Mechanism of Stimulation of Osteoclastic Bone Resorption through Gas6 / Tyro 3, a Receptor Tyrosine Kinase Signaling, in Mouse Osteoclasts

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SUMMARY

The signaling through receptor tyrosine kinases (RTKs) expressed on mature osteoclasts has recently been suggested to be involved in osteoclastic bone resorption. This study investigated the mechanism and the possible physiological relevance of Gas6 / Tyro 3, an RTK signaling pathway in osteoclasts in stimulating osteoclastic bone resorption using several mouse culture systems. Gas6, expressed ubiquitously in bone cells, did not affect the differentiation or the survival of osteoclasts, but stimulated osteoclast function to form resorbed pits on a dentine slice. The expression of its receptor, Tyro 3, was seen only in mature osteoclasts among bone cells. Gas6 upregulated the phosphorylation of cellular proteins including p42/p44 mitogen-activated protein kinase (MAPK), but not p38 or JNK MAPK, and increased the kinase activity of immunoprecipitated Tyro 3 in isolated osteoclasts. The ability of Gas6 to stimulate pit formation resorbed by osteoclasts was abrogated by PD98059, a specific inhibitor of p42/p44 MAPK. In addition, the Gas6 mRNA level in bone marrow was upregulated by ovariectomy and was reduced by estrogen replacement. These results strongly suggest that Gas6 acts directly on mature osteoclasts through activation of Tyro 3 and p42/p44 MAPK, possibly contributing to the bone loss by estrogen deficiency.
INTRODUCTION

Osteoclastic bone resorption is regulated by the differentiation, function, and survival of osteoclasts. The tumor-necrosis-factor-family molecule receptor activator of NF-κB ligand /osteoclast differentiation factor (RANKL/ODF) was identified as the membrane-associated molecule regulating osteoclast differentiation (1-3); however, the signaling pathways in regulating mature osteoclast function and survival are still controversial. Recent study of random sequence analysis of PCR-amplified cDNA clones identified 14 distinct kinase-related genes in purified rabbit mature osteoclasts, and 8 of them were identified as receptor tyrosine kinases (RTKs) (4). RTKs expressed on mature osteoclasts include fibroblast growth factor receptor type 1 (FGFR1), c-Fms, and Tyro 3, whose ligands: FGF-2, macrophage-colony stimulating factor (M-CSF), and the growth arrest-specific gene 6 (Gas6), respectively, are known to regulate osteoclast differentiation, function, or survival. We recently reported that FGF-2 stimulates mature osteoclast function directly through the activation of FGFR1 on mature osteoclasts (5, 6). M-CSF is also reported to stimulate the survival and chemotactic behavior of osteoclasts through the activation of its receptor, c-Fms, on osteoclasts (7-10). Another RTK, Tyro 3, is the RTK most frequently cloned in isolated rabbit osteoclasts in the random sequence study above (4). Tyro 3, also known as Sky, Rse, Brt, and Tif, is a member of the AUS (Axl, Ufo and Sky) family RTKs (11-14) containing characteristic extracellular ligand binding domain composed of two immunoglobulin-like domains and two fibronectin Type III repeats (15, 16). The ligand for Tyro 3 is known to be Gas6 which is a vitamin K-dependent protein acting as a growth-potentiating factor for thrombin-induced cell proliferation (17-19).
We now propose the possibility that signaling through RTKs on osteoclasts contributes not only to osteoclastic function but also to the pathophysiology of osteopenic disorders. In this regard, we recently reported that endogenous FGF-2 in the synovial fluid contributes to joint destruction in rheumatoid arthritis (RA) patients through a direct action on mature osteoclasts (5, 6, 20). M-CSF has been implicated in the pathophysiology of the bone loss of ovariectomy (21-24). Hence, in this study we examined the mechanism whereby Gas6 / Tyro 3 signaling stimulates osteoclastic bone resorption using several mouse culture systems, and investigated the possible physiological relevance of this signaling to the bone loss of estrogen deficiency.
EXPERIMENTAL PROCEDURES

Materials

Neonatal ddY mice, 5-week- and 8-week-old ddY mice were purchased from Shizuoka Laboratories Animal Center (Shizuoka, Japan). Rat recombinant Gas6 and human recombinant FGF-2 were generously provided by Shionogi Research Laboratory (Osaka, Japan) and Kaken Pharmaceutical Co., Ltd. (Kyoto, Japan), respectively. Alpha modified-minimum essential medium (αMEM) was purchased from Gibco BRL (Rockville, MD), and fetal bovine serum (FBS) was from the Cell Culture Laboratory (Cleveland, OH). Recombinant human M-CSF was purchased from R&D Systems Inc. (Minneapolis, MN). Recombinant human soluble RANKL/ODF was purchased from PeproTech, Inc. (London, UK). Bacterial collagenase, 1,25(OH)2 vitamin D3, and ISOGEN were purchased from Wako Pure Chemicals Co. (Osaka, Japan), and dispase from Nitta Gelatin Co. (Osaka). Monoclonal mouse antibody against phosphotyrosine was obtained from UBI (Lake Placid, NY), and monoclonal mouse antibody against p60V-src (mAb 327) from Oncogene Research Products (Cambridge, MA). This antibody recognizes specifically both p60V-src and p60c-src, and has been used to determine the expression of p60c-src in various primary cells and clonal cell lines. Polyclonal goat antibody against mouse Tyro 3 and nonimmune IgG as well as blocking peptides for respective antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [32P]dCTP and [γ-32P]-ATP were obtained from Amersham LIFE SCIENCE (Buckinghamshire, UK). Herbimycin A and 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) were from Alexis Biochemicals (San Diego, CA).
2-amino-3-methoxyflanone (PD98059), monoclonal mouse antibody against phospho-
p44/42 MAPK, polyclonal rabbit antibody against phospho-p38 MAPK, mouse monoclonal
against phospho-JNK MAPK, were obtained from New England Biolabs, Inc. (Beverly,
MA). 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole
(SB203580) was purchased from Calbiochem-Novobiochem Co. (La Jolla, CA). Other
chemicals were obtained from Sigma Chemical Company (St.Louis, MO).

Mouse primary osteoblast culture
All animal experiments were performed according to the guidelines of the International
Association for the Study of Pain (25). In addition, the experimental work was reviewed by
the committee of Tokyo University charged with confirming ethics. Calvariae dissected from
1 to 4 day-old mice were washed in phosphate buffered saline (PBS) and digested with 1 ml
of trypsin / EDTA (GIBCO BRL) containing 10 mg collagenase (Sigma, type 7) for 10 min X
5 times, and cells from fractions 3 to 5 were pooled. Cells were plated in 6-multiwell dishes
at a density of 5,000 cells / cm² and grown to confluence in αMEM containing 10% FBS.

Resorbed pit formation assay in the coculture of mouse bone marrow cells and osteoblasts
Mouse osteoblasts (1 X 10⁶ cells/dish) prepared as described above and bone marrow cells (2
X 10⁷ cells /dish) from tibiae of 8-week-old ddY mice were cocultured on 10-cm culture
dishes coated with 0.24% collagen gel matrix (Nitta Gelatin, Tokyo) containing αMEM with
10% FBS, 1,25(OH)₂ vitamin D₃ (10⁻⁸ M), and PGE₂ (10⁻⁶ M) for 6 days with a medium
change every 3 days, and for 1 additional day in αMEM with 10% FBS. After culture for 7
days, non adherent cells were washed with PBS and adherent cells were stripped by 0.2% bacterial collagenase. An aliquot of crude osteoclast preparation (0.1 ml) was further cultured on a dentine slice placed in each well of 96-well dishes containing αMEM / 10% FBS in the presence or absence of Gas6 (10^{-14}-10^{-8} M) and/or PD98059 (1-30 µM) and SB203580 (30 µM). After 48 h of culture, cells were removed with 1N NH₄OH solution, and stained with 0.5% toluidine blue. Total area was estimated under a light microscope with a micrometer to assess osteoclastic bone resorption using an image analyzer (System Supply Co., Nagano, Japan). At the same time, cells on a dentine slice in the independent culture were fixed with 3.7% (vol/vol) formaldehyde in PBS and ethanol-acetone (50:50, vol:vol), and stained at pH 5.0 in the presence of L(+)-tartaric acid using naphthol AS-MX phosphate (Sigma) in N,N-dimethyl formamide as the substrate. Tartrate resistant acid phosphatase (TRAP) positive-multinucleated cells containing more than three nuclei were counted as osteoclasts.

**TRAP-positive multinucleated cell formation assay in the mouse coculture system**

Mouse osteoblasts (3 X 10⁴ cells /well) and bone marrow cells (1 X 10⁶ cells/well) were cocultured in 24-multiwell dishes containing αMEM / 10% FBS in the presence and /or absence of Gas6 (10^{-14}-10^{-8} M) for 6 days with a medium change at 3 days. After 6 days of culture, the cells were fixed and stained for TRAP as described above. TRAP positive-multinucleated cells containing more than three nuclei were counted as osteoclasts.

**Analysis of osteoclast survival**
Coculture of mouse osteoblasts and marrow cells was performed on 10-cm culture dishes coated with 0.24% collagen gel matrix to obtain osteoclasts as described above. After 7 days of culture, dishes were treated with 4 ml of 0.2% bacterial collagenase in αMEM for 20 min at 37°C, and collected cells were suspended in 170 ml of αMEM / 10% FBS. An aliquot of crude osteoclast preparation (2 ml) was replaced in 12-well dishes, and further cultured. After incubation for 2 h, the plates were treated with PBS containing 0.001% pronase E and 0.02% EDTA to remove stromal cells. After purification, osteoclasts were cultured in the presence or absence of Gas6 (10^{-8} M) or M-CSF (100 ng/ml) for various periods up to 72 h, stained with trypan blue and TRAP. Trypan blue-negative and TRAP-positive cells were counted as living osteoclasts.

**Reverse transcriptase-PCR (RT-PCR) for Gas6 and Tyro 3**

Mouse osteoblasts and marrow cells were cocultured in 10-cm dishes as described above. After 6 days of culture, osteoclasts were formed and isolated by 0.001% pronase E and 0.02% EDTA in PBS. In addition, to obtain osteoclastic cells in various differentiation stages, spleen cells (2 X 10^8 cells/dish) from 8-week-old mice were cultured on 10-cm culture dishes containing αMEM / 10% FBS with soluble RANKL/ODF (100 ng/ml) and M-CSF (10 ng/ml) for 6 days. Total RNA was extracted from mouse brain, bone marrow cells, osteoblasts, osteoclasts, and cultured spleen cells harvested every day using ISOGEN following the manufacturer’s instructions, and 2 µg of RNA was reverse transcribed and amplified by PCR using Amplitaq Gold (PERKIN ELMER, Branchburg, NJ). The primers
for Gas6 were: sense, 5′-CCATCAACCACGGCATGTGG-3′; antisense, 5′-
TCGCACACCTTGATTCCAT-3′ and Tyro 3 were: sense, 5′-
GGAAGAGACGCAAGGAGAC-3′; antisense, 5′-ATGGGAATGGGGAGACGAC-3′.
The cycling parameters were 1 min at 96°C, 30 sec at 58°C, 1 min 30 sec at 72°C for 25
cycles. The PCR products for Gas6 and Tyro 3 were 589 bp and 445 bp, respectively.

**Western blot analysis for Tyro 3**

Mouse osteoclasts and osteoblasts were lysed with TNE buffer (10 mM Tris-HCl, 150 mM
NaCl, 1% NP-40, 1 mM EDTA, 10 mM NaF, 2 mM Na3VO4, 1 mM aminoethyl-
benzenesulfonyl fluoride, and 10 µg/ml aprotinin). The protein concentration in the cell
lysate was measured using a Protein Assay Kit II (BIO-RAD). Equivalent amounts (60 µg)
of cell lysates were electrophoresed by 8% SDS-PAGE, and transferred to nitrocellulose
membrane. After blocking nonspecific binding with 5% skim milk, Tyro 3 containing
proteins were stained using the ECL chemiluminescence reaction (Amersham Co., Arlington
Heights, IL) following the manufacture’s instructions. After this visualization, the antibodies
on the membrane were stripped in a buffer consisting of 62.5 mM Tris-HCl (pH 6.7), 2%
SDS and 100 mM 2-ME at 50°C for 40 minutes. To ascertain the specificity of these
blottings, the stripping membrane was further immunoreacted with polyclonal anti-Tyro 3
and respective blocking peptide, and the immunoreactive bands were again visualized under
the above conditions. The immunoreactivity to anti-Tyro 3 was not lost by this stripping
procedure.

**Assay for tyrosine phosphorylation of cellular proteins**
Isolated mouse osteoclasts were incubated in αMEM / 0.1% FBS for 2 h and treated with Gas6 (5 X 10^{-9} M) for various periods (0-10 min). Cell lysates containing equal amounts of protein (20 µg) were analyzed by Western blot as described above. After blocking with 5% BSA, the membrane was incubated with monoclonal mouse antibody against phosphotyrosine and with peroxidase-conjugated anti-mouse IgG antibody. Phosphotyrosine-containing proteins were visualized using the ECL chemiluminescence reaction following the manufacturer’s instructions. After the antibody was stripped from the membrane, membranes were incubated with 5% skim milk to block nonspecific binding, and then with monoclonal mouse antibody against p60^{v-src}, monoclonal mouse antibodies against phospho-p44/42 MAPK, -JNK MAPK, polyclonal rabbit antibodies against phospho-p38 MAPK, and the immunoreactive bands were visualized as described above.

**In vitro kinase assay**

Equal amounts of protein (100 µg) from osteoclasts stimulated with Gas6 for various periods were immunoprecipitated with 1 µg of polyclonal goat antibody against mouse Tyro 3 for 4h at 4°C, and the immune complexes were recovered with protein G-Sepharose (GIBCO BRL). The immunocomplex was washed three times with TNE buffer and three times with kinase buffer (20 mM HEPES-NaOH (pH 7.4), and 10 mM MgCl$_2$); the samples were then resuspended in 60 µl of kinase buffer with 1 µCi (37kBq) of $[^{32}\text{P}-\alpha\text{-}]\text{-ATP}$, and incubated for 15 minutes at 30°C. The reaction was stopped by adding 20 µl of 4 X sample buffer [250 mM Tris-HCl (pH 6.8), 8 mM EDTA, 12% SDS, 500 mM 2-mercaptoethanol, 15% glycerol, and 0.01% Bromophenol blue], and subjected to 10% SDS-PAGE under reducing conditions.
followed by autoradiography.

**Northern blotting for bone and bone marrow cells of sham-operated, ovariectomized (OVX), and estrogen-replaced OVX mice**

Eight-week-old female ddY mice were subjected to either dorsal ovariectomy or sham operation under general anesthesia. The OVX mice were implanted with either a placebo pellet (OVX, n=6) or a slow release (21 d) pellet containing 10 µg of 17ß-estradiol (Innovative Research of America, Toledo, OH) (OVX+E, n=6). The sham-operated mice were also implanted with a placebo pellet (Sham, n=6). Mice were sacrificed 3 weeks after surgery, and the whole tibiae and femora were excised. To extract RNA from cells of bone marrow cells and residual bone from which bone marrow was removed, epiphyses at both ends were cut off, bone marrow was flushed with PBS, collected cells were suspended in ISOGEN extraction buffer, and total RNA was extracted. The residual bones were washed with PBS and immediately put into ISOGEN extraction buffer. Twenty µg and 5 µg of total RNA from bone marrow cells and residual bones, respectively, were run on a 1.2% agarose-2.2 M formaldehyde gel, transferred to a nitrocellulose membrane by positive pressure, and fixed to the membrane by ultraviolet irradiation. After 1 hour of prehybridization in GMC buffer (0.5 M Na₂HPO₄, 1% BSA, 1 mM EDTA and 7% SDS, pH 7.2) at 60°C, filters were hybridized overnight in GMC buffer at 65°C with a random primer [³²P]dCTP-labeled cDNA probe for Gas6, Tyro 3, FGF-2, FGFR1, M-CSF, and c-Fms. cDNA probes were generated by RT-PCR using the template of total RNA from mouse brain. The primers for probes were as follows:
Gas6: sense, 5’-CCATCAACCACGGCATGTGG-3’
; antisense, 5’-TCGCACACCTTGATTTCAT-3’.
Tyro3: 5’-GGAAGAGACGCAAGGAGAC-3’
; antisense, 5’-ATGGGAATGGGGAGACGAC-3’.
FGF-2: 5’-CAAGCAGAAGAGAGGAGGAGTTGTGTC-3’
; antisense, 5’-CAGTTCGTTCAGTGCCACATACC-3’.
FGFR1: 5’-TGGAGTTCTATGTGCAAGGTG-3’
; antisense, 5’-ATAGAGAGGACCATCCTGTG-3’.
M-CSF: 5’-ACAACACCCCCAATGCTAAC-3’
; antisense, 5’-ACTGCCTGCGTCCTCTATGC-3’.
c-Fms: 5’-TGCTAAAGTCCACGGCTCAT-3’
; antisense, 5’-CAGTCCAAAGTCCCAATCT-3’.

Filters were washed in 1 X SSC (0.15 M NaCl, 15 mM Na3 citrate, pH 7.0)-0.1% SDS twice for 15 min at 65°C, then once for 15 min in 0.1 X SSC-0.1% SDS at 65°C. Signals were quantitated by densitometry (Bio-Rad Laboratories, Richmond, CA). Filters were stripped by boiling in 0.1% SDS + 0.1 x SSC between hybridizations.

**Statistical analysis**

Means of groups were compared by ANOVA and significance of differences was determined by post-hoc testing using Bonferroni’s method.
RESULTS

Effects of Gas6 on the function, differentiation, and survival of osteoclasts

To examine the effect of Gas6 on the function of mouse osteoclasts, the pit area on a dentine slice resorbed by crude osteoclastic cells formed in the coculture of mouse osteoblasts and bone marrow cells in the presence of 1,25(OH)\(_2\) vitamin D\(_3\) and PGE\(_2\) was measured. Gas6 (≥10\(^{-11}\) M) dose dependently stimulated the resorbed pit area up to 2.1-fold of the control culture (Fig. 1, top panel). This stimulation was not due to the increase in the number of osteoclasts but due to the activation of each osteoclast function because the number of TRAP-positive multinucleated osteoclasts on a dentine slice was not affected by Gas6 (Fig. 1, middle panel). In fact, the effect of Gas6 on the pit area per osteoclast (resorbed pit area / osteoclast number in a dentine slice) showed a similar pattern to that on the resorbed pit area (Fig. 1, bottom panel).

To confirm the action of Gas6 on osteoclast differentiation, dose response of effects of Gas6 (10\(^{-14}\)-10\(^{-8}\) M) on TRAP-positive multinucleated osteoclast formation in the coculture for 6 days on a plastic dish was examined. Gas6 did not increase osteoclast formation at any concentration while FGF-2 and 1,25(OH)\(_2\) vitamin D\(_3\), positive controls, stimulated it potently (Fig. 2A). Gas6 also did not affect TRAP-positive multinucleated osteoclast formation in the culture of mouse bone marrow cells alone although FGF-2 and 1,25(OH)\(_2\) vitamin D\(_3\) did (data not shown).
We further investigated the effect of Gas6 (10^{-8} M) on the survival of osteoclasts. Mouse osteoclasts isolated from the coculture were cultured on a plastic dish for up to 72 hours with or without Gas6 and M-CSF (100 ng/ml) as a positive control (Fig. 2B). The survival rates decreased similarly with time in the control and Gas6 treated cultures. At 24 h only 32.5 % and 22.5 % of initially-surviving cells still adhered to the dish in control and Gas6 treated cultures, respectively, and by 72 h all cells had died in both cultures. On the contrary, M-CSF increased the survival rate of osteoclasts as reported previously (6-10); the survival rates were 83.3% at 24 h and 17.8% of cells were still alive after 72 h. Hence, these studies using mouse culture systems revealed that Gas6 did not affect the differentiation or survival of osteoclasts, but did stimulate their bone resorptive function.

**Expression pattern of Gas6 and Tyro 3 in bone cells**

To study the expression of Gas6 and Tyro 3 in cells of osteoblastic and osteoclastic lineages, mRNA levels in osteoblasts, osteoclasts, spleen cells, bone marrow cells, and brain were examined by RT-PCR. Gas6 was expressed in all cells examined; however, the expression of Tyro 3 was detected only in osteoclasts and brain (Fig. 3A). Western blot analysis confirmed that the protein level of Tyro 3 was detected in osteoclasts, but not in osteoblasts (Fig. 3B). To investigate the expression of Tyro 3 during the differentiation of osteoclastic cells, Tyro 3 mRNA levels were examined in spleen cells cultured in the presence of soluble RANKL/ODF and M-CSF without support of osteoblastic / stromal cells (Fig. 3C). Because we extracted mRNA from cultured spleen cells every day, various differentiation stages of osteoclastic cells were assumed to be included. TRAP-positive multinucleated osteoclasts became detectable at 5 days of culture and increased at 6 days, and Tyro 3 expression was
detected slightly at 5 days and abundantly at 6 days of culture. These findings confirm that Tyro 3 is expressed predominantly in mature osteoclasts, but not in osteoclast precursors. We therefore propose that this localization of Tyro 3 may explain the selective action of Gas6 on the function of mature osteoclasts.

**Intracellular signaling through Gas6 / Tyro 3 in isolated osteoclasts**

To learn the mechanism of Gas6 / Tyro 3 signaling in mature osteoclasts, we examined the time course of effects of Gas6 (5 X 10^{-9} M) on tyrosine phosphorylation of cellular proteins in isolated mouse osteoclasts (Fig. 4A). Several proteins were phosphorylated by Gas6 as early as 1 min and this activation was maintained for 10 min. The c-Src signal was used as an internal control. Western blot analyses using antibodies against specific proteins related to MAPKs revealed that phosphorylation of p42/p44 MAPK was seen at 1 min and maintained for 10 min, while neither p38 nor JNK MAPK phosphorylation was seen (Fig. 4A). To investigate the autophosphorylation of Tyro 3 by Gas6, the kinase activity of immunoprecipitated Tyro 3 was examined by *in vitro* kinase assay (Fig. 4B). Gas6 induced the kinase activity of Tyro 3 at 1 min, reached maximum at 2 min, and decreased considerably after 5 min.

To examine the functional relevance of the activation of p42/p44 MAPK by Gas6 in osteoclasts, PD98059, a specific inhibitor of upstream kinase of p42/p44 MAPK (26, 27), was added to the pit formation assay system (Fig. 4C). PD98059 (1-30 µM) dose dependently inhibited the stimulation of Gas6 on pit formation resorbed by mouse osteoclasts to the levels of the control culture, while SB203580 (30 µM), a specific inhibitor of p38 MAPK (28, 29),
did not affect the Gas6 stimulation. Although PD98059 at the highest concentration (30 µM) did not decrease the resorbed pit formation in the control culture, inhibitors of src kinase, herbimysin (1 µM) and PP1 (10 µM), abrogated pit formation not only in the Gas6-stimulated culture but also in the control culture (data not shown), suggesting the essential role of src kinase signaling in the basal function of osteoclasts.

**Messenger RNA levels of RTKs and their ligands in bone and bone marrow of sham-operated (Sham), OVX, and estrogen-replaced OVX (OVX+E) mice**

The physiological relevance of this Gas6 / Tyro 3 signaling to the pathophysiology of bone loss by estrogen deficiency was investigated by examination of mRNA levels of Gas6 and Tyro 3 in cells of bone marrow and residual long bones of Sham, OVX, and OVX+E mice (Fig. 5). Since we assume it possible that signaling through RTKs on osteoclasts may contribute to the pathophysiology of osteopenic disorders, we also examined mRNA levels of other RTKs and their ligands: FGFR1 & FGF-2, and c-Fms & M-CSF, which are known to regulate osteoclast function or survival (5-10). Among them, Gas6 mRNA level in bone marrow cells was upregulated by OVX and was reduced by estrogen replacement. No Tyro 3 expression could be detected by Northern blotting either in bone or bone marrow of any mice although it was detected as a single band when mRNA from mouse brain was used (data not shown). Among other RTKs and ligands, M-CSF was upregulated by OVX as previously reported (21-24) although it was little reduced by estrogen replacement. Messenger RNA levels of other molecules were not regulated by OVX or estrogen replacement. It is therefore proposed that the upregulation of Gas6 expression in bone marrow may possibly contribute to the bone resorptive status by estrogen deficiency.
DISCUSSION

This study demonstrated that Gas6, although ubiquitously expressed in bone cells, did not affect the differentiation or survival of osteoclasts, but stimulated osteoclast bone resorptive function using several mouse culture systems. This may be due to the restricted localization of its receptor Tyro 3 on mature osteoclasts. Gas6 was further shown to activate osteoclasts through the phosphorylation of Tyro 3 and p42/p44 MAPK in mature osteoclasts. This Gas6/Tyro 3 signaling in osteoclasts was suggested to be involved in bone loss by estrogen deficiency because Gas6 mRNA level was upregulated by OVX and reduced by estrogen replacement.

Gas6 is reported to be expressed ubiquitously in heart, lung, stomach, kidney, muscle, brain, spleen, liver, ovary, and testis (30), although little is known about its function in these organs. It is reported to increase the proliferation and the survival of fibroblasts and vascular smooth muscle cells (31, 32). The expression pattern of Tyro 3, on the other hand, is limited to the central nervous system, kidney, ovary, and testis (11-16). The downstream pathway of Gas6/Tyro 3 signaling has been little investigated. The possible mediation by p42/p44 MAPK shown here in osteoclasts has also been reported in a previous study using human embryonic kidney 293 cells (33), while that of phosphatidylinositol 3 (PI3) kinase pathway is indicated in NIH3T3 cells (34). However, functional or physiological relevance of the signaling through Gas6/Tyro 3 in these cells remains elusive.

FGF-2, M-CSF, and Gas6 are potent regulators of osteoclastic bone resorption whose
receptors are RTKs expressed on mature osteoclasts. We recently reported that FGF-2 acts directly on mature osteoclasts through the signaling pathway similar to that of Gas6: activation of its receptor FGFR1 and p42/p44 MAPK (6). M-CSF is reported to activate its receptor, c-Fms, followed by the activation of not only p42/p44 MAPK pathway (10) but also c-src-dependent pathway (8). c-Src, a ubiquitous cellular tyrosine kinase which is highly expressed in osteoclasts, is essential for osteoclasts to form a ruffled border and to resorb bone (35), and the contribution of c-src kinase to Gas6 / Tyro 3 signaling has been suggested (36). In this study, inhibitors of the src family kinases, herbimycin and PP1, abrogated the osteoclast function in control cultures as well as in Gas6-stimulated cultures. Hence, we assume that the src kinase signal may be essential for the basal osteoclast function, while p42/p44 MAPK is the major pathway for the Gas6 action.

Although the essential component of signaling regulating osteoclast differentiation and function is controversial, the roles of nuclear factor (NF)-κB and JNK have been extensively investigated (37). The stimulation by RANKL/ODF has been demonstrated to be mediated by the activation of NF-κB which is dependent on the interaction with tumor necrosis factor receptor-associated factor 6 (TRAF6) or TRAF2 (38). In fact, knockout mice of both NF-κB1 and NF-κB2, and of TRAF6 exhibited severe osteopetrosis due to the impaired osteoclast differentiation and function (39, 40). Although RANKL/ODF also activates JNK in osteoclasts (37), the role of osteoclast function is still controversial. In this study, JNK does not seem to mediate the Gas6 effect judging from the lack of JNK phosphorylation by Gas6. The essential signal pathway for the survival of osteoclasts is also controversial. NF-κB pathway (9) and p42/p44 MAPK pathway (10) have been reported to be important in
sustaining the survival of osteoclasts. In the present study, although Gas6 induced activation of p42/p44 MAPK, it did not promote the survival of osteoclasts. This result, however, cannot rule out possibility of involvement of p42/p44 MAPK in osteoclast survival because the p42/p44 MAPK activation by Gas6 might be insufficient to sustain the survival while sufficient to activate osteoclast function.

In regard to the possible physiological relevance, this study demonstrated that OVX increased and estrogen replacement decreased the Gas6 mRNA expression in bone marrow. OVX animal is a model for the postmenopausal osteoporosis caused by estrogen withdrawal in humans. Joint destruction in RA patients is also accompanied by the acceleration of osteoclastic bone resorption. We recently reported that increased FGF-2 in the synovial fluid contributed to joint destruction through a direct action on mature osteoclasts (20). Expressions of Gas6 and Tyro3 have been recently demonstrated in the synovium of RA patients (41). These observations suggest that Gas6 as well as FGF-2 play some roles in the pathological bone disorders in these diseases. In addition, Gas6 and FGF-2 have a common action in induction of angiogenesis. Regarding other angiogenic growth factors besides Gas6 and FGF-2, vascular endothelial growth factor (VEGF) has been reported to enhance the osteoclastic bone resorption directly and indirectly in cultures (42-44). In our recent study to identify RTKs expressed on rabbit mature osteoclasts, we cloned an RTK, TIE, whose ligands are angiopoietins, other potent angiogenic growth factors (4). These findings indicate that several angiogenic factors have a common action to stimulate osteoclastic function as well as angiogenesis, suggesting a positive interrelationship between the bone resorption and the invasion of blood vessels during bone modeling and remodeling.
The results in this study demonstrate that Gas6 / Tyro 3, an RTK signaling, may play an important role in the mature osteoclast function. This direct action of Gas6 on osteoclasts might possibly have a key function in bone loss by estrogen deficiency. Further studies will reveal the contribution of RTK signalings like Gas6 / Tyro 3 to the pathophysiology of osteopenic disorders.
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FIGURE LEGENDS

Figure 1. Dose response of effects of Gas6 on resorbed pit area (top), osteoclast number (middle), and the pit area per osteoclast (bottom) on a dentine slice

Osteoblasts from neonatal mouse calvariae and bone marrow cells from 8-week-old mice were cocultured on a collagen gel to form osteoclasts. A crude fraction of cells including osteoclasts was then released from the gel and further cultured on a dentine slice with or without Gas6 (10^{-14}-10^{-8} M). After 48 h of culture, the total pit area and the osteoclast number were measured by toluidine blue and TRAP staining, respectively.

Data are expressed as means (bars) ± SEMs (error bars) for 8 cultures / group.

*P<0.01, significantly different vs. control.

Figure 2. Effects of Gas6 on the differentiation (A) and the survival (B) of osteoclasts

(A) Dose response of effects of Gas6, FGF-2, and 1,25(OH)_{2} D_{3} (Vit.D_{3}) on TRAP positive-multinucleated cell formation in the coculture system.

Mouse bone marrow cells and osteoblasts were cocultured with or without Gas6 (10^{-14}-10^{-8} M), FGF-2 (10^{-8} M), and Vit.D_{3} (10^{-8} M) on plastic dishes for 6 days. TRAP positive-multinucleated cells containing more than three nuclei were counted as osteoclasts.

Data are expressed as means (bars) ± SEMs (error bars) for 8 cultures / group.

(B) Effect of Gas6 and M-CSF on the survival of isolated osteoclasts

Mouse osteoclasts isolated from the coculture on a plastic dish were further cultured with or without Gas6 (10^{-8} M) or M-CSF (100 ng/ml) for various periods up to 72 hours. Trypan
blue-negative and TRAP-positive osteoclasts were counted as living osteoclasts.

Data are expressed as means (symbols) ± SEMs (error bars) for 6 cultures / group.

*P<0.01, significant difference from control culture at each time point.
Figure 3. Expression patterns of Gas6 and Tyro 3 in bone cells.

(A) Messenger RNA levels of Gas6 and Tyro 3 in mouse osteoblasts (OB), osteoclasts (OCL), spleen cells, bone marrow, and brain (RT-PCR).

(B) Protein levels of Tyro 3 in mouse osteoclasts (OCL) and osteoblasts (OB) (Western blotting).

(C) Tyro 3 mRNA level and TRAP-positive osteoclast formation during differentiation of osteoclastic cells in the mouse spleen cell culture (RT-PCR).

Total RNA was extracted from osteoblasts, osteoclasts, spleen cells, bone marrow, and brain as described in Experimental Procedures. The PCR products for Gas6 and Tyro 3 were 589 bp and 445 bp, respectively. For Western blotting, cellular proteins extracted with TNE buffer were subjected to SDS-PAGE, and immunoblotted with polyclonal anti-goat Tyro 3 antibody or non-immune IgG. To confirm the specificity of these blottings, stripped membranes were immunoreacted with each polyclonal anti-Tyro 3 and respective blocking peptide. For spleen cell cultures, spleen cells (2 X 10^8 cells/dish) were cultured in the presence of soluble RANKL/ODF and M-CSF for 6 days, and mRNA was extracted every day. TRAP positive-multinucleated cells containing more than three nuclei were counted as osteoclasts (# of OCL).
Figure 4. Intracellular signaling through Gas6 / Tyro 3 in isolated osteoclasts

(A) Effects of Gas6 on phosphorylation of cellular proteins and MAPKs in isolated osteoclasts.

Mouse osteoclasts isolated from the coculture were cultured with or without Gas6 (5 X 10^{-9} M) for the indicated period (0-10 min) and lysed with TNE buffer. Twenty µg of cell lysates was subjected to 7.5% SDS-PAGE, and immunoblotted with antibodies against phosphotyrosine, src, phospho-p44/42 MAPK, phospho-p38 MAPK, and phospho-JNK MAPK. Arrow head indicates 170 kD protein (the same size as Tyro 3).

(B) Tyrosine kinase activity of immunoprecipitated Tyro 3 in isolated osteoclasts

Isolated osteoclasts were cultured with and without Gas6 (5 X 10^{-9} M) for various periods (1-10 min), lysed with TNE buffer, and 100 µg of cell lysates was immunoprecipitated with polyclonal anti-Tyro 3 antibody. The samples were incubated in kinase buffer with [γ-32P]-ATP, and subjected to SDS-PAGE.

(C) Effects of PD98059 (PD) and SB203580 (SB) on resorbed pit formation stimulated by Gas6 on a dentine slice

Osteoblasts from neonatal mouse calvariae and bone marrow cells from 8-week-old mice were cocultured on a collagen gel to form osteoclasts. Crude osteoclastic cells released from the coculture on a collagen gel were further cultured on a dentine slice with or without Gas6. Gas6 (10^{-8} M), PD98059 (1, 3, 10 and 30 µM) and SB203580 (30 µM) were added to the culture at 1 hour after the seeding. After 48 h of culture, total pit area in a dentine slice was measured.

Data are expressed as means (bars) ± SEMs (error bars) for 8 cultures / group.
*P<0.01, significant stimulation by Gas6; #P<0.01, significant inhibition by PD98059.
Figure 5. Messenger RNA levels of RTKs and their ligands in bone and bone marrow of sham-operated (Sham), OVX, and estrogen-replaced OVX (OVX+E) mice

Eight-week-old mice were subjected to either ovariectomy (n=12) or sham operation (n=6). The OVX mice were implanted with either placebo pellets (n=6) or slow release pellets of 17β-estradiol (n=6). Mice were sacrificed 3 weeks after surgery, the whole tibiae and femora were excised, and total RNA was extracted from bone marrow and the residual bone as described in Experimental Procedures. Twenty μg and 5 μg of total RNA from bone marrow and residual bone, respectively, were run on a gel, and mRNA levels of Gas6, Tyro 3, FGF-2, FGFR1, M-CSF, and c-Fms were analyzed by Northern blotting using cDNA probes produced by PCR. The number under each band is the treated / control ratio of the intensity of each band normalized to that of G3PDH measured by densitometry.
| Gene       | Bone Marrow | Residual Bone |
|------------|-------------|---------------|
| Gas6       | 1.0 3.8 2.0 | 1.0 1.2 0.9   |
| Tyro 3     |             |               |
| FGF-2      | 1.0 0.8 0.7 | 1.0 1.1 1.1   |
| FGFR1      | 1.0 0.9 0.8 | 1.0 0.7 0.9   |
| M-CSF      | 1.0 2.1 1.7 | 1.0 1.0 1.2   |
| c-Fms      | 1.0 1.1 0.9 | 1.0 0.6 0.6   |
| G3PDH      |             |               |
Mechanism of Stimulation of Osteoclastic Bone Resorption through Gas6 / Tyro 3, a Receptor Tyrosine Kinase Signaling, in Mouse Osteoclasts
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