKL-induced stimulation was examined. As shown to Fig. 5A, the phosphorylation of ERK in pepstatin A–treated cells decreased to less than that in non-treated cells, although IκB and Akt showed almost no change. Since NFATc1 has been reported to be one of the master genes in the osteoclastogenesis after RANKL stimulation (*23*, *24*), we examined the expression of NFATc1. The induction of NFATc1 was suppressed by pepstatin A (Fig. 5, B and C), which resulted in inhibition of osteoclastogenesis. However, it is not clear whether these effects of pepstatin A on the phosphorylation of ERK and the expression of NFATc1are direct or indirect. As pepstatin A did not affect the expression of TRAF6 (not shown), it may participate in the signals downstream of TRAF6. As NFATc1 is regulated by the intracellular calcium concentration, it is possible that pepstatin A affects regulation of the calcium concentration.

The proximal events in RANKL-mediated transcription factor activation depend on the TRAF family, especially TRAF-2 and 6. TRAF-2 and 6 mediate activation of the NF-κB pathway via RANKL. However, it was recently reported that CD40-NF-κB is blocked by pepstatin A in KMH2 cells, the Reed-Sternberg (RS) cells in Hodgkin disease (*43*). CD40 activates NF-κB, which is mediated by the proteolysis of TRAF3. After the TRAF-associated activator of NF-κB (TANK) has bound to TRAF3, TRAF3 is degraded by aspartic proteinase. As a result, TANK binds to TRAF2, which results in NF-κB activation. Consistent with this, Hauer et al. reported that TRAF3 specifically blocked the NF-κB activation via TRAF2/5 in 293T cells, a human embryonic kidney cell line (*44*). As this pathway can be blocked by treatment with pepstatin A, but not with ritonavir or indinavir, which are other aspartic proteinase inhibitors, we examined the possibility that pepstatin A inhibited the degradation of TRAF3 during osteoclastogenesis. According to the results of Western blot analysis involving a bone marrow culture, no degradation of TRAF3 was observed in pepstatin A-treated osteoclasts (not shown). This result suggests that the decrease in osteoclastogenesis caused by pepstatin A is not due to NF-κB activation brought out by the degradation of TRAF3, not like in the case of CD40.

A number of studies have revealed that cysteine proteinases are involved in osteoclast formation and bone resorption (*30*, *32–35*). Among them, it is thought that CK is the most important enzyme, and the resulting CK-deficiency causes impaired bone resorption in mouse and man (*16*, *17*). Brage et al. reported that cystatin C and other cysteine proteinase inhibitors such as leupeptin, E-64 and benzyloxycarbonyl-Phe-Ala-diazomethane ($Z\text{-FA-CHN}_2$) inhibit osteoclastogenesis by interfering with the expression of RANK and NFATc1 (*35*). In the present study, we demonstrated that pepstatin A, an aspartic proteinase inhibitor, suppresses osteoclast formation by interfering with both the phosphorylation of ERK and the expression of NFATc1, and that this inhibition is independent of the proteinase activity that is associated with an aspartic proteinase, CD.

This work was supported by a Grant-in-Aid for research fellows of the Japan Society for the Promotion of Science from the Ministry of Education, Science, and Culture of Japan. We are grateful to the members of the Division of Oral and Maxillofacial Surgery, Graduate School of Biomedical Sciences, Nagasaki University, and Yuka Narita, a student of Nagasaki University School of Dentistry, for the helpful suggestions.

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