Guggulsterone Inhibits Osteoclastogenesis Induced by Receptor Activator of Nuclear Factor-κB Ligand and by Tumor Cells by Suppressing Nuclear Factor-κB Activation

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Abstract

Bone resorption is commonly associated with aging and with certain types of cancer, including multiple myeloma and breast cancer. What induces bone resorption is not fully understood, but the role of osteoclasts is well established. Recently, receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), a member of the tumor necrosis factor superfamily, was implicated as a major mediator of bone resorption, suggesting that agents that can suppress RANKL signaling have the potential to inhibit bone resorption or osteoclastogenesis. Guggulsterone [4,17(20)-pregnadiene-3,16-dione], isolated from the guggul tree Commiphora mukul and used to treat osteoarthritis and bone fractures, was recently shown to antagonize the farnesoid X receptor, decrease the expression of bile acid–activated genes, and suppress the NF-κB activation induced by various carcinogens. We investigated whether guggulsterone could modulate RANKL signaling and osteoclastogenesis induced by RANKL or tumor cells. We found that treatment of monocytes with guggulsterone suppressed RANKL-activated NF-κB activation (as indicated by gel-shift assay) and that this suppression correlated with inhibition of IκB kinase and phosphorylation and degradation of IκBα, an inhibitor of NF-κB. Guggulsterone also suppressed the differentiation of monocytes to osteoclasts in a dose-dependent and time-dependent manner. Suppression of osteoclastogenesis by the NF-κB-specific inhibitory peptide implies a link between NF-κB and osteoclastogenesis. Finally, differentiation to osteoclasts induced by coincubating human breast tumor cells (MDA-MB-468) or human multiple myeloma (U266) cells with monocytes was also completely suppressed by guggulsterone. Collectively, our results indicate that guggulsterone suppresses RANKL and tumor cell–induced osteoclastogenesis by suppressing the activation of NF-κB.

Osteoclasts are multinucleated cells belonging to the monocyte macrophage lineage that form through the fusion of their mononuclear precursors. This multistep differentiation process is under the control of the bone microenvironment, which includes stromal cells, osteoblasts, and local factors (1). One of the key factors mediating osteoclastogenesis is receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL; ref. 2), a member of the tumor necrosis factor (TNF) family that has also been called osteoclast differentiation factor (3). TNF-related activation–induced cytokine (4), and osteoprotegerin ligand (5). RANKL, which is expressed on the surface of osteoblast/osteoclast cells, is directly involved in the differentiation of monocyte macrophages into osteoclasts (3, 5, 6). Mice with disruptions in the RANKL gene show a lack of osteoclasts, severe osteoporosis, and defective tooth eruption, indicating that RANKL is essential for osteoclast differentiation (7). RANKL-induced osteoclastogenesis is mediated through the cell surface receptor RANK. The interaction of RANKL with RANK leads to recruitment of TNF receptor–associated factor (8–11). Selective modulation of RANKL signaling pathways may have important therapeutic implications for the treatment of bone diseases associated with enhanced bone resorption, such as osteoporosis, osteoarthritis, and cancer-induced bone loss. Thus, agents that can suppress RANKL signaling may be able to suppress osteoclastogenesis-induced bone loss.

A phytochemical that has aroused considerable interest is guggulsterone [4,17(20)-pregnadiene-3,16-dione], a plant sterol derived from the gum resin (guggulu) of the tree Commiphora mukul. The resin has been used in Ayurvedic medicine for centuries to treat a variety of ailments, including obesity, bone fractures, arthritis, inflammation, cardiovascular disease, and lipid disorders (12, 13). The anti-arthritis and anti-inflammatory activity of gum guggul was shown as early as 1960 by Gujral et al. (14) followed by a report of activity in experimental arthritis induced by mycobacterial adjuvant (15).
and another on the effectiveness of guggul for treating osteoarthritis of the knee (16). Recent studies have shown that guggulsterone is an antagonist for the bile acid receptor farnesoid X receptor (17, 18). Other studies have shown that guggulsterone enhances transcription of the bile salt export pump (19), thereby regulating cholesterol homeostasis. Our laboratory reported that guggulsterone suppressed the DNA binding of NF-κB induced by various carcinogens and inflammatory agents (20).

Whether guggulsterone can suppress RANKL-induced NF-κB activation and osteoclastogenesis induced by RANKL and by tumor cells was investigated. We show that guggulsterone suppressed the RANKL-induced NF-κB activation pathway by inhibiting IκBα kinase (IKK); moreover, this effect correlated with the suppression of osteoclastogenesis induced by RANKL or by breast cancer or multiple myeloma cells.

Materials and Methods

Materials. The rabbit polyclonal antibodies to IκBα p50 and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against phospho-IκBα was purchased from Cell Signaling Technology (Beverly, MA). Anti-IKK-α and anti-IKK-β antibodies were kindly provided by Imgenex (San Diego, CA). Goat anti-rabbit horseradish peroxidase conjugate was purchased from Bio-Rad Laboratories (Hercules, CA); goat anti-mouse horseradish peroxidase and BioCoat Osteologic Bone Cell Culture System were from BD Biosciences (San Jose, CA); and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide was from Sigma-Aldrich (St. Louis, MO). Guggulsterone was obtained from Steroid, Inc. (Newport, RI) and dissolved in DMSO as a 100 mmol/L stock solution and stored at −20°C. DMEM/F12 medium, fetal bovine serum, 0.4% trypan blue vital stain, and an antibiotic-antimycotic mixture were obtained from Invitrogen (Carlsbad, CA).

Protein A/G-Sepharose beads were obtained from Pierce (Rockford, IL). [γ-32P]ATP was from ICN Pharmaceuticals (Costa Mesa, CA). Highly purified recombinant murine TNF-α was provided by Genentech (South San Francisco, CA). The p65 peptide conjugated with the delivery peptide was kindly provided by Imgenex.

Cell lines. The mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and antibiotics. This cell line has been shown to express RANK and, when cultured with soluble RANKL, to differentiate into tartrate resistance acid phosphatase (TRAP)–positive, functional osteoclasts when exposed to RANKL. We used electrophoretic mobility shift assays to determine NF-κB activation (22) by using an acid phosphatase kit, and the total TRAP expression (22) was determined by trypan blue exclusion method.

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Results

The aim of this study was to investigate the effect of guggulsterone on RANKL-induced activation of the NF-κB pathway and on osteoclastogenesis induced by the cytokine and by tumor cells. The murine monocytic cell line RAW 264.7 was used because these cells have been shown to undergo differentiation to osteoclasts when exposed to RANKL.

Guggulsterone inhibits RANKL-induced NF-κB activation. Previous studies from our laboratory have indicated that stable transfection of RAW cells with a super-repressor IκBα plasmid abolishes both NF-κB activation and osteoclastogenesis induced by RANKL (26), thus implicating NF-κB in osteoclastogenesis. To investigate whether guggulsterone modulates RANKL-induced NF-κB activation in RAW 264.7 cells, we incubated these cells with guggulsterone and RANKL, prepared nuclear extracts, and assayed NF-κB activation by electrophoretic mobility shift assays in the nuclei. RANKL activated NF-κB maximally within 30 minutes, and guggulsterone completely abrogated the RANKL-induced NF-κB activation (Fig. 1A). The extent of inhibition of NF-κB by guggulsterone increased with dose, with maximum inhibition observed at 50 μmol/L guggulsterone (Fig. 1B). Treatment of cells with 50 μmol/L guggulsterone for 4 hours had no effect on the cell viability as determined by trypan blue exclusion method.

www.aacrjournals.org ClinCancerRes2006;12(2)January15,2006663
Supershift assay of NF-κB-DNA probe binding showed that RANKL-activated NF-κB consisted of p65 and p50 subunits (Fig. 1C). The specificity of the RANKL-induced formation of the NF-κB/DNA complexes was further confirmed by showing that binding was abolished by the presence of a 100-fold excess of unlabeled κB-oligonucleotides but not by the mutated oligonucleotide (Fig. 1C).

Guggulsterone inhibits RANKL-induced IκBα phosphorylation and degradation through inhibition of IKK activity. Activation of NF-κB by most agents requires phosphorylation and degradation of its inhibitory subunit IκBα. To investigate the mechanism involved in the inhibition of NF-κB activation by guggulsterone, we first checked the effects of guggulsterone treatment on the levels of IκBα by using Western blot analysis. In cells treated with RANKL, IκBα levels dropped within 10 minutes and returned to normal within 60 minutes (Fig. 2A, left). In contrast, cells pretreated with guggulsterone showed suppressed RANKL-induced degradation of IκBα (Fig. 2A, right).

Next, we investigated the effect of guggulsterone on the RANKL-induced phosphorylation of IκBα, which occurs before its ubiquitination, and degradation of IκBα (27). We used the proteasome inhibitor ALLN to prevent RANKL-induced degradation of IκBα (28). Western blot analysis for phospho-IκBα in Fig. 2B clearly indicates that RANKL induced IκBα phosphorylation in RAW 264.7 cells, and that guggulsterone eliminated this effect. Treatment of cells with guggulsterone alone did not result in phosphorylation of IκBα. Notably, the content of IκBα in the guggulsterone-treated samples was less than that in the control; quantification of the IκBα/β-actin ratio indicated that guggulsterone treatment down-regulated the expression of IκBα and inhibited the RANKL-induced degradation of IκBα.

Because IKK phosphorylates IκBα (29), we next checked whether guggulsterone alters the activity or the levels of IKK.
With immunoprecipitation followed by in vitro IKK assay, cells treated with RANKL showed a sharp increase in IKK activity as indicated by the phosphorylation of IκBα within 10 minutes. In contrast, cells pretreated with guggulsterone could not phosphorylate GST-IκBα upon RANKL treatment (Fig. 2C, top). To check whether the apparent loss of IKK activity was due to the loss of IKK protein expression, the expression levels of the IKK subunits IKK-α and IKK-β were tested by Western blot analysis. Results in Fig. 2C clearly showed that guggulsterone treatment did not alter the expression of IKK-α and IKK-β.

**Guggulsterone inhibits RANKL-induced osteoclastogenesis in RAW 264.7 cells.** Next, we investigated the effect of guggulsterone on osteoclastogenesis. RAW 264.7 cells were incubated with different concentrations of guggulsterone in the presence of RANKL and allowed to grow and differentiate into osteoclasts. Figure 3A illustrates that RANKL induced osteoclasts in the absence of guggulsterone, but its presence significantly decreased the differentiation. Quantitation revealed that the number of osteoclasts decreased with increasing concentration of guggulsterone (Fig. 3B). These results show that 5 μmol/L guggulsterone was sufficient to suppress osteoclastogenesis by >90%. Treatment of cells with 5 μmol/L guggulsterone for 5 days had no effect on the growth and survival of cells as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method.

**Guggulsterone 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 guggulsterone acts, we treated the RAW 264.7 cells with RANKL, added guggulsterone on different days, and then checked its effect on osteoclast formation. Guggulsterone inhibited osteoclastogenesis even when the cells were exposed 24 hours after the RANKL treatment (Fig. 4A). However, the inhibitory effect decreased significantly when cells were treated with guggulsterone 4 days after RANKL treatment (Fig. 4B).

**Activation of NF-κB is critical for RANKL-induced osteoclastogenesis.** Besides NF-κB activation, RANKL is known to activate several other signals in the cell. It is possible that guggulsterone inhibits RANKL-induced osteoclastogenesis by suppressing signals other than NF-κB. To establish that guggulsterone

![Fig. 3. Guggulsterone inhibits RANKL-induced osteoclastogenesis. RAW 264.7 cells (1 x 10⁶) were incubated with or without RANKL (5 nmol/L) and with or without the indicated concentration of guggulsterone (GS) for 5 days and stained for TRAP expression. A, photograph of TRAP-positive cells. Original magnification, x100. B, multinucleated (three nuclei) osteoclasts were counted.](attachment:image)

![Fig. 4. Guggulsterone effectively inhibits RANKL-induced osteoclastogenesis 24 hours after stimulation. RAW 264.7 cells (1 x 10⁶) were incubated with or without RANKL (5 nmol/L), and guggulsterone (GS; 5 μmol/L) was added either at the same time or after the indicated intervals. Cells were cultured for 5 days after RANKL treatment and stained for TRAP expression. A, photographs of cells. Original magnification, x100. B, multinucleated (three nuclei) osteoclasts were counted.](attachment:image)
suppressed osteoclastogenesis by inhibiting NF-κB activation, we treated RAW 264.7 cells with the p65 inhibitory peptide recently described from our laboratory (30). Neither the delivery peptide (PTD) nor the p65 inhibitory peptide alone significantly inhibited osteoclastogenesis in RAW cells (Fig. 5A and B). However, the conjugate of the delivery peptide with the p65 inhibitory peptide (PTD-p65) significantly suppressed osteoclastogenesis. These results suggest that NF-κB activation has critical role in RANKL-induced osteoclastogenesis.

Coincubation of monocytes with tumor cells leads to osteoclastogenesis. Osteoclastogenesis is commonly associated with breast cancer (31, 32) and with multiple myeloma (33). Both multiple myeloma U266 and breast cancer MDA-MB-468 cells are known to express constitutive NF-κB (34, 35) and express RANKL (36, 37). Whether these tumor cells can induce osteoclastogenesis is not known. We found that incubating monocytes with breast cancer MDA-MB-468 cells (Fig. 6A) or with multiple myeloma U266 cells (Fig. 6B) induced osteoclast differentiation, and that guggulsterone suppressed this differentiation. These results indicate that osteoclastogenesis induced by tumor cells is significantly suppressed by the presence of guggulsterone. Treatment of cells with 5 μmol/L guggulsterone for 5 days had no effect on the growth and survival of cells as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method.

Discussion

Guggulsterone, commonly used to treat osteoarthritis, bone fractures, and arthritis, acts through a mechanism that is not understood. The present study was designed to investigate the role of guggulsterone on bone resorption. We showed that guggulsterone suppressed NF-κB activation, IKK, and phosphorylation and degradation of IκBα induced by RANKL, a bone-resorbing cytokine. Guggulsterone also suppressed the RANKL-induced monocyte differentiation to osteoclast. Osteoclastogenesis induced by human breast tumor cells or human multiple myeloma was also abrogated by guggulsterone.

In the present study, we used a homogeneous, clonal population of murine monocytic cells RAW 264.7 to define the direct effect of guggulsterone on osteoclast development induced by RANKL. The advantage of this system is that it does not contain any osteoblast/bone marrow stromal cells or cytokine like macrophage-colony-stimulating factor and allows us to focus on RANKL signaling in preosteoclast cells. Our results indicate that RANKL activates NF-κB in osteoclastic precursor cells through the activation of IKK and subsequent...
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IκB phosphorylation and degradation. These results are in agreement with those of Wei et al. (21). We also showed that guggulsterone inhibits RANKL-induced IKK activation, leading to the suppression of NF-κB activation. The mechanism of NF-κB activation induced by RANKL differs from that of TNF. For instance NF-κB-inducing kinase (also called NIK), although required for RANKL-induced NF-κB activation (38), is dispensable for TNF-induced NF-κB activation (39). Osteoclastogenesis is regulated by primarily by canonical pathway. Although NIK is needed for RANKL signaling, genetic deletion of NIK, p100, or p52 has no effect on osteoclastogenesis, suggesting non-canonical pathway is less important. NF-κB is regulated by primarily by canonical pathway. Although NIK

activation by guggulsterone correlated with inhibition of NF-κB activation, leading to suppression of NF-κB activation in agreement with our previous report in which we showed that this steroid can suppress TNF-induced IKK activation (20).

We found that suppression of RANKL-induced NF-κB activation by guggulsterone correlated with inhibition of osteoclastogenesis. The interaction of RANKL with RANK results in a cascade of intracellular events, including the activation of NF-κB (8, 9, 43). NF-κB signaling has been shown to play an important role in osteoclastogenesis (44). NF-κB is activated by RANKL both in RAW 264.7 cells and in monocytes (5, 9, 45, 46) and is required in vivo for osteoclast formation (41). p50 and p52 expression are essential for RANK-expressing osteoclast precursors to differentiate into TRAP⁺ osteoclasts in response to RANKL and other osteoclastogenic cytokines (47). Therefore, suppression of NF-κB activation would play an important role in osteoclast formation.

It is possible that the inhibitory effect of guggulsterone on osteoclastogenesis is not mediated through suppression of NF-κB. This is unlikely, however, as we found that p65 inhibitory peptide, which inhibits activate NF-κB induced by various activators (48), inhibited osteoclastogenesis induced by RANKL in RAW 264.7 cells. Additionally, we have previously shown that suppression of NF-κB by DN-IκBα abrogates osteoclastogenesis (26). Thus, it is very likely that NF-κB suppression shown in this study is involved in the inhibition of osteoclastogenesis by guggulsterone.

Breast cancers commonly cause osteolytic metastases in bone, a process that depends on osteoclast-mediated bone resorption (49), but the mechanism responsible for tumor-mediated osteoclast activation has not yet been clarified. We showed in this study that breast cancer cell induced osteoclastogenesis, and guggulsterone inhibited it. Bone resorption has also been associated with multiple myeloma (49). We found that multiple myeloma cell-induced osteoclastogenesis was also suppressed by the guggulsterone. Both of these tumors have been shown to express RANKL (37, 50) and exhibit constitutive NF-κB activation (51, 52). Thus, it is very likely that these tumors activate osteoclastogenesis through RANKL expression. This implies that guggulsterone could be used in the treatment of secondary bone lesions associated with cancer and those associated with nonmalignant diseases like postmenopausal osteoporosis, Paget’s disease, and rheumatoid arthritis.

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Acknowledgments

We thank Christine Wogan for her critical review of this article and Dr. Bryant Darnay (Department of Experimental Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, TX) for supplying the RANKL protein.
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