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Simvastatin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, suppresses osteoclastogenesis induced by receptor activator of nuclear factor-κB ligand through modulation of NF-κB pathway

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Simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, is a cholesterol-lowering drug that may play a role in bone metabolism through a mechanism that is not fully understood. Recently, receptor activator of NF-κB ligand (RANKL), a member of the TNF superfamily, has emerged as a major mediator of bone loss *via* activation of osteoclastogenesis. The latter is also associated with certain cancers such as multiple myeloma and breast cancer. Whether simvastatin can modulate RANKL-induced or cancer induced osteoclastogenesis was investigated. The effect of simvastatin on RANKL signaling and consequent osteoclastogenesis was investigated. RANKL induced NF-κB activation, whereas pretreatment with simvastatin completely suppressed such activation and correlated with suppression of RANKL-induced activation of IκBα kinase, IκBα phosphorylation and IκBα degradation. Similarly, RANKL induced the differentiation of monocytic cells to osteoclasts, whereas simvastatin suppressed it. The inhibition was maximal when cells were exposed to both simvastatin and RANKL simultaneously and minimal when simvastatin was added 1 day after RANKL treatment. Simvastatin also inhibited the osteoclastogenesis induced by human breast cancer and by multiple myeloma cells. Together, our results indicate that simvastatin inhibits the RANKL-induced NF-κB activation pathway that leads to suppression of osteoclastogenesis induced by RANKL and by tumor cells, thereby suggesting its therapeutic potential in osteoporosis and in cancer-related bone loss.

Key words: simvastatin; receptor activator of nuclear factor-κB ligand; osteoclastogenesis; nuclear factor-κB; IκBα; kinase

Osteoclasts and osteoblasts are instrumental in controlling the amount of bone tissue in the body. Osteoclasts (from the Greek words for "bone" and "broken") are bone cells that resorb bone tissue by removing the bone's mineralized matrix. Osteoclasts are multinucleated cells (3–100 nuclei per cell) formed by the fusion of cells of the monocyte–macrophage lineage.[1] These cells are characterized by high expression of tartrate-resistant acid phosphatase (TRAP) and cathepsin K.[2] Numerous inflammatory cytokines have been linked to the formation of osteoclasts including receptor activator of nuclear factor κB ligand (RANKL), TNF-α, interleukin-1 (IL-1), IL-6 and macrophage-colony stimulating factor (M-CSF).[3–5] RANKL is a member of the TNF superfamily and plays an important role in osteoclastogenesis.[6] RANKL, its receptor RANK, and osteoprotegerin (OPG), a soluble receptor that binds RANKL, have been shown to control osteoclast formation in mice.[7,8] For instance, RANKL knockout mice exhibit a phenotype of osteopetrosis and tooth eruption defects and an absence or deficiency of osteoclasts.[9,10] RANKL can activate mature osteoclasts *in vitro* and can lead rapidly to the resorption of bone *in vivo* by activating preexisting osteoclasts.[11,12]

Several types of cancer, both solid and hematopoietic are deeply linked with the skeleton and cause an increase in osteoclast formation, either systemically as in humoral hypercalcemia of malignancy (HHM) or locally as in bone metastases. HHM is commonly regulated by the hormonal action of parathyroid hormone-related protein (PTHrP), which greatly induces bone resorption and overrides normal calcium homeostasis.[13,14] Moreover, various tumor cells can induce osteoclastogenesis by secreting RANKL,[15–23] which suggests that the selective suppression of RANKL signaling pathways may have important therapeutic implications for cancer-induced bone loss, as well as osteoporosis and osteoarthritis.

After screening more than 30,000 compounds from natural sources, Mundy *et al.*[24] found that lovastatin and simvastatin enhance new bone formation in part through increased expression of bone morphogenic protein-2 (BMP-2). This effect was shown to be mediated through the action of statins on osteoblast.[25] Moreover, other investigators have shown that statins may also mediate their effect on bone through osteoclasts, thus causing suppression of bone resorption.[26]

Since statins affect osteoblasts and osteoclasts, and osteoblasts regulate osteoclast activity through the secretion of RANKL, we examined the effect of simvastatin on RANKL-stimulated signaling and osteoclastogenesis. Our results demonstrated that simvastatin suppresses RANKL-induced NF-κB activation through inhibition of IκBα kinase (IKK) and inhibits osteoclastogenesis induced by RANKL and by different human tumor cells.

Material and methods

Materials

A solution of 100% dimethyl sulfoxide and 50 mM simvastatin (LKT Laboratories, St. Paul, MN) was prepared and stored as small aliquots at −20°C and then diluted as needed in cell culture medium. DMEM-F12, FBS, 0.4% trypan blue vital stain and antibiotic–antimycotic mixture were obtained from Invitrogen (Carlsbad, CA). Antibodies against IκBα and LDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific anti-IκBα (Ser32/36) was purchased from Cell Signaling (Beverly, MA). Anti-IKK-α and anti-IKK-β antibodies were kindly provided by Imgenex (San Diego, CA). Goat anti-rabbit HRP conjugate and goat anti-mouse HRP were purchased from Bio-Rad (Hercules, CA). 3-(4,5-Dimethyltriazole)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). Protein A/G-agarose beads were obtained from Pierce (Rockford, IL). [γ-^{32}P]ATP was obtained from ICN Pharmaceuticals (Costa Mesa, CA).

Cell lines

RAW 264.7 (mouse macrophage), MCF-7 (human breast adenocarcinoma) and U266 (multiple myeloma) cells were obtained

Abbreviations: ALLN, *N*-acetyl-leu-leu-norleucinal; IκB, inhibitory subunit of NF-κB; IKK, IκBα kinase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; TRAP, tartrate-resistant acid phosphatase.

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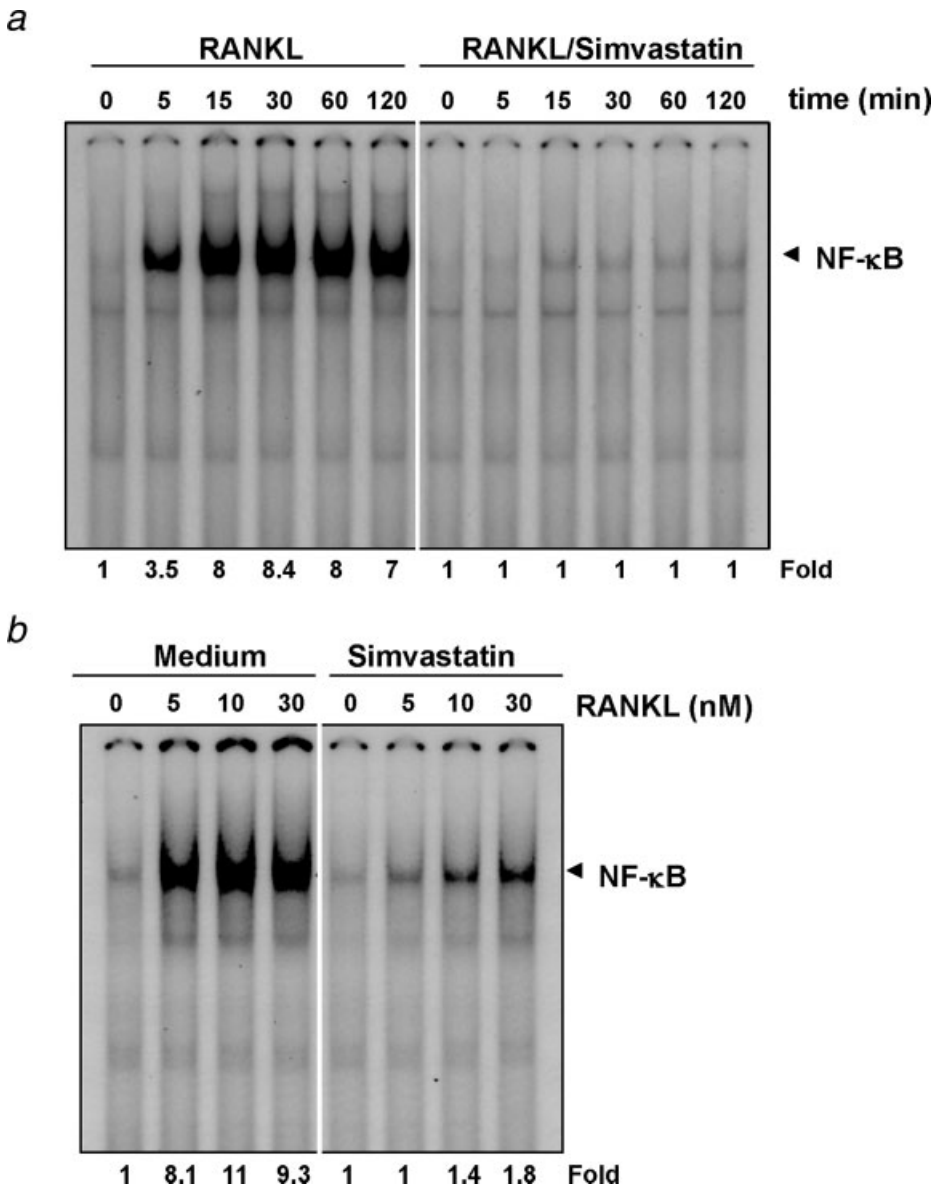

FIGURE 1 – Simvastatin inhibits RANKL-dependent NF-κB activation. (*a*) RAW 264.7 cells (1×10^6 cells), either alone (left panel) or in the presence of simvastatin (50 μM) (right panel), were treated with RANKL (10 nM) for different times. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA as described in Material and Methods. (*b*) RAW 264.7 cells (1×10^6 cells) were incubated with 50 μM simvastatin for 6 hr and then treated with the indicated concentrations of RANKL for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA as described in Material and Methods. Fold value is based on the value for medium (control), arbitrarily set at 1.

from the American Type Culture Collection (Manassas, VA). The RAW 264.7 cell line is a well established osteoclastogenic cell system that has been shown to express RANK and differentiate into functional TRAP-positive osteoclasts when cocultured with soluble receptor activator of nuclear factor-κB ligand (RANKL).[27] Moreover, RANKL has been shown to activate NF-κB in RAW 264.7 cells.[28]

Cell culture and staining

RAW 264.7 cells were cultured in DMEM-F12 supplemented with 10% FBS and antibiotics. MCF-7 (human breast adenocarcinoma) and U266 (multiple myeloma) cells were cultured in RPMI 1640 medium with 10% FBS supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. All cell lines were subjected to TRAP staining using a leukocyte acid phosphatase kit (387-A) from Sigma-Aldrich.

Preparation of nuclear extracts and electrophoretic mobility shift assay for NF-κB

To determine the NF-κB activation status of cells, electrophoretic mobility shift assays (EMSAs) were performed as described previously.[29] The dried gels were visualized using a Storm 820 optical scanner and radioactive bands were quantified using Imagequant software (Amersham, Piscataway, NJ).

Western blot analysis

To determine the levels of protein expression in the cytoplasm and nucleus of cells, extracts were prepared[30] and then resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, probed with each antibody and detected by incubation with ECL reagent (Amersham, Piscataway, NJ).

IKK assay

To determine the effect of simvastatin on RANKL-induced IKK activation, we analyzed IKK by a method essentially as described previously.[30] Briefly, the IKK complex from whole-cell extracts was precipitated with IKKα and treated with protein A/G-agarose beads (Pierce Chemical, Rockford, IL). After 2 hr, the beads were washed with lysis buffer and resuspended in a kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM dithiothreitol, 20 μCi [γ-^{32}P] ATP, 10 μM unlabeled ATP and 2 μg

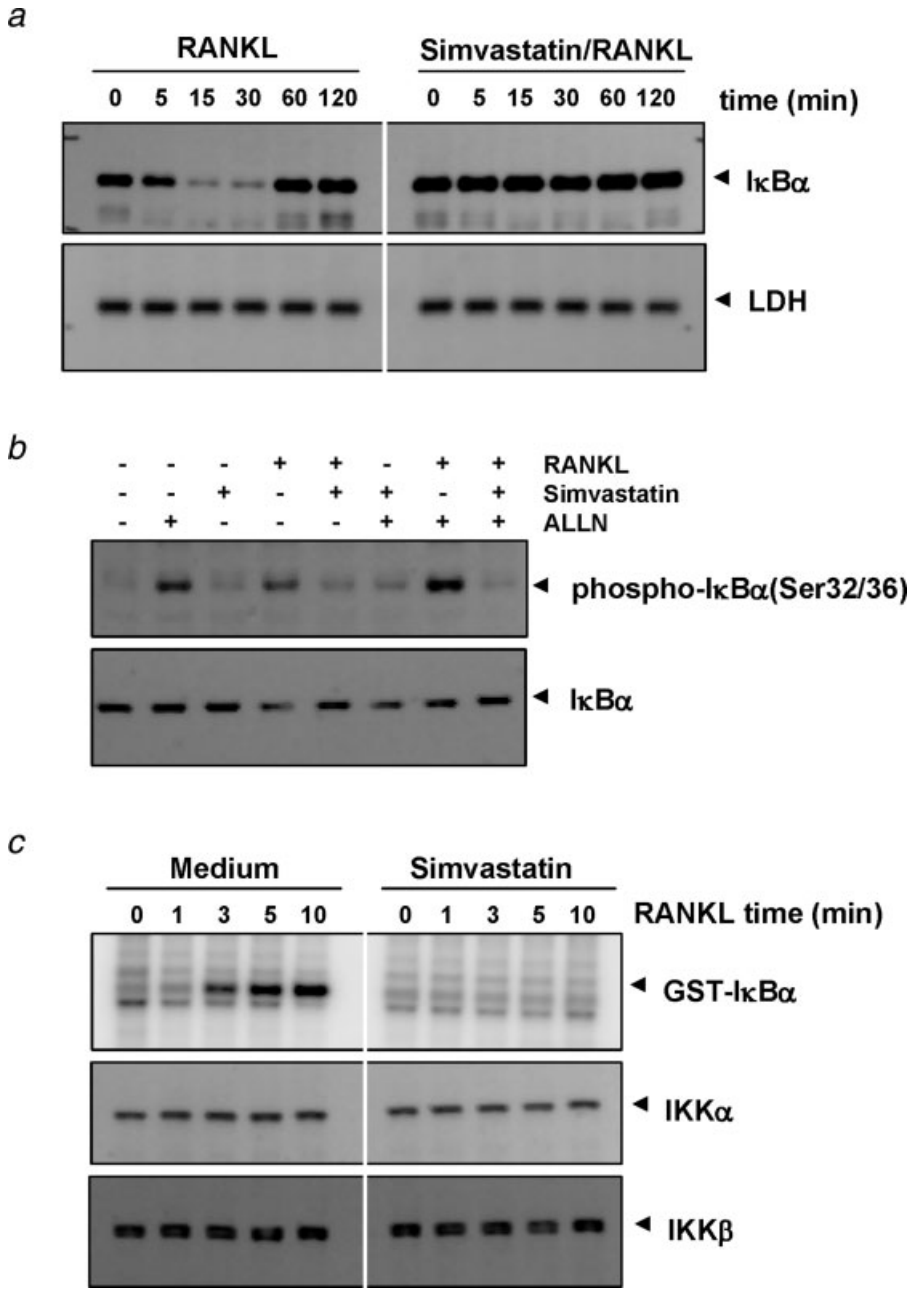

FIGURE 2 – Simvastatin suppresses RANKL-induced IκBα degradation (*a*) and phosphorylation (*b*) through inhibition of IKK activity (*c*). (*a*) RAW 264.7 cells (1×10^6 cells) were incubated with RANKL (10 nM) either alone (left panel) or in the presence of simvastatin (50 μM) (right panel) for the indicated times. Cytoplasmic extracts were prepared, fractionated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed with anti-IκBα. (*b*) RAW 264.7 cells (1×10^6 cells) were incubated with 50 μg/mL ALLN for 30 min, simvastatin (50 μM) for 6 hr, RANKL (10 nM) for 15 min or the indicated combinations. Cytoplasmic extracts were prepared, fractionated by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Western blot analysis was performed using either anti-phosphospecific IκBα (upper panel) or anti-IκBα (lower panel). (*c*) RAW 264.7 cells (4×10^6 cells) were incubated with 50 μg/mL ALLN for 30 min and then incubated with RANKL (10 nM) either alone (left panel) or in the presence of simvastatin (50 μM) (right panel) for the indicated times. Whole-cell extracts were immunoprecipitated using antibody against IKK-α and analyzed by an immune complex kinase assay using recombinant GST-IκBα as described in "Material and methods". To examine the effect of simvastatin on the level of IKK proteins, whole-cell extracts were fractionated by 7.5% SDS-PAGE and examined by western blot analysis using anti-IKK-α (middle panel) and anti-IKK-β (bottom panel) antibodies.

of substrate GST-IκBα (amino acids 1–54) and incubated at 30°C for 30 min. The reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried and the radioactive bands were visualized with a Storm 820. To determine the total amounts of IKK-α and IKK-β in each sample, 30 μg of whole-cell proteins were resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane and then blotted with either anti-IKK-α or anti-IKK-β antibodies.

Osteoclast differentiation assay

RAW 264.7 cells were cultured in 24-well dishes at a density of 1×10^4 cells per well and allowed to adhere overnight. DMEM-F12 was then replaced, and the cells were treated with 5 nM (100 ng/mL) RANKL. At Day 5, cultures were stained to detect TRAP expression as described previously[31] using an acid phosphatase kit, and the total number of TRAP-positive multinucleated osteoclasts (3 nuclei) per well were counted.

Results

Using a well-established model of osteoclastogenesis (*i.e.*, the murine monocytic RAW 264.7 cell system),[32,33] the effects of simvastatin on RANKL-induced NF-κB signaling pathway and on osteoclastogenesis induced by both RANKL and tumor cells were determined.

Simvastatin abolishes RANKL-induced NF-κB activation

To determine the effect of simvastatin on the RANKL-induced NF-κB activation in RAW 264.7 cells, cells were either pretreated with simvastatin for 6 hr or untreated, exposed to 10 nM RANKL for 0, 5, 15, 30, 60 and 120 min, processed to obtain nuclear extracts and finally assayed for NF-κB activation by EMSA.

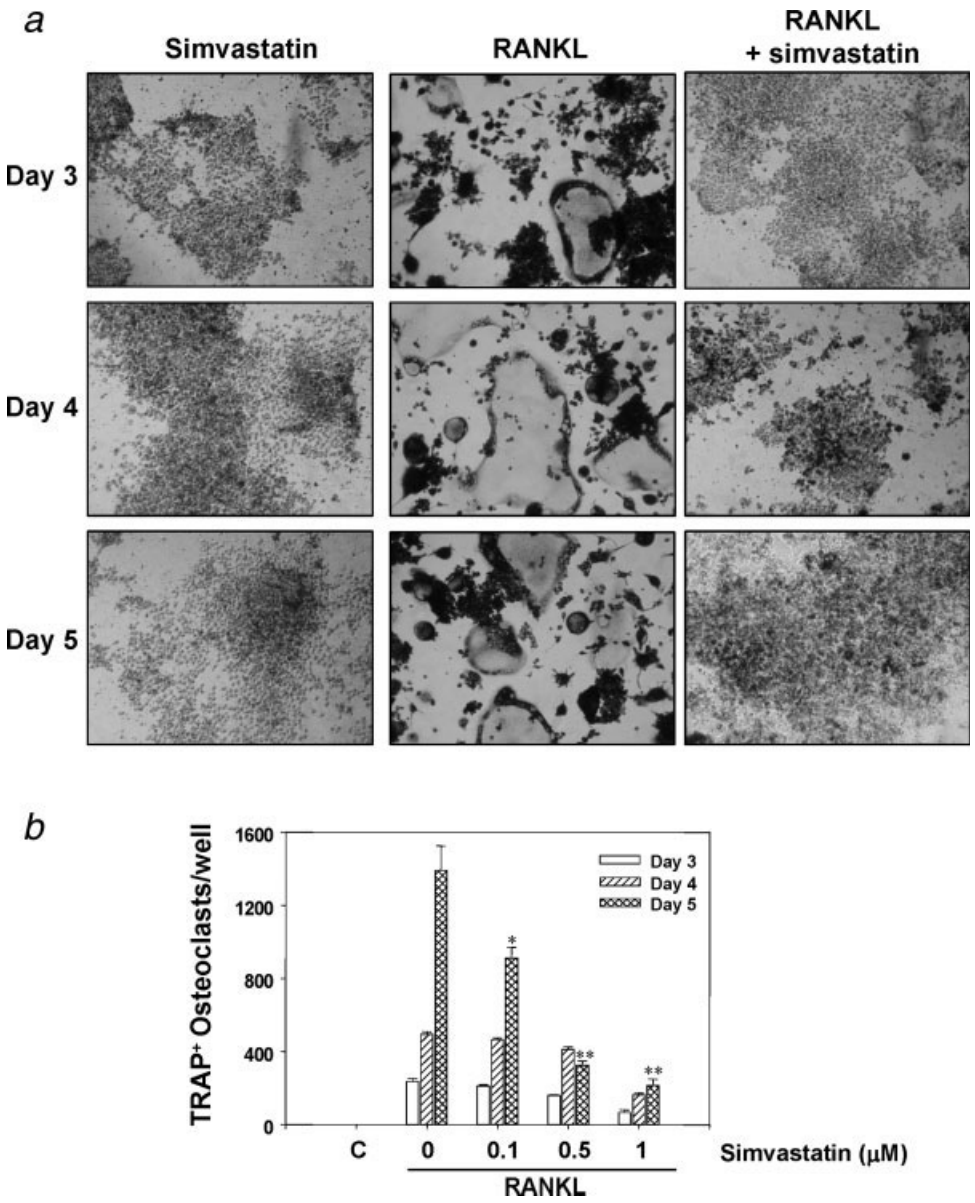

FIGURE 3 – Simvastatin inhibits RANKL-induced osteoclastogenesis. RAW 264.7 cells (1×10^4 cells) were incubated with or without RANKL (5 nM) and with or without the indicated concentrations of simvastatin for 5 days and then stained for TRAP expression. (*a*) TRAP-positive cells were photographed (original magnification, $\times 100$). (*b*) Multinucleated osteoclasts (*i.e.*, those containing 3 nuclei) were counted. "C" stands for cells exposed to medium alone (control). Data represent the mean of 3 measurements ± SD. $^*p < 0.05$; $^{**}p < 0.01$.

Although the activation of NF-κB by RANKL was time dependent, the activation of NF-κB induced by RANKL was clearly suppressed in simvastatin-pretreated cells (Fig. 1*a*).

To determine whether simvastatin inhibits activation of NF-κB by higher concentrations of RANKL, simvastatin-treated and untreated cells were exposed to different concentrations of RANKL for 30 min. Cells were then processed to obtain nuclear extracts and assayed for NF-κB activation by EMSA. RANKL administered at a concentration of 10 nM induced the maximum NF-κB activation. A slight decline in NF-κB activation was noted at the higher RANKL concentration. Simvastatin treatment, however, significantly suppressed the activation induced by either concentrations of RANKL (Fig. 1*b*). Unlike Figure 1*a*, however, the suppression of NF-κB activation induced by 10 nM RANKL was not complete.

Simvastatin blocks RANKL-induced IκBα degradation

In most cases, activation of NF-κB requires degradation of its inhibitory subunit IκBα.[34] To identify the mechanism of action of simvastatin involved in the inhibition of NF-κB activation, IκBα levels were assessed by Western blot analysis. In cells not pretreated with simvastatin, IκBα levels greatly decreased within 15 min after treatment with RANKL but returned to normal levels within 60 min (Fig. 2*a*, left). In contrast, RANKL-induced IκBα degradation was completely suppressed in cells pretreated with simvastatin (Fig. 2*a*, right). Meanwhile, the levels of an unrelated protein (LDH) did not change, indicating that the effect was specific.

Simvastatin inhibits RANKL-induced IκBα phosphorylation

The ubiquitination and degradation of IκBα by most stimuli requires the phosphorylation of IκBα.[34] Treatment with the proteasome inhibitor *N*-acetyl-leu-leu-norleucinal (ALLN) prevents such degradation. As shown by Western blot analysis, treatment with ALLN alone increased the levels of phosphorylated IκBα in RAW 264.7 cells, and the addition of RANKL further enhanced them (Fig. 2*b*). However, pretreatment with simvastatin abolished IκBα phosphorylation. Treatment with simvastatin alone did not induce phosphorylation of IκBα at all.

Simvastatin inhibits RANKL-induced IKK activation

Because IKK causes IκBα phosphorylation,[35] the ability of simvastatin to alter the activity or expression of IKK was evaluated. Immunocomplex kinase assays of cells treated with RANKL showed a sharp rise in IKK activity, as indicated by the phosphorylation of GST-IκBα within 3 min. In contrast, assays of IKK in cells pretreated with simvastatin showed the suppression of GST-IκBα phosphorylation on RANKL treatment (Fig. 2*c*, upper panel). To determine whether the apparent loss of IKK activity was because of the loss of IKK protein expression, the levels of the IKK subunits IKK-α and IKK-β were analyzed by Western blotting. As clearly shown in Figure 2*c*, simvastatin treatment did not alter the protein expression level of either subunit.

Simvastatin inhibits RANKL-induced osteoclastogenesis

Since RANKL signaling is critical for osteoclastogeneis, simvastatin's effect on RANKL-induced osteoclastogenesis was examined. RAW 264.7 cells were incubated with different concentrations of simvastatin in the presence of RANKL and allowed to differentiate into osteoclasts. As illustrated in Figure 3*a*, RANKL induced the differentiation of osteoclasts in the absence of simvastatin but to significantly lesser extent in the presence of simvastatin. In addition, the number of osteoclasts decreased with increasing concentration of simvastatin (Fig. 3*b*). As little as 0.1 μM simvastatin had a significant effect on RANKL-induced osteoclast formation. Nonetheless, the viability of cells under these conditions was not affected (data not shown).

Simvastatin acts early in the pathway leading to RANKL-induced osteoclastogenesis

It normally takes up to 5 days for RAW 264.7 cells to differentiate into osteoclasts in response to RANKL. To determine how early in this pathway simvastatin acts, RAW 264.7 cells were treated initially with RANKL, treated with simvastatin on 1, 2, 3 and 4 days after RANKL addition and finally analyzed for the effect on osteoclast formation. RANKL induced formation of approximately 1,000 osteoclasts and simvastatin inhibited 83% of osteoclast formation when administered 24 hr after RANKL treatment; (Figs. 4*a* and 4*b*). However, the inhibitory effect decreased significantly in a time-dependent manner when cells were treated with simvastatin after RANKL addition (Fig. 4*b*).

Simvastatin inhibits osteoclastogenesis induced by tumor cells

Osteoclastogenesis is commonly associated with breast cancer,[36,37] multiple myeloma[38] and squamous cell carcinoma[39] *via* the secretion of RANKL. Whether simvastatin also inhibits tumor cell-induced osteoclastogenesis was therefore investigated. Incubation of macrophages with multiple myeloma cell line induced formation of approximately 180 osteoclasts and it was significantly inhibited on treatment with simvastatin (Fig. 5*a*). Similarly, incubation with breast tumor cell line also induced formation of approximately 70 osteoclasts and it was significantly inhibited by simvastatin (Fig. 5*b*).

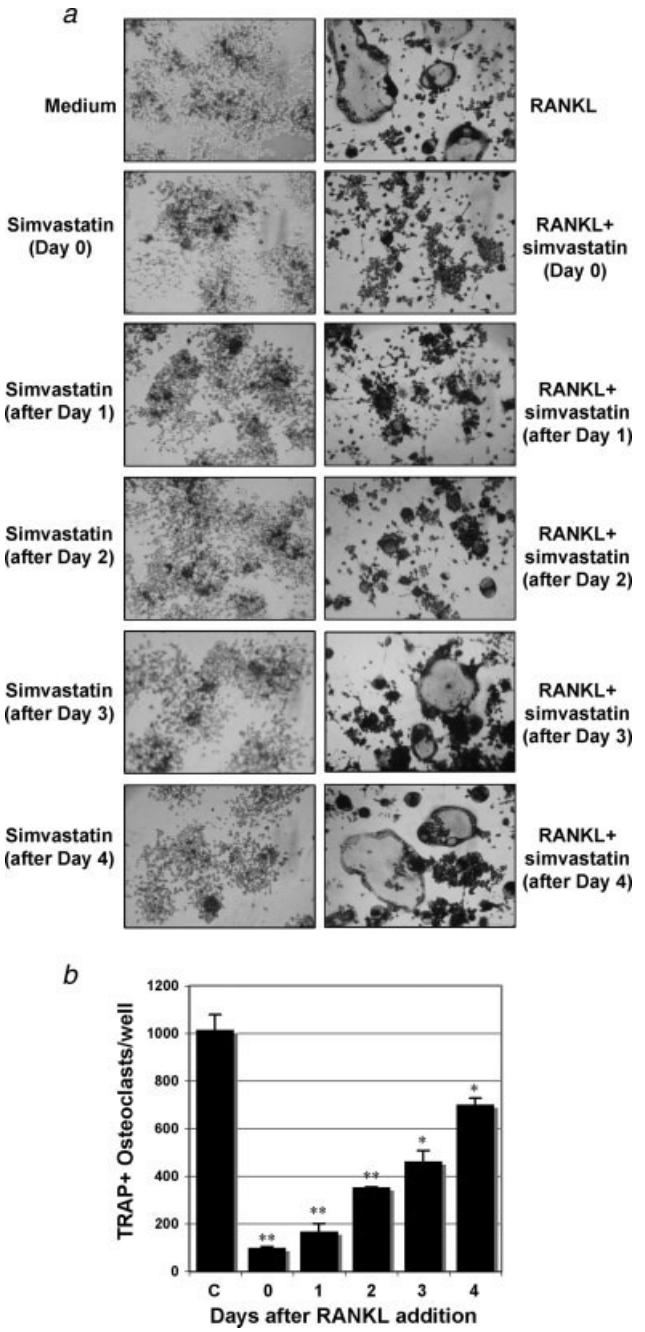

Figure 4 – Simvastatin inhibits RANKL-induced osteoclastogenesis 24 hr after stimulation. RAW 264.7 cells (1×10^4 cells) were incubated with RANKL (5 nM) and simvastatin (1 μM) for indicated time periods. (*a*) Cells were cultured for 5 days after RANKL treatment and stained for TRAP expression. (*b*) The total number of TRAP-positive multinucleated osteoclasts (*i.e.*, those containing 3 nuclei) per well were counted. "C" stands for cells treated with medium alone (control). Data represent the mean of 3 measurements ± SD $*p < 0.05$; $**p < 0.01$.

Discussion

The aim of this study was to investigate the effects of simvastatin on RANKL-induced NF-κB activation and on osteoclastogenesis induced by both RANKL and tumor cells. Using the well-established osteoclastogenic murine monocytic (RAW 264.7) cell system,[32,33] we showed that simvastatin inhibited RANKL-induced NF-κB activation through the suppression of IκBα phosphorylation, IκBα degradation and IκBα kinase activity, thus preventing osteoclast formation. Moreover, we found that simvastatin not only inhibited the initial phase of cell differentiation and fusion induced by RANKL and the formation of multinucleated cells, but also suppressed the osteoclastogenesis induced by breast cancer and multiple myeloma cells.

Besides their beneficial cholesterol-lowering effects, statins also seem to exert other effects. Statins can stimulate bone formation partly by inducing osteoblast differentiation. Atorvastatin has been shown to stimulate the production of BMP-2[24] and OPG[40] from osteoblasts. Although some studies have suggested that statins can also suppress bone resorption, the mechanism underlying this statin-induced effect is not well understood. Both mevastatin

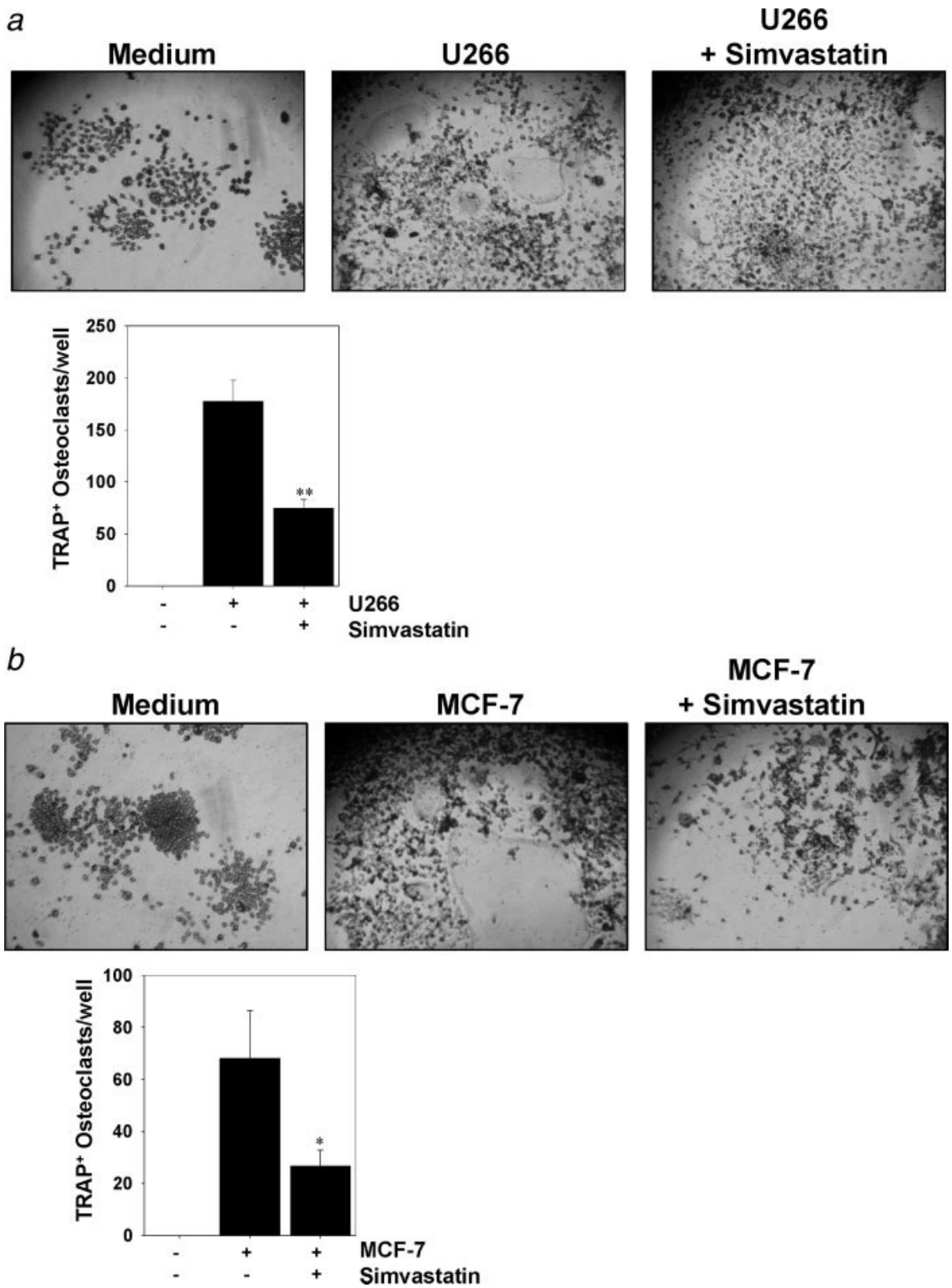

FIGURE 5 – Simvastatin inhibits osteoclastogenesis induced by tumor cells. RAW 264.7 cells (1×10^4 cells) were incubated in the presence of (*a*) U266 cells (1×10^3 cells) or (*b*) MCF-7 (1×10^3 cells) for 24 hr, then exposed to simvastatin (0.1 μM) for 5 days, and finally stained for TRAP expression. Multinucleated osteoclasts (*i.e.*, those containing 3 nuclei) in cocultures were counted. Data represent the mean of 3 measurements ± SD. *$p < 0.05$; **$p < 0.01$.

and simvastatin have been found to increase the level of OPG mRNA and inhibit the level of RANKL mRNA in mouse bone cell cultures.[41] OPG can stimulate bone-formation by inhibiting RANKL-induced osteoclastogenesis. In the present study, we employed a homogeneous, clonal population of RAW 264.7 cells to show the direct effect of simvastatin on osteoclast formation induced by RANKL. Because this cell system does not interfere with any osteoblast/bone marrow stromal cells or cytokines like

M-CSF, it allowed us to focus on RANKL signaling in preosteoclasts.

Our present data indicate that RANKL induces NF-κB activation in osteoclastic precursor cells through the activation of IKK and the subsequent phosphorylation and degradation of IκBα, a finding in agreement with that of Wei *et al*.[28] Our data also indicate that simvastatin abolishes the RANKL-induced IKK activation that is required for the phosphorylation of IκBα.[34] A more recent study showed that IKKs are potent regulators of cytokine-induced osteoclastogenesis and inflammatory arthritis.[42] Although simvastatin's ability to suppress TNF-induced IKK activation has been established,[30] our present report is the first to document simvastatin's suppression of RANKL-induced IKK activation and consequently of NF-κB activation.

RANKL activates NF-κB and nuclear factor of activated T cells (NFATc1) through the cell surface receptor RANK. NF-κB is activated almost immediately after RANKL and RANK begin to interact. Osteoclast differentiation is inhibited by OPG, which binds to RANKL and thereby prevents its interaction with RANK. RANKL-induced osteoclastogenesis is mediated through RANK. Through the recruitment of the adapter proteins TNF receptor-associated factor (TRAF) 2, 3, 5 and 6 and NIK, RANK activates NF-κB and c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and p44/p42 MAPK signaling pathways.[27,43–45] Recently, molecular adapter Grb-2-associated binder-2 (Gab2) was found to associate with RANK and to mediate RANK-induced activation of NF-κB, Akt and JNK.[46] More important, Gab2 was found to be crucial to the differentiation of human progenitor cells into osteoclasts. In short, the interaction between RANKL and RANK results in a cascade of intracellular events, including the activation of NF-κB and the protein kinase JNK.[27,45,47]

NF-κB signaling has been shown to play an important role in osteoclastogenesis.[48] For example, NF-κB $p50^{-/-}$ and $p52^{-/-}$ double knockout mice suffer severe osteopetrosis caused by the failure of osteoclast formation.[49,50] This is understandable given that p50 and p52 expression are necessary for the differentiation of RANK-expressing-osteoclast precursors into TRAP-positive osteoclasts in response to RANKL and other osteoclastogenic cytokines.[51] Several reports have noted that NF-κB is stimulated by RANKL in both RAW 264.7 cells and monocytes[8,27,52,53] and that it is required for osteoclast formation *in vivo*.[49] Therefore, suppression of NF-κB activation might play a critical role in osteoclast formation. Indeed, our present observation that suppression of NF-κB activation by simvastatin correlated with inhibition of osteoclastogenesis suggests that NF-κB activation is critical for RANKL-induced osteoclastogenesis.

Various tumors metastasize to bone and induce bone-loss.[38,54–56] Breast cancers commonly cause osteolytic metastases in bone, a process that is dependent on osteoclast-mediated bone resorption.[57] However, the mechanism responsible for tumor-mediated osteoclast activation has not yet been clarified. In the present study, we showed that simvastatin inhibited the osteoclastogenesis induced by breast cancer cells and by multiple myeloma cells. Both breast cancer and multiple myeloma tumors are known to express RANKL[38,57] and to exhibit constitutive NF-κB activation,[58,59] thus implicating them in the induction of osteoclastogenesis *via* the expression of RANKL. Consequently, our results suggest that simvastatin could be used in the treatment of secondary bone lesions associated with cancer and with nonmalignant diseases.

Statins are generally well tolerated and have become some of the most commonly prescribed drugs in the United States. In the present study, we have demonstrated that at least 1 statin (*i.e*., simvastatin) can exert an anti-osteoclastogenic effect and that this effect is most likely mediated through the suppression of RANKL-induced NF-κB activation. It is very likely that other statins exhibit similar activity. Together, our present finding provides the theoretical basis for a novel statin-based approach to treating osteoporosis and cancer-associated bone lesions.

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