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ORIGINAL ARTICLE

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A novel role of L-serine (L-Ser) for the expression of nuclear factor of activated T cells (*NFAT)2* in receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis in vitro

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Abstract Multinucleated cell formation is crucial for osteoclastogenesis, and the expression of nuclear factor of activated T cells *(NFAT)2 (NFATc1)* is essential for this process. We previously found, using mouse RAW264 cells, that culture at high cell density blocked progression to the multinucleated cell stage induced by stimulation with receptor activator of nuclear factor κB ligand (RANKL). Here, we have confirmed this finding in a bone marrow cell system and extended the analysis further. A high cell density appeared to cause a change in the composition of the culture medium accompanying downregulation of NFAT2 expression, and we identified L-serine (L-Ser) as essential for the expression of NFAT2 induced by RANKL. Namely, culture at high cell density caused a depletion of L-Ser in the medium. Consequently, L-Ser appeared to exert its effect at an early stage under the regular conditions used for inducing the expression of c-Fos, an upstream regulator of *NFAT2*. D-Ser, an enantiomer of L-Ser, showed no NFAT2-inducing activity. The expression of NFAT2, using a retrovirus vector, could compensate for the depletion of L-Ser and resume the progression to the multinucleated cell stage. These results demonstrate a novel role for L-Ser in RANKL-induced osteoclastogenesis in vitro.

Key words c-Fos · L-Ser · *NFAT2* · osteoclasts · RANKL

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Introduction

Osteoclasts are multinucleated (MN) giant cells with bone-resorbing activity [1]. They are believed to be derived from hematopoietic cells, in particular, from the monocyte/macrophage lineage [2–4]. A unique feature of this differentiation process is cell fusion, which eventually produces giant cells containing up to 100 nuclei. We previously reported, using an *in vitro* osteoclastogenesis system consisting of mouse RAW264 cells and recombinant receptor activator of nuclear factor (NF)-κB ligand (RANKL), that the expression of nuclear factor of activated T cells *(NFAT)2 (NFATc1)* induced by stimulation with RANKL is essential for progression to the MN cell stage [5]. An essential role for *NFAT2* as a downstream effector of c-*fos* in osteoclastogenesis has been observed using knockout mice as well [6,7]. We also found that the number of RAW264 cells seeded was crucial to obtain full differentiation. Namely, few MN cells were observed in densely plated cultures, and the expression of *NFAT2* was suppressed in those cells. These observations consequently raised questions as to what changes high cell density caused in the culture system and how *NFAT2* became suppressed.

Cell density has been reported to affect proliferation and/or differentiation in culture systems of certain cell types. Contact inhibition of cell growth, in which high cell density inhibits growth/proliferation, for example, has been well recognized as a general rule in the field of cell biology. Moreover, when mouse embryonic stem (ES) cells were induced to undergo neural differentiation, culture at low cell density was found to be needed [8,9], and high cell density, conversely, was shown to inhibit the differentiation, accompanying the retardation of nuclear β–catenin protein expression [10]. High cell density, in another case, was reported to induce programmed cell death in mammary gland epithelial cells [11], and Id-1 was implicated in this process [12]. In any case, the importance of cell-cell/matrix contact has been suggested [10,11].

In the present study, we confirmed the cell density-dependent suppression of MN cell formation in a mouse

bone marrow cell system, as well as in mouse RAW264 cells, and extended our previous analysis [5] further. We revealed a novel role of L-serine (L-Ser) in inducing the expression of c-Fos and NFAT2, and found that culture at high cell density caused a depletion of L-Ser. The findings here thus highlight a novel role for L-Ser in RANKL signaling in osteoclastogenesis.

Materials and methods

Cell culture and osteoclastogenesis in vitro

The RAW264 mouse monocyte/macrophage cell line was maintained in Eagle's medium (Nissui, Tokyo, Japan) supplemented with 0.15% $NaHCO_3$, 1% nonessential amino acids (NEAA) (GIBCO-BRL, NY, NY, USA), 2 mM glutamine (Nissui), and 10% fetal bovine serum (FBS) at 37°C, as described previously [13]. Dialyzed FBS was prepared by dialysing FBS against 0.15 M NaCl for 24 h at 4°C. When the different NEAA were used individually, they were purchased from Nacalai (Tokyo, Japan) and used at the same concentrations as those found in the mixture of NEAA purchased from GIBCO-BRL. D-Ser was obtained from Sigma (St. Louis, MO, USA). [^{3}H]-L-Ser was purchased from Amersham Bioscience (Arlington, Heights, IL, USA). Soluble recombinant RANKL was expressed as a glutathione S-transferase (GST)-fused protein and finally purified free of lipopoly succharide (LPS), using Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, IL, USA). For osteoclastogenesis *in vitro*, RAW264 cells were plated at the density indicated in figure legends for each experiment. RANKL and GST were added to final concentrations of 500 ng/ml and 200 ng/ml, respectively, and incubated with the cells for a specific period in each experiment. The cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity on plastic dishes as described previously [14]. Values for quantified results represent the means ± SD of at least triplicate experiments. Osteoclast precursors were generated from mouse bone marrow cells by treatment with macrophage colony-stimulating factor (M-CSF), as described previously [14]. For osteoclast differentiation, osteoclast precursors were plated at a density of 1×10^4 per well in a 24-well plate and cultured overnight, then stimulated with 500 ng/ml of RANKL and 100 ng/ml of M-CSF. The medium was changed after 3 days together with RANKL and M-CSF, as described above. The formation of osteoclasts was monitored the next day. A methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was carried out with an MTT cell growth kit (Chemicon International, Temecula, CA, USA).

Constructs, transfection, and retroviral gene transfer

Green fluorescent protein (GFP) and *NFAT2* cDNA were subcloned into the retroviral vector pCX4*puro* [15]. To prepare the retrovirus, the plasmid was introduced into packaging cell line Platinum-E (plat-E) cells [16] with the vectors pE-eco and pGP (TaKaRa, Kyoto, Japan) and cultured for 48 h. The supernatant was saved, filtered through a 0.22-μm pore filter, and used as a retrovirus stock. For infection, osteoclast precursors were cultured with the retrovirus overnight in α-MEM, then stimulated with RANKL and M-CSF as described above.

Western blot analysis and reagents

Western blotting was carried out essentially as described previously [5]. In brief, cells were lysed in EBC buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 1 mM ethylene diamine tetraacetic acid [EDTA], 0.5% Nonidet P-40 [NP40] (Roche Diagnostics, Indianapolis, IN, USA), 20 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.1% aprotinin, and 2 mM Na_3VO_4), and centrifuged at 10000 *g* for 5 min at 4°C. The supernatant was used as the total cell lysate. Immunoblot detection was performed with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Mouse anti-NFAT2 monoclonal antibody 7Ab (sc-7294) and anti-c-Fos (cs-52) rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin monoclonal antibody (AC-74, Sigma) was used.

Recovery of conditioned medium

Cells were inoculated as required for each experiment and incubated for 24 h in the presence of RANKL under standard conditions. Media were recovered and passed through a membrane filter (0.2-μm pore). When the media thus prepared were used for another culture as conditioned media, one-tenth the volume of FBS and a defined amount of RANKL were added.

Results

Cell density is important for MN cell formation in RAW264 cells, as well as in bone marrow cells

We previously reported that the number of cells to be inoculated was crucial to the expression of NFAT2 protein and the efficiency of MN cell formation in an RAW264 cell-system [5]. We here used osteoclast precursors prepared from mouse bone marrow cells and examined the effect of cell density. Under regular conditions, we seeded 1×10^4 bone marrow cells in 24-well plates and incubated them for 4 days in the presence of RANKL and M-CSF, obtaining plates fully covered with TRAP-positive MN cells (Fig. 1). However, when the number to be inoculated was either decreased to half or increased to 32-fold, the number of MN cells formed was found to be significantly reduced, as shown in Fig. 1, suggesting that cell density was critical for efficient MN cell formation in bone marrow cells, as well as in RAW264 cells.

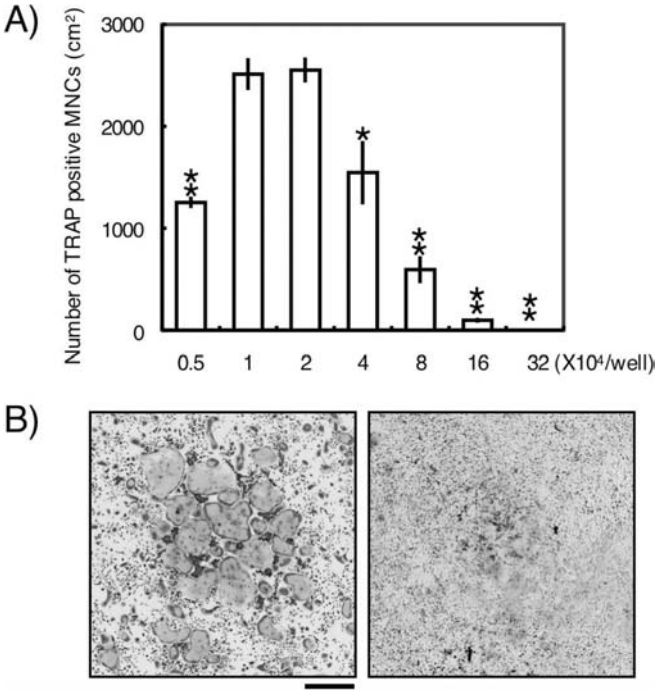

Fig. 1. Cell density-dependent multinucleated cell formation in osteoclast precursors. Cells prepared from mouse bone marrow were inoculated on 24-well plates at the indicated cell numbers and subjected to stimulation with receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), as described in "Materials and methods". **A** Numbers of tartrate-resistant acid phosphatase (*TRAP*)-positive multinucleated cells (*MNCs*) were counted. *Columns with asterisks* show significant differences compared with 1 or 2×10^4 cells/well (Student's *t*-test, $*P < 0.05$; $**P < 0.01$). **B** The morphology of TRAP-stained cells was examined. *Left panel*, low cell density (1×10^4 cells/well); *right panel*, high cell density (8×10^4 cells/well). Bar, 500 μm

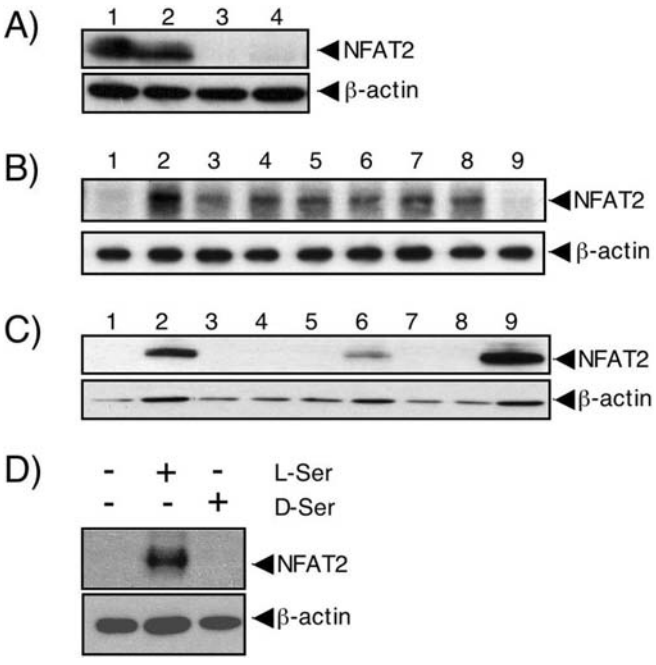

Fig. 2. Requirement of nonessential amino acids (NEAA) and identification of L-serine (*L-Ser*) as an essential component. **A** Effects of adding NEAA on nuclear factor of activated T cells 2 (*NFAT2*) expression. RAW264 cells were cultured for 24 h in the following conditions, and cell lysates were immunoblotted with anti-NFAT2 antibody. *Lane 1*, low cell density with fresh medium; *lane 2*, low cell density with conditioned medium recovered from a high-density culture and supplemented with NEAA; *lane 3,* the same conditions as those in *lane 2*, without supplementary NEAA; *lane 4*, high cell density with fresh medium. Anti-β-actin monoclonal antibody was used for monitoring the amount of protein applied. **B** L-Ser is an essential component of NEAA. RAW264 cells were cultured, using conditioned medium at low cell density, with supplementary RANKL and dialyzed fetal bovine serum (FBS) together with NEAA, added in a combinatorial manner. Conditioned media were recovered from the high-cell-density culture for 24 h. *Lane 1*, without NEAA; *lane 2*, complete NEAA (Ala, Asn, Asp, Glu, Gly, Pro, and Ser); *lane 3*, NEAA lacking Ala; *lane 4*, NEAA lacking Asp; *lane 5*, NEAA lacking Glu; *lane 6*, NEAA lacking Gly; *lane 7*, NEAA lacking Asn; *land 8*, NEAA lacking Pro; *lane* 9, NEAA lacking Ser. Cell lysates were prepared and immunoblotted with anti-NFAT2 antibody. β–Actin was blotted to quantitate the protein applied. **C** L-Ser alone can substitute for an entire set of NEAA. RAW264 cells were cultured, using fresh media, at low cell density with supplementary RANKL and dialyzed FBS, except that a single amino-acid component was added instead of the entire set of NEAA. NFAT2 was examined, as in **B**. *Lane 1*, no supplement; *lane 2*, complete NEAA; *lane 3*, Ala; *lane 4*, Asp; *lane 5*, Glu; *lane 6*, Gly; *lane 7*, Asn; *lane 8*, Pro; *lane 9*, Ser. **D** Effects of L-Ser and D-Ser on NFAT2 expression. RAW264 cells were cultured for 24 h in medium containing dialyzed FBS and lacking NEAA (*lane 1*). Instead, L-Ser (*lane 2*) or D-Ser (*lane 3*) was added, at equimolar concentrations. Cell lysates were prepared and immunoblotted as in **A**

Nutrient was a major determinant of and NEAA restored the inducing activity of NFAT2

A prominent phenomenon was that the expression of NFAT2 protein was undetectable, even when cells were cultured at low density, if the medium recovered from high-density plates was used (Fig. 2A). We subsequently noticed that when we added NEAA to the recovered medium prior to the subsequent cultures, the medium significantly restored the level of NFAT2 (Fig. 2A), implicating an important role for NEAA in the expression of NFAT2. The NEAA used in the present system contained seven amino acids (Ala, Asn, Asp, Glu, Gly, Pro, and Ser), and so we examined which amino acid was important for the induction of NFAT2. To this end, we prepared a series of NEAA mixtures in which single amino acids were depleted one by one, and we tested the additive effect on NFAT2 using conditioned medium produced from densely plated cells. As shown in Fig. 2B, the depletion of no single amino acid affected the expression of NFAT2: the one exception was L-Ser, the depletion of which resulted in a significant decrease in the level of NFAT2 protein. Conversely, when individual amino acids were added to the regular medium without NEAA, the addition of L-Ser resulted in the expression of NFAT2 at a level comparable to that obtained with NEAA; the other amino acids had no effect, except for Gly, which had weak activity (Fig. 2C). Therefore, L-Ser was found to be indispensable for the induction of NFAT2 expression induced by stimulation with RANKL under the present culture conditions. Regarding this, L-Ser in cells is known to be converted to D-Ser and Gly by serine racemase and serine hydroxymethyl transferase, respectively. Therefore, it may be that L-Ser exerted its effect after being converted to D-Ser or Gly. However, the addition of D-Ser, instead of

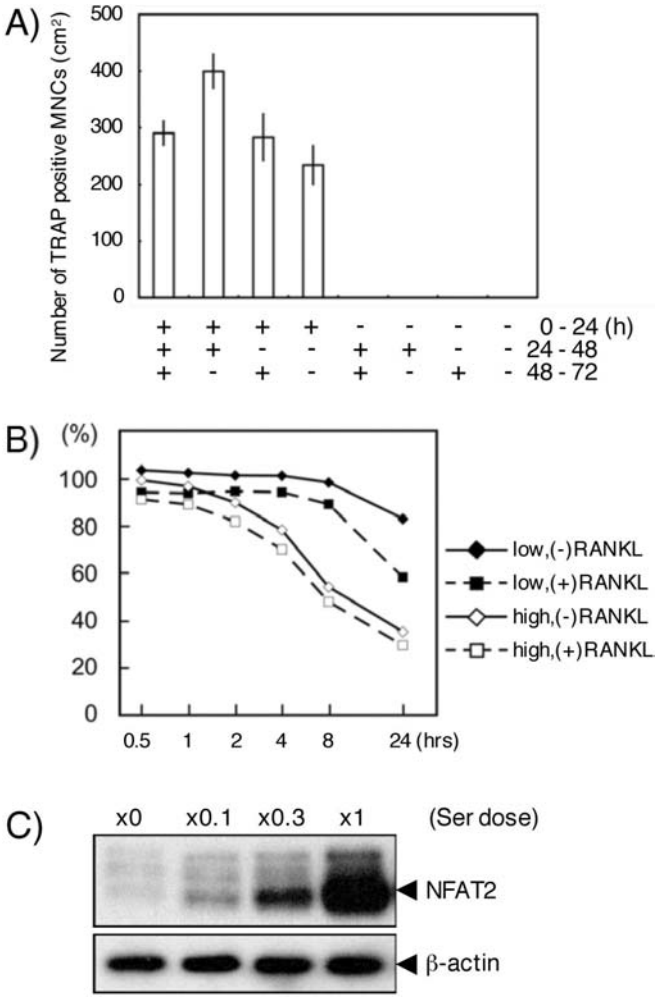

Fig. 3. Function and depletion of L-Ser. **A** Effects of adding L-Ser at different periods on MN cell formation. RAW264 cells were seeded at low density, using dialyzed FBS and fresh medium without L-Ser, and cultured for 96 h in the presence of RANKL. L-Ser (0.1 mM) was added at the periods indicated at the bottom of the graph. TRAP-positive MN cells were enumerated at 96 h. **B** Depletion of L-Ser. RAW264 cells were cultured at low or high density in the presence or absence of RANKL for the indicated periods, using the regular medium containing [^{3}H]-L-Ser (10 μCi/ml). Ten microliters of the medium was taken from each plate at the indicated time points and the radioactivity was measured using a scintillation counter. **C** L-Ser dose dependency. RAW264 cells were cultured for 2 h under regular conditions with dialyzed FBS and varying amounts of L-Ser; *X1* is equivalent to the regular concentration. The amount of NFAT2 protein was monitored by Western blotting with anti-NFAT2 antibody

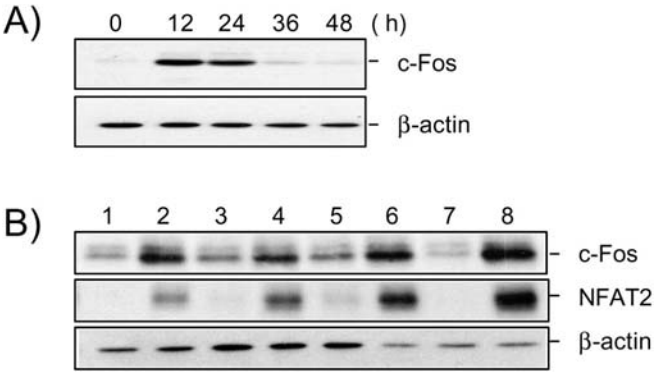

Fig. 4. Effects of L-Ser on c-Fos expression. **A** Expression of c-Fos in cells cultured at low density. RAW264 cells were cultured in the presence of RANKL for the indicated periods, and extracts were prepared for Western blot analysis with anti-Fos antibody. Anti-β-actin monoclonal antibody was used for monitoring the amount of protein applied. **B** Effects of NEAA on c-Fos and NFAT2 expression. RAW264 cells were cultured at low density for 24 h, using dialyzed FBS and different combinations of NEAA. Cell lysates were immunoblotted with anti-Fos (*top column*) and anti-NFAT2 (*middle column*) antibodies. *Lane 1*, without NEAA; *lane 2*, Ser; *lane 3*, Gly; *lane 4*, Ser and Gly; *lane 5*, NEAA lacking Ser; *lane 6*, NEAA lacking Gly; *lane 7*, NEAA lacking Ser and Gly; *lane 8*, complete NEAA. β-actin was monitored, as in **A** (bottom column)

L-Ser, showed no effect on NFAT2 expression, at least at an equimolar concentration (Fig. 2D), suggesting that L-Ser can specifically function as it is.

L-Ser was essential for the expression of c-Fos and NFAT2 induced by RANKL

We then examined when L-Ser was needed by dividing the differentiation process into intervals of 24 h until 72 h. Regarding this, the nuclear localization of the NFAT2 protein at 48–72 h was observed to be crucial for MN cell formation [5]. The presence of L-Ser within the first 24 h appeared to be sufficient for MN cells to form, while its addition or depletion after 24 h had no effect (Fig. 3A), suggesting that L-Ser was required only in the early stage of the osteoclastogenesis. In fact, when the concentration of L-Ser in the medium was analyzed at high or low cell density together with either RANKL or GST, the disappearance of L-Ser was much more rapid and significant at high density in the first 8 h (Fig. 3B), and the level of NFAT2 protein was found to be correlated with L-Ser in a concentration-dependent manner (Fig. 3C).

Because it is now well established that *NFAT2* acts downstream of c-*fos* in osteoclastogenesis [6,7] and because we observed that c-Fos expression was also downregulated in densely plated cells (data not shown), we then examined the expression of c-Fos under the same conditions as those used to examine NFAT2 expression. As shown in Fig. 4A, the expression of c-Fos appeared to respond to RANKL within 12 h, and then declined to the baseline level at 36 h. Moreover, the depletion or addition of L-Ser had the same effect on c-Fos as on NFAT2 (Fig. 4B). Together, these results suggest that L-Ser is required early on for the induction of c-Fos expression.

Retroviral transfer of NFAT2 compensates for the depletion of L-Ser in the bone marrow cell system

The requirement of L-Ser for MN cell formation was further assessed using bone marrow cells with dialyzed FBS. L-Ser has been considered a nonessential amino acid. In fact, the depletion of L-Ser from NEAA or the addition of various concentrations of L-Ser alone instead of NEAA did not essentially affect the proliferation of bone marrow cells, as

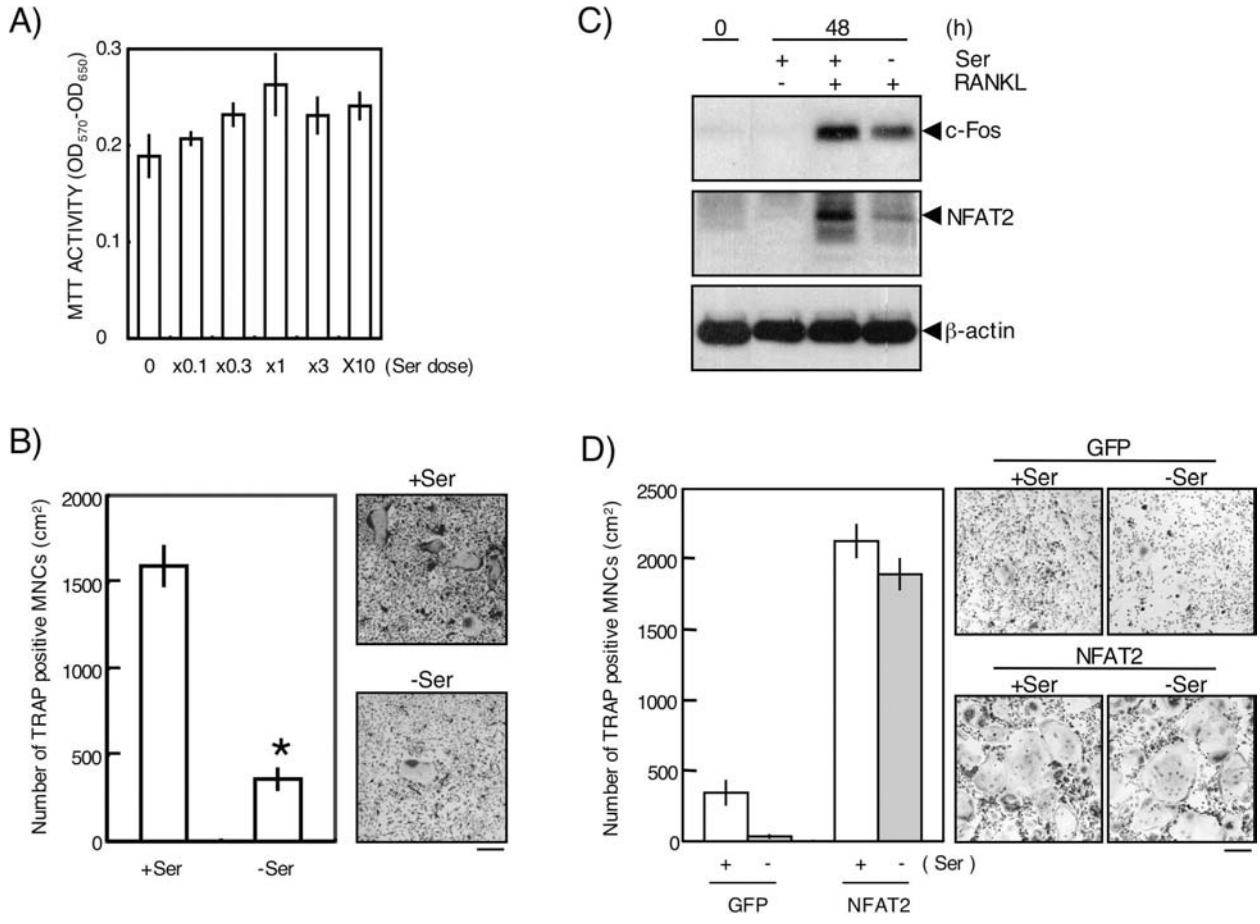

Fig. 5. Effects of L-Ser on the osteoclastogenesis of bone marrow cells. **A** Effects of L-Ser on the proliferation of bone marrow cells. Bone marrow cells were prepared and cultured for 3 days, using dialyzed FBS, as described in "Materials and methods", except that the amount of L-Ser was varied as indicated; *X1* was equimolar to that used in the regular medium. Cell viability was monitored by measuring MTT activity. **B** Effects of L-Ser on MN cell formation in bone marrow cells. Bone marrow cells were prepared and induced to undergo osteoclastogenesis, as described in "Materials and methods", using dialyzed FBS in the presence of L-Ser alone (+ *Ser*) or NEAA lacking L-Ser (– *Ser*). Cells were TRAP-stained after 5 days. *Upper panel* shows quantification of MN cells formed and *lower panel* shows the morphology of the cells. Student's *t*-test, $*P < 0.05$. Bar, 200 μm. **C** Effects of L-Ser on c-Fos and NFAT2. Cell lysates were prepared 48 h after stimulation with RANKL and M-CSF, and the expression of c-Fos and NFAT2 was monitored by Western blotting, using anti-Fos and anti-NFAT2 antibody, respectively. **D** Effects of retroviral transfer of *NFAT2* on L-Ser-depleted bone marrow cells that had been prepared as described in **B**. The morphology of cells expressing green fluorescent protein (GFP; *upper panel*) and NFAT2 (*lower panel*) is shown *on the (right)*. Quantification of MN cells formed is shown *on the left.* Bar, 200 μm

measured with the MTT assay (Fig. 5A). However, the depletion of L-Ser caused a significant reduction in the formation of MN cells and most cells stayed as TRAP-negative mononuclear cells (Fig. 5B), implying that L-Ser is essential for the progression to the MN cell stage for osteoclast precursors, as well as for RAW264 cells. The reduction in the level of c-Fos and NFAT2 under L-Ser-depleted conditions was also confirmed (Fig. 5C). Although the above results suggested the necessity of L-Ser for the expression of c-Fos/NFAT2 and the progression to the MN cell stage, it was not clear whether or not the induction of *NFAT2* expression was sufficient for this progression. To clarify this point, we introduced *NFAT2* into bone marrow cells, using retroviral vectors under L-Ser-depleted conditions. Infection with a vector encoding GFP did not affect the observations in uninfected cells, whereas the expression of NFAT2 induced progression to the TRAP-positive MN cell stage at a level comparable to that obtained under the regular conditions (Fig. 5D), supporting further the idea that *NFAT2* plays a crucial role in the progression to the MN cell stage as a downstream effector of c-*fos*.

Discussion

In this study, we newly identified an indispensable role for L-Ser in RANKL-induced osteoclastogenesis, on the basis of the finding that the progression of osteoclast precursors to the MN cell stage was inhibited at high cell density. A novel and significant finding here was that the conditioned medium recovered from such high cell-density cultures lost the potential to generate MN cells, making it possible for us to identify the important role of L-Ser in the expression of

NFAT2, which had previously been recognized as a key regulator of MN cell formation [5]. The depletion of L-Ser in the medium was evident in densely plated cells at 2 h after plating. Moreover, conditioned medium containing a reduced amount of L-Ser was found not to induce the expression of NFAT2, even at low cell density, and the degree of L-Ser depletion in the medium and the degree of the suppression of NFAT2 showed a strong correlation, suggesting that the concentration of L-Ser in extracellular fluid may be a principal factor in the phenomenon reported here. It is not yet clear, however, whether the reduction simply reflected the difference in cell numbers or whether some other mechanism(s) was involved. Regarding this, a reduction in the concentration of L-Ser in the medium was also observed in GST-treated cells, suggesting that, early on, this phenomenon was not specific to RANKL-treated cells. In fact, when the same medium as that used for the RAW264 cell system was applied to densely-plated cells of other types, such as NIH3T3 and COS7, the recovered medium had the same effect on RAW264 cells (data not shown), suggesting that the depletion of L-Ser due to high cell density is not specific to RAW264 cells, but, rather, it is a general mechanism. Moreover, when cells were inoculated at high density and differentiated under the regular condition, except for the presence of a tenfold higher concentration of L-Ser, little expression of NFAT2 or formation of TRAP-positive MN cells was observed (data not shown). Therefore, although L-Ser appears to be an essential component for the expression of NFAT2, culture at high cell density may induce another effect that suppresses the function of L-Ser. We presume that densely plated cells cause a more profound and/or rapid depletion of L-Ser in their microenvironment than cells inoculated at low density. The precise mechanism underlying this effect, however, remains elusive.

L-Ser has been considered a nonessential amino acid, and, in fact, was not essential for cell proliferation in the present systems. Evidence is accumulating that free amino acids have physiological roles, in addition to acting as intermediates in metabolism, in various tissues [17,18]; indeed, some amino acids are known to function through extracellular receptors. L-Glu, for example, has been extensively analyzed as an excitatory amino acid neurotransmitter in the central nervous system. L-Glu exerts its effects through two types of receptor, metabotropic (m)GluRs and ionotropic (i)GluRs [17,19]. The extracellular condensation of L-Glu is also well controlled [20]. Furthermore, a role for L-Ser as a key mediator of neutrophic support has been proposed; namely, L-Ser is released by glial cells and, in cultured neurons, is essential for survival, neuritogenesis, and functional voltage responses [21]. The level of D-3-phosphoglyceride dehydrogenase (*phgdh*), which catalyzes the first step in the biosynthesis of L-Ser, was very low in neurons, and it was necessary for L-Ser to be supplied by astrocytes [11,22,23]. In fact, when *phgdh* was deleted in mice, early embryonic lethality was observed, with multiple developmental defects, particularly in the central nervous system [24].

The findings in the present study suggest the existence of a novel mechanism which links L-Ser and RANKL for full activation of the signaling cascade, leading to the expression of c-Fos/NFAT2 in osteoclast precursors and then to the multinucleated cell stage. Now that the role of c-*fos* as a regulator of *NFAT2* has become evident [6,7] and now that it has been shown that L-Ser appeared to be required for the induction of c-Fos, it is necessary to identify molecules functioning upstream of c-*fos* induced by stimulation with RANKL. In this regard, RANKL signaling is known to be mediated by the recruiting of tumor necrosis factor-associated factor (TRAF) protein(s) that activate mitogen-activated protein (MAP) kinase cascades, and these signaling cascades, in turn, could regulate two distinct transcription factors, NF-κB and activator protein-1 (AP-1) [25–27]. However, three subgroups of the MAP kinase family, p38, ERK, and JNK, were found to become activated by RANKL signal without supplementary L-Ser in the present systems (data not shown), suggesting that L-Ser may act either downstream of these MAP kinases or that it may act in an as yet unidentified signaling cascade(s). Moreover, the expression of c-Fos is known to be regulated at multiple steps [28,29], and c-Fos was further shown to be under the regulation of the ubiquitin-proteasome proteolytic pathway [30]. Therefore, although we observed an elevated expression of c-Fos in the presence of L-Ser, the precise mechanism(s) underlying this observation needs to be clarified, and this will provide an important clue for the understanding not only of osteoclastogenesis but also of the physiological significance of L-Ser signaling.

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