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Activin A Stimulates IκB-α/NFκB and RANK Expression for Osteoclast Differentiation, but not AKT Survival Pathway in Osteoclast Precursors

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Abstract Recent studies have reported that activin A enhances osteoclastogenesis in cultures of mouse bone marrow cells stimulated with receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). However, the exact mechanisms by which activin A functions during osteoclastogenesis are not clear. RANKL stimulation of RANK/TRAF6 signaling increases nuclear factor-κB (NFκB) nuclear translocation and activates the Akt/PKB cell survival pathway. Here we report that activin A alone activates IκB-α, and stimulates nuclear translocation of NFκB and receptor activator of nuclear factor-κB (RANK) expression for osteoclastogenesis, but not Akt/PKB survival signal transduction including BAD and mammalian target of rapamycin (mTOR) for survival in osteoclast precursors in vitro. Activin A alone failed to activate Akt, BAD, and mTOR by immunoblotting, and it also failed to prevent apoptosis in osteoclast precursors. While activin A activated IκB-α and induced nuclear translocation of phosphorylated-NFκB, and it also enhanced RANK expression in osteoclast precursors. Moreover, activin A enhanced RANKL- and M-CSF-stimulated nuclear translocation of NFκB. Our data suggest that activin A enhances osteoclastogenesis treated with RANKL and M-CSF via stimulation of RANK, thereby increasing the RANKL stimulation. Activin A alone activated the NFκB pathway, but not survival in osteoclast precursors in vitro, but it is, thus, insufficient as a sole stimulus to osteoclastogenesis. J. Cell. Biochem. 90: 59–67, 2003.

Key words: activin A; NFκB; RANK; osteoclast differentiation; survival; AKT; mTOR

INTRODUCTION

There is evidence that activin A, a member of the transforming-growth factor (TGF)-β superfamily originally identified as an erythroid differentiation factor, has diverse functions [Eto et al., 1987; Feijen et al., 1994]. Activin proteins are produced from two gene products, activin βA and activin βB that dimerise to form activin A, activin B, and activin AB [Roberts et al., 1991; Roberts and Barth, 1994; Ferguson et al., 2001]. Activins signal via cell surface serine-threonine kinase receptors that include type 1 ligand-binding receptors and type 2 signaling receptors [Attisano et al., 1992; Zimmerman and Mathew, 1996]. It has also been known that activins play multiple roles including ability to act as mesoderm-inducing factors in amphibian development, induction of *Xenopus laevis* embryos, cell cycle arrest and apoptosis [Woodruff, 1998].

Bone is a major storage site for TGF-β superfamily members, including TGF-β, bone morphogenetic proteins (BMPs), and activin A. It is believed that these cytokines are released from bone during bone resorption. Recent studies have reported that activin A is involved in bone cell biology [Fuller et al., 2000a; Itoh et al., 2001; Murase et al., 2001; Gaddy-Kurten et al., 2002; Koseki et al., 2002]. The periosteal injection of activin A stimulates bone formation [Oue et al., 1994], ectopic bone formation induced by BMP is enhanced by activin A [Ogawa et al., 1992],

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and local administration of activin promotes fracture healing in vivo [Sasaki et al., 1999]. Moreover, activin-$\beta A^{-/-}$ mice have a defect in craniofacial development [Matzuk et al., 1995]. Thus, activin A is essential for bone development in vivo. In vitro, activin A supports osteoblast formation by alkaline phosphatase-positive and mineralized colony formation [Gaddy-Kurten et al., 2002]. It also stimulates chondrogenesis because it enhances the size of precartilaginous condensations and the cartilage phenotype [Jiang et al., 1993]. However, it has reported that activin A has an inhibitory effect on chondrogenic differentiation [Luyten et al., 1994].

It has also been known that activin A stimulates osteoclastogenesis [Fuller et al., 2000a; Itoh et al., 2001; Murase et al., 2001; Gaddy-Kurten et al., 2002; Koseki et al., 2002]. Sasaki et al. [1993] first reported that activin A enhances osteoclast-like cell formation supported by 1α,25-dihydroxyvitamin D_3 or PTH, but not osteoclast activation in murine bone marrow cultures. Recently, multiple laboratories also have demonstrated that activin A stimulates osteoclast differentiation from bone marrow macrophages (BMMs) or RAW 264.7 cells (a murine macrophage line also capable of RANKL mediated osteoclastogenesis) supported by RANKL and M-CSF (9-13). BMP-2 and TGF-β1 also enhance osteoclastogenesis stimulated with RANKL and M-CSF in vitro [Galvin et al., 1999; Fuller et al., 2000b; Kaneko et al., 2000; Itoh et al., 2001; Yan et al., 2001]. Moreover, BMP-2 and BMP-4 activate bone resorption by these BMPs-stimulated cathepsin K expression [Kaneko et al., 2000].

The signaling cascade downstream of RANK/RANKL including the activation of NFκB and the JNK/AP-1 pathways is essential for osteoclastogenesis [Wagner and Karsenty, 2001]. Activin A enhances osteoclastogenesis, however, it fails to stimulate NFκB and JNK activation in RAW 264.7 cells [Koseki et al., 2002]. BMP-2 also stimulates osteoclastogenesis [Kaneko et al., 2000; Itoh et al., 2001], whereas it fails to stimulate NFκB, JNK activation, and RANK expression during osteoclastogenesis treated with RANKL and M-CSF [Itoh et al., 2001; Koseki et al., 2002]. Thus, cytokines-stimulated osteoclastogenesis is not understood fully as yet in terms of mechanism. In the present study, we explored the role of activin A on osteoclastogenesis and survival. Activin A activated IκB-α and induced nuclear translocation of phosphorylated-NFκB, and it also induced RANK expression in osteoclast precursors. Activin A alone failed to activate Akt, BAD, and mTOR, which are essential for survival, and it also failed to prevent apoptosis in osteoclast precursors. Activin A alone also failed to activate the JNK/AP-1 pathway. The present findings suggest that activin A is co-factor during osteoclastogenesis supported by RANKL and M-CSF, increasing RANK expression and NFκB, and but not survival in osteoclast precursors.

MATERIALS AND METHODS

Materials

Polyclonal anti-Akt, anti-phospho-Akt threonine (Thr) 308, anti-phospho-Akt serine (Ser) 473, anti-IκB-α, anti-phospho-IκB-α (Ser 32), anti-Bad, anti-phospho-Bad (Ser 136), anti-mTOR, anti-phospho-mTOR (Ser 2448), anti-phospho NFκB RelA/p65 (Ser 536), and rapamycin (mTOR inhibitor) were purchased from New England Biolabs (Beverly, MA). Polyclonal anti-RANK was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant mouse M-CSF and activin A were purchased from R&D Systems, Inc. (Minneapolis, MN). All other chemicals were purchased from Sigma (St. Louis, MO).

Mouse Bone Marrow Macrophages Cultures

BMMs prepared from the femur and tibia of 4-to 6-week-old C57BL/6 mice and incubated in tissue culture dishes (100 mm dishes) at 37°C in 5% CO_2 in the presence of recombinant mouse M-CSF (100 ng/ml). After 24 h in culture, the non-adherent cells were collected and layered on Histopaque gradient, and the cells at the gradient interface were collected. The cells were replated (60 mm dishes) at 65,000/cm^2 in αMEM, supplement with 10% heat-inactivated FBS at 37°C in 5% CO_2 in the presence of M-CSF (100 ng/ml). After 3 days in culture, cells were harvested for immunoblotting.

Preparation of Cell Lysates and Immunoblotting

The cells were starved for 2 h in αMEM serum-free media. For RANKL (100 ng/ml), M-CSF (20 ng/ml), or activin A (10 ng/ml)

stimulation experiments, RANKL, M-CSF, or activin A were added to the serum-free medium, and incubated. To visualize phosphorylated and total Akt, mTOR, IκB-α, Bad, and phospho-NFκB RelA/p65 and RANK, the cells were then washed once with ice-cold PBS and lysed in a cell lysis buffer (New England Biolabs) to prepare whole-cell lysates and lysates were clarified by centrifugation at 14,000*g* for 10 min. For the detection of phospho-NFκB RelA/p65, nuclear extracts were used instead of whole-cell lysates. Cells for the detection of phospho-NFκB RelA/p65 resuspended in hypotonic lysis buffer A (10 mM HEPES (pH 7.8), 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, and 5 μg/ml leupeptin) and incubated on ice for 15 min and nuclei were pelleted. Nuclei were washed and then resuspended in nuclear extraction buffer B (20 mM HEPES (pH 7.8), 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin) and rotated for 30 min at 4°C. The samples were then centrifuged, and nuclear proteins in the supernatant were transferred to the fresh tubes. Protein concentrations of whole-cell lysates and nuclear extraction in the supernatants were measured using the Bio-Rad protein assay reagent kit (Bio-Rad, Hercules, CA). Proteins were resolved by SDS–PAGE, electrobloted to PVDF membrane (Millipore, Bedford, MA), blocked in 5% skim milk, 1× PBS, 0.05% Tween-20, and probed with primary antibodies. Following incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (New England Biolabs), bound immunoglobulins were detected using enhanced chemiluminescence (Pierce, Rockford, IL).

Osteoclastogenic Cultures

BMMs were cultured in 24-well dishes (65,000/cm^2) for 5 d with vehicle or rapamycin (10, 50, and 100 nM) in the presence of RANKL (100 ng/ml), M-CSF (20 ng/ml), and activin A (10 ng/ml) in αMEM containing 10% heat-inactivated FBS at 37°C in 5% CO_2. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts) [Itoh et al., 2001]. TRAP-positive multinucleated cells (MNCs) containing more than three nuclei were counted as osteoclasts under microscopic examination [Itoh et al., 2001].

Apoptosis Assay

After treated with rapamycin, RANKL, M-CSF, or activin A for the indicated time, BMMs were harvested. Then, whole-cell lysates were prepared as above described. Lysates were clarified by centrifugation at 14,000*g* for 10 min, and the supernatant fraction was harvested. Caspase-3 activity assay of cell extracts measured using kit (CaspACE™ assay System; Upstate Biotech, Lake Placid, NY) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Previous reports demonstrate that activin A stimulates osteoclastogenesis [Fuller et al., 2000a; Itoh et al., 2001; Murase et al., 2001; Gaddy-Kurten et al., 2002; Koseki et al., 2002]. Here, we also demonstrated that activin A enhanced osteoclastogenesis stimulated with RANKL and M-CSF, and it induced osteoclastogenesis. The effects of activin A on osteoclastogenesis were time-dependent and present by day 3 of culture when compared to RANKL and M-CSF-treated cells (Fig. 1). To investigate the mechanisms by which activin A functions in osteoclastogenesis, we first examined whether activin A alone supported osteoclast precursors survival signal transduction including activation of Akt, mTOR, and BAD. Akt, also known as PKB (protein kinase B), is a serine/threonine protein kinase that has been shown to regulate cell survival signaling in response to growth factors, cytokines [Scheid and Woodgett, 2001]. It has been reported that Akt is activated by RANKL and M-CSF [Kelley et al., 1999; Wong et al., 1999; Sugatani et al., 2003]. Here we demonstrated, RANKL activation of Akt phosphorylation at Thr 308 at 5 min, but not Akt phosphorylation at Ser 473 (Fig. 2) in agreement with previous studies [Sugatani et al., 2003]. M-CSF activated phosphorylation of Akt at both Thr308 and Ser473 (Fig. 2) whereas activin A failed to activate Akt (Fig. 2). The dose range of activin A from 10 to 100 ng/ml also failed to stimulate Akt phosphorylation (data not shown). Next, we investigated activation of mTOR and BAD, by the same set of cytokines. BAD is a distant member of the Bcl-2 family that promotes cell death [del Peso et al., 1997].

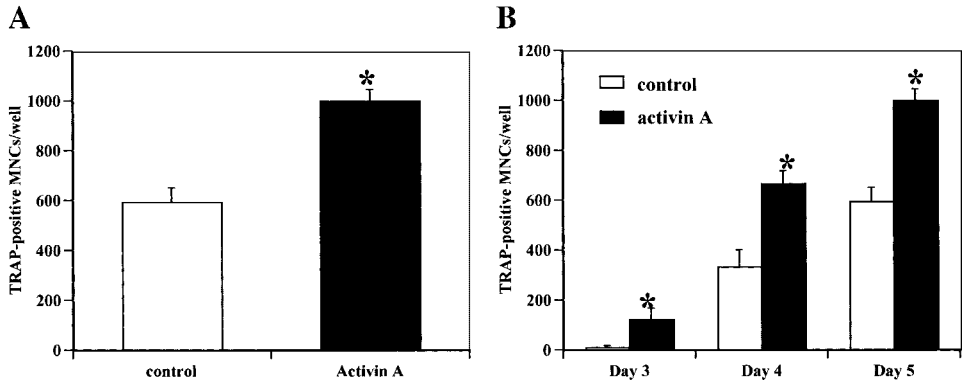

Fig. 1. Activin A enhances osteoclastogenesis in cultures of mouse bone marrow cells stimulated RANKL and M-CSF. **A, B**: Mouse bone marrow cells were cultured with M-CSF (100 ng/ml) for 3 days. Cells were further cultured for 3 days with RANKL (100 ng/ml) and M-CSF (20 ng/ml) in the absence (control) or presence of activin A (10 ng/ml). Cells were then fixed and stained for tartrate-resistant acid phosphatase (TRAP), and the number of TRAP-positive MNCs was scored. Values are expressed as the mean ± SEM of quadruplicate cultures. Similar findings were obtained in four independent sets of experiments. $^*P < 0.01$ as compared with control.

Phosphorylation of BAD prevents this [del Peso et al., 1997]. This proapoptotic function of BAD is regulated by the phophatidyl inositol 3-kinase (PI3-kinase)-Akt pathway [del Peso et al., 1997]. In fact, RANKL phosphorylated Akt and BAD and prevented apoptosis in RAW 264.7 cells [Sugatani et al., 2003]. mTOR (also, known as FRAP and RAFT-1) is a member of the phosphoinositide kinase related kinase family [Gingras et al., 2001]. The mTOR has serine/threonine kinase activity and mediates the cellular response to mitogens through signaling to p70s6 kinase ($p70^{s6k}$) and 4E-BP1, resulting in an increase in translation of subsets of cellular mRNA [31]. $p70^{s6k}$ and 4E-BP1 are also regulated, in part, through the PI3-kinase/Akt signaling pathway [Gingras et al., 2001]. That mTOR is phosphorylated by Akt raises the possibility of a direct signaling pathway from PI3-kinase/Akt to mTOR [Gingras et al., 2001]. Recent studies have reported that mTOR, but not BAD, is essential for cell survial in hematopoetic cells [Bao et al., 1999; Hinton and Welham, 1999]. In agreement with these results, we found that rapamycin, an inhibitor of mTOR, markedly induced apoptosis by 6 h in osteoclast precursors treated with RANKL, M-CSF, and activin A, and rapamycin also completely blocked osteoclastogenesis (Fig. 3A,B). Moreover, RANKL and M-CSF, but not activin A, activated mTOR (Fig. 3C), and none of the these cytokines phosphorylated BAD (data not shown). To further examine whether activin A alone induced osteoclast precursors survival, we performed apoptosis assays on cells maintained in culture media with 10% FBS. RANKL and M-CSF alone prevented apoptosis (Fig. 3D). However, activin A alone failed to prevent apoptosis (Fig. 3D). These results indicate that activin A fails to prevent apoptosis compatible with its failure to activate the Akt/PKB and mTOR survival signaling pathway in osteoclast precursors. RANKL and M-CSF prevented apoptosis by stimulated Akt and mTOR activation, but not BAD activation.

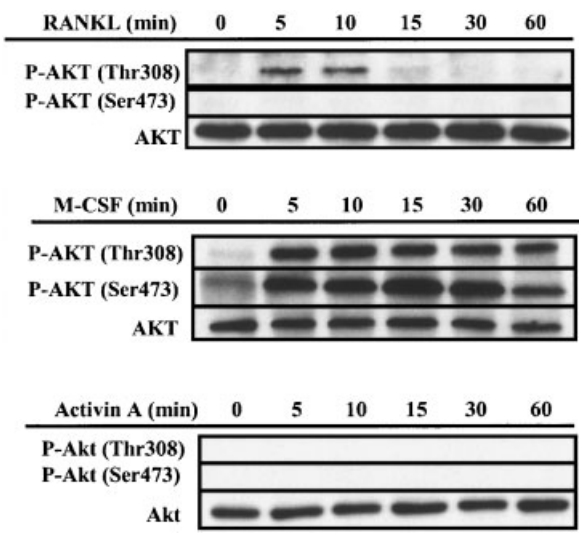

Fig. 2. Activin A fails to activate Akt in osteoclast precursors. Bone marrow macrophages (BMMs) were treated with RANKL (100 ng/ml), M-CSF (20 ng/ml), or activin A (10 ng/ml) for indicated time, whole-cell extracts were electrophoresed and analyzed by immunoblotting with antibodies against phospho-Akt Thr308 and Ser473.

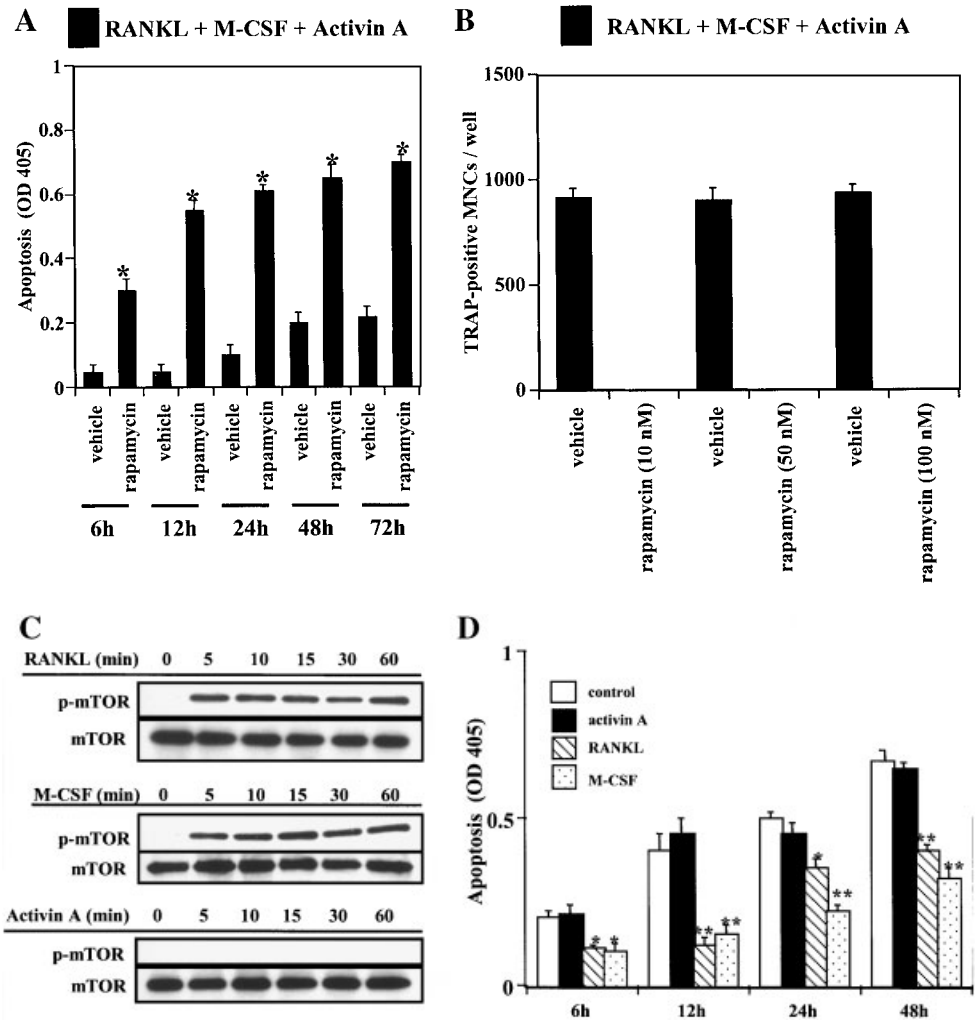

Fig. 3. Activin A fails to activate Akt survival pathway including mTOR and BAD and prevent apoptosis in osteoclast precursors. **A**: BMMs were treated with RANKL (100 ng/ml), M-CSF (20 ng/ml), and activin A (10 ng/ml) in the presence or absence of rapamycin (50 nM) for indicated time, and cells were harvested, then whole-cell extracts were prepared for caspase-3 activity assay. Data represent mean ± SD of three experiments in duplicate. *$P < 0.01$ compared with vehicle (DMSO). **B**: Mouse bone marrow cells were cultured with M-CSF (100 ng/ml) for 3 days. Cells were further cultured for 3 days with RANKL (100 ng/ml), M-CSF (20 ng/ml), and activin A (10 ng/ml) in the presence or absence of rapamycin (10, 50, 100 nM). Cells were then fixed and stained for TRAP, and the number of TRAP-positive MNCs was scored. Values are expressed as the mean ± SEM of quadruplicate cultures. Similar findings were obtained in four independent sets of experiments. Vehicle: DMSO. **C**: BMMs were treated with RANKL (100 ng/ml), M-CSF (20 ng/ml), or activin A (10 ng/ml) for indicated time, whole-cell extracts were electrophoresed and analyzed by immunoblotting with antibodies against phospho-mTOR, mTOR. **D**: BMMs were treated with RANKL (100 ng/ml), M-CSF (20 ng/ml), and activin A (10 ng/ml) in the culture media with 10% FBS for indicated time, and cells were harvested, then whole-cell extracts were prepared for caspase-3 activity assay. Data represent means ± SD of three experiments in duplicate. * $P < 0.05$, ** $P < 0.01$ compared with vehicle (10% FBS).

NFκB, classically a heterodimer composed of the p50 and p65 submits, is a transcription factor whose activity is tightly regulated at multiple levels [Karin and Ben-Neriah, 2000]. NFκB is normally sequestered in the cytoplasm as an inactive complex bound by an inhibitor known as IκB [Karin and Ben-Neriah, 2000]. Following cellular stimulation, IκB proteins become phosphorylated by the IκB kinase, which subsequently targets IκB for ubiquitination and degradation through the 26S proteasome [Karin and Ben-Neriah, 2000]. The degradation of IκB proteins liberates NFκB, allowing this transcription factor to translocate to the nucleus. In addition to regulation by IκB, NFκB is also regulated by phosphorylation events that positively up-regulate the transactivation potential of NFκB subunit [Karin and

Ben-Neriah, 2000]. Recently, multiple laboratories have demonstrated that the PI3-K/Akt pathway provides cell survival signals, in part, through the activation of the NFκB transcription factor [Ozes et al., 1999; Madrid et al., 2000; Mayo et al., 2002]. Recent studies have reported that BMP-2 and activin A fail to activate NFκB in BMMs and RAW 264.7 cell by a gel shift assay and luciferase assay, respectively [Itoh et al., 2001; Koseki et al., 2002]. We examined whether activin A alone stimulates IκB-α activation and nuclear translocation of NFκB by immunoblotting in osteoclast precursors. Activin A alone activated IκB-α and induced nuclear translocation of phosphorylated-NFκB, and RANKL and M-CSF also stimulated both of them (Fig. 4A,B). Moreover, nuclear translocation of phosphorylated-NFκB was strongly enhanced in BMMs treated with RANKL, M-CSF, and activin A compared with cells treated with RANKL and M-CSF (5, 10, and 15 min) (Fig. 4B). We further examined whether activin A alone enhances RANK expression in osteoclast precursors. RANK, the surface receptor for RANKL, initiates osteoclastogenic signal transduction after ligation with RANKL [Wagner and Karsenty, 2001]. RANK is present on chondrocytes and osteoclasts and their precursors [Hsu et al., 1999], and it can be upregulated by M-CSF [Arai et al., 1999]. Like osteoprotegrin, soluble recombinant RANK suppresses osteoclast differentiation, and antibodies to RANK can stimulate osteoclast formation [Hsu et al., 1999]. Moreover, mice lacking RANK are osteopetrotic [Dougall et al., 1999]. Therefor, RANK is essential for osteoclastogenesis. Recently, Yan et al. [2001] reported that TGF-β1 stimulate RANK expression in RAW 264.7 cells. As predicted, in this study, M-CSF alone markedly enhanced RANK expression at 24 h, and activin A alone also induced RANK expression at 48 h on the culture media with 10% FBS (Fig. 4C). These data indicate that activin A stimulates osteoclastogenesis supported by RANKL and M-CSF, and this is accompanied by increased nuclear translocation of NFκB phosphorylation and RANK expression in osteoclast precursors.

The model in Figure 5 illustrated the role of RANKL, M-CSF, and activin A for osteoclastogenesis and survival in osteoclast precursors. Activin A fail to activate Akt survival signaling pathway including Akt, mTOR, and BAD. In contrast, RANKL and M-CSF alone activates

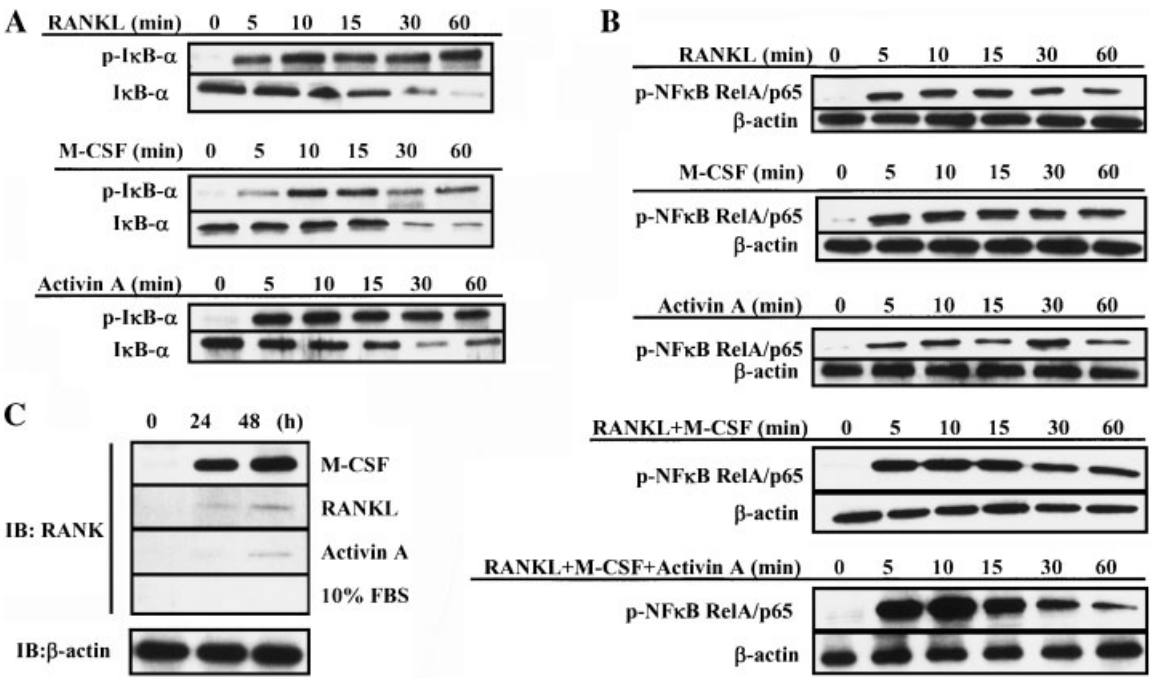

Fig. 4. Activin A stimulates IκB-α activation, nuclear translocation of phosphorylated-NF-κB RelA/p65, and RANK expression in osteoclast precursors. BMMs were treated with RANKL (100 ng/ml), M-CSF (20 ng/ml), or activin A (10 ng/ml) for indicated time without (**A**) and with 10% serum (**C**). Whole-cell extracts were electrophoresed and analyzed by immunoblotting with antibodies against phospho-IκB-α, IκB-α, phospho-NF-κB RelA/p65, and RANK (A and C). For the detection of phospho-NF-κB RelA/p65, nuclear extracts were used instead of whole-cell lysates (**B**).

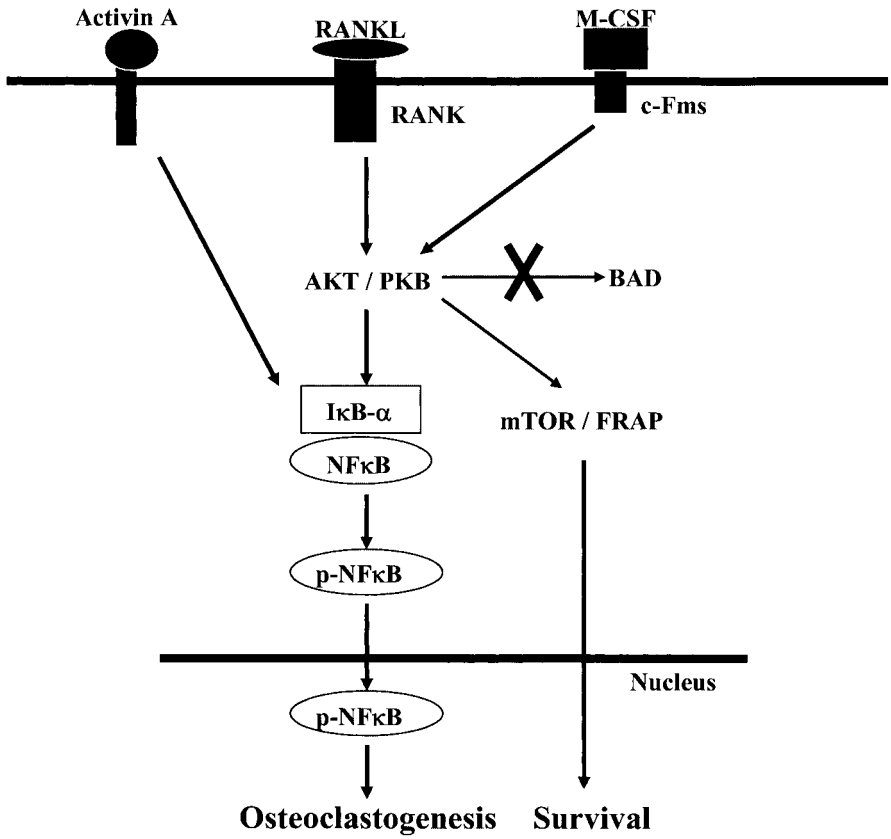

Fig. 5. The model in this figure illustrates the role of activin A for osteoclastogenesis and survival in osteoclast precursors. Activin A activates IκBα-/NF-κB pathway for osteoclastogenesis, but not Akt survival pathway in osteoclast precursors.

Akt and mTOR for survival, but not BAD. In fact, RANKL and M-CSF prevented apoptosis in osteoclast precursors. Moreover, activin A activates IκB-α activation not via Akt signaling pathway, and it induces nuclear translocation of phosphorylated-NFκB for osteoclastogenesis. RANKL activates JNK/AP-1(c-*fos*) pathway [Wagner and Karsenty, 2001]. However, activin A alone failed to activate JNK/AP-1(c-*fos*) pathway (data not shown).

In conclusion, we demonstrated, at least at part, that the role of activin A for osteoclastogenesis and survival in osteoclast precursors. However, the molecular mechanism by which TGF-β superfamily members potentiate the RANK-mediated signals is completely understood. Bone is a major storage site for TGF-β superfamily members including TGF-β, BMPs, and activin A. Therefore, further studies are needed to determine the molecular mechanism of the cross-communication between TGF-β superfamily members and RANKL in osteoclastogenesis and survival.

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