Bisphosphonates inhibit phosphorylation of signal transducer and activator of transcription 3 and expression of suppressor of cytokine signaling 3: implications for their effects on innate immune function and osteoclastogenesis

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Objective. This study tested the effects of bisphosphonates (BPs) on the suppressor of cytokine signaling 3 (SOCS3) protein in macrophages. SOCS3 has been shown to regulate cell differentiation and survival; however, its potential role in mediating the effects of BPs has not been explored.

Study design. The cell viability of murine RAW 267.4 macrophages was assessed after culturing with control medium or media containing increasing concentrations of 2 BPs (ibandronate or clodronate) for 24, 48, and 72 hours. The phosphorylation status of signal transducer and activator of transcription 3 (STAT3) and the expression of SOCS3 protein levels were determined by Western blot analysis.

Results. In control cultures, STAT3 phosphorylation and STAT3 and SOCS3 protein levels increased within 5 minutes after the addition of fresh medium. This increase was inhibited in cultures treated with both BPs. Macrophage cell viability also decreased after BP treatment.

Conclusions. These data demonstrate that, in addition to their effects on macrophage viability, BPs can decrease STAT3 and SOCS3 expression, which are important modulators of immune responses and bone homeostasis. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:196-204)

Bisphosphonates (BPs) are a class of drugs commonly used to treat osteoporosis, Paget disease, multiple myeloma, and hypercalcemia of malignancy. To date, at least 5 nitrogen-containing members (pamidronate, alendronate, ibandronate, risedronate, and zoledronic acid) are commonly used to treat skeletal fractures associated with these diseases, with their most recent use including pain reduction. The therapeutic effects of these medications derive from their ability to inhibit the resorptive capacity of osteoclasts. However, since 2003, a number of reports have been published associating use of these compounds with the development of avascular osteonecrosis of the mandible and/or maxilla: bisphosphonate-related osteonecrosis of the jaw (BRONJ).5-10

Bisphosphonates are divided into 2 structural groups based on the presence or absence of a nitrogen atom contained within their molecules. This structural difference also correlates with a difference in the mechanism of action, although both classes are capable of inducing cellular apoptosis.11 Clodronate does not contain a nitrogen chain in its aliphatic carbon chains and promotes cell death via its metabolism into cytotoxic compounds or by inhibition of protein tyrosine phosphatases.12 It has been used extensively as a macrophage depletion agent to study the contributions of these cells to the inflammatory process in a variety of experimental models.13-15 In contrast, ibandronate (Boniva) is a third-generation representative of the nitrogen-containing...
subcellular. Members of this class inhibit farnesyl diphosphate synthase, an enzyme of the mevalonate pathway, and interfere with subsequent posttranslational isoprenylation of small guanosine triphosphatases (GTPases), an important modification necessary for protein–protein interactions in the maintenance of cytoskeletal stability and cellular function.\textsuperscript{16,17} Ibandronate can be administered as an oral tablet or intravenous injection for the treatment and prevention of postmenopausal osteoporosis. It is the first oral BP formulated for once-monthly use, and its quarterly administered injectable form has been shown to increase patient compliance and improve therapeutic outcomes.\textsuperscript{18} Furthermore, it is one of the most commonly prescribed drugs for the treatment of osteoporosis with isolated reports implicating this compound in the development of BRONJ.\textsuperscript{19-21}

The symptoms of BRONJ consist of pain, bone exposure, soft-tissue swelling, and infection which may lead to inflammation and complement activation.\textsuperscript{22,23} Termination of inflammatory cytokine production and signaling in immune cells can be achieved by transcription of the suppressor of cytokine signaling 3 (SOCS3) protein. Binding of inflammatory cytokines (e.g., interleukin [IL] 6, leptin) to their receptors leads to phosphorylation of the signal transducer and activator of transcription 3 (STAT3), which dimerizes and then translocates to the nucleus to initiate SOCS3 transcription. SOCS3 then serves as a regulator for cytokine receptor signaling pathways\textsuperscript{24-26} and decreases cytokine signaling by binding and inhibiting cytokine receptor–associated Janus kinases (JAKs) or competing for phosphoryrosine binding sites on cytokine receptors. For example, SOCS3 binding to signal transducer element tyrosine-759 of glycoprotein-130 prevents further signaling of the cytokine IL-6.\textsuperscript{26-29} Recently, the absence of SOCS3 has also been linked to increased macrophage activation, osteoclast generation, and bone destruction,\textsuperscript{30} and SOCS3 antisense was shown to suppress receptor activator of nuclear factor κB ligand (RANKL)–mediated osteoclast formation in vitro.\textsuperscript{31} These data indicate that this molecule may have key roles in osteoclastogenesis\textsuperscript{32,33} and osteonecrosis. Yet, there are no reports demonstrating the effects of BPs on SOCS3. In the present study, we demonstrate for the first time that 2 structurally distinct BPs (clodronate and ibandronate) inhibit STAT3 phosphorylation and downregulate SOCS3 expression in macrophage cells.

MATERIAL AND METHODS

Cell culture

Murine RAW 264.7 macrophage (ATCC) cell cultures were grown in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C (humidified incubator) with 5% CO\textsubscript{2} in accordance with the ATCC protocol. Our modified media also contained HEPES, 2% L-glutamine, and 0.001% amphotericin. For the Western blot experiments, macrophages were plated at a density of $2.5 \times 10^5$ cells/cm\textsuperscript{2} with 1 mL growth medium per well in a 24-well culture dish and allowed to attach overnight. After 24 hours, the medium was removed and replaced with either fresh growth medium (control) or medium containing BPs. For the time course experiments, cultures were treated with clodronate (200 nmol/L) or ibandronate (60 nmol/L) for 0, 5, 10, 15, 30, 60, 120, and 240 minutes. For the dose–response experiments, cultures were treated with clodronate (100, 200, and 400 nmol/L) or ibandronate (30, 60, and 120 nmol/L) for 24, 48, and 72 hours.

Western blot analysis

Control and BP-treated cells were extracted for whole cell lysis with T-PER containing a Pierce Halt protease inhibitor cocktail (ThermoScientific, Rockford, IL). An equal amount (30 μg) of protein from each cell lysate sample was separated on a NuPAGE 4%-12% bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride membrane. The membranes were incubated at room temperature for 60 minutes with blocking buffer containing 5% nonfat milk in Tris-buffered saline solution with 0.1% Tween-20 (TBST: 25 mmol/L Tris [pH 8.0], 137 mmol/L NaCl, 2.7 mmol/L KCl, and 0.1% Tween-20). After being washed with TBST 3 times, the membranes were hybridized overnight with anti-STAT3 or anti-SOCS3 primary antibodies at 4°C. After being washed again with TBST 3 times, the blots were incubated with blocking buffer containing horseradish peroxidase–c-onjugated antirabbit secondary antibody (Cell Signaling Technology, Boston, MA) at room temperature for 2 hours and then washed again with TBST 3 times followed by 2 washes with water. The membranes were then incubated with enhanced chemiluminescence reagents for 5 minutes at room temperature (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer’s instructions and exposed to HyBlot CL film (Denville Scientific, Metuchen, NJ). The rabbit primary antibodies used were generated against phosphorylated STAT3, nonphosphorylated STAT3, SOCS3 (Cell Signaling Technology), and β-actin (Abcam, Cambridge, MA). The blots were re-probed after detection by incubating the membrane in stripping buffer (ReBlot; ThermoScientific, Rockford, IL) for 15 minutes at room temperature and rebloking the membrane. The results shown represent the 3 separate experiments.
MTT assay

Macrophage cell viability was determined by monitoring the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to the manufacturer’s protocol (Tox-1 Kit; Sigma, St. Louis, MO). Viable cells contain dehydrogenases that cleave this compound to an insoluble formazan derivative. Increases in cytotoxicity result in a decrease in the amount of formazan produced, which can be measured with spectrophotometry. In brief, RAW 264.7 cells were seeded into a 96-well microplate at a density of 2.0 × 10^5 cells/well containing growth medium and allowed to attach overnight. After attachment, the cells were incubated with growth medium (control) or medium containing various concentrations of clodronate (100, 200, or 400 nmol/L) or ibandronate (30, 60, or 120 nmol/L) for 24, 48, or 72 hours. The initial dose for these experiments was chosen based on each compound’s published half maximal inhibitory concentration for inhibition of farnesyl diphosphate synthase and increased to simulate serum concentrations after intravenous injections. After the initial incubation, reconstituted MTT (20 μL) was added to each well, and the plates were returned to the incubator for 3 hours at 37°C. The supernatant was then discarded, and 200 μL MTT solubilization solution was added to each well. The plates were gently mixed in a gyratory shaker to enhance dissolution and read using a Molecular Devices (Sunnyvale, CA) plate reader at wavelengths of 570 nm (absorbance) and 650 nm (background subtraction). The cell survival rates were expressed as a percentage of the mean absorbance of BP-treated cells divided by the mean absorbance of the control. The results shown are the mean ± SEM of experiments done in triplicate and represent 3 separate experiments. Lipopolysaccharide (LPS; 10 ng/mL) treatment was used as an additional control for cell death.

Statistical analysis

All values were expressed as the mean ± SEM. Significance was assigned where P < .05. The datasets were analyzed using ANOVA with individual group means evaluated with post hoc Dunnett multiple comparisons test (Prism; Graphpad, La Jolla, CA).

RESULTS

Effects of BPs on STAT3 protein levels and phosphorylation

We investigated the effects of 2 chemically distinct BPs on cytokine production using cultured macrophages. Using Western blot analysis, time course experiments of STAT3 protein expression and phosphorylation in macrophage cultures were performed after BP treatment (Fig. 1). β-Actin was used as a loading control. The addition of fresh control growth medium increased the STAT3 protein levels and phosphorylation (pSTAT). In contrast, the pSTAT level decreased with BP treatment compared with cells treated with control media alone. This phenomenon was evident within 5 minutes in cultures treated with both clodronate and ibandronate. This reduction was sustained for up to 4 hours in the clodronate-treated cultures and for 4 hours in the ibandronate-treated cells. Overall, these results suggest that BPs may modulate the cellular effects of STAT signaling by decreasing both its protein expression and level of phosphorylation.

Effects of BPs on SOCS3 expression

Because STAT3 signaling has been implicated in the induction of SOCS3, we hypothesized that the SOCS3 protein levels would also decrease in response to BP treatment. SOCS3 expression was down-regulated after treatment with either BP. In the time course experiment, SOCS3 expression increased within 5 minutes after the addition of the control cell growth medium. In cells treated with clodronate, this increase was delayed for 60 minutes and abrogated in cells treated with ibandronate (Fig. 2, A). Compared with the control cells, decreased SOCS3 levels were sustained for up to 48 hours in cells treated with varying concentrations of ibandronate (Fig. 2, B). The greater effect observed with ibandronate was not surprising, because of its substantially increased potency relative to clodronate.

Fig. 1. Bisphosphonates decrease STAT3 phosphorylation and protein level. RAW 264.7 macrophage cells were incubated with or without bisphosphonates for up to 4 hours at the indicated time points (5, 10, 15, 30, 120, and 240 minutes). Total cell lysates were subjected to Western blot analysis. Protein expression was evaluated using the following antibodies: polyclonal rabbit anti-STAT3, anti–phosphorylated STAT3 (pSTAT3), or β-actin. The blots represent 3 separate experiments. CON, Control; CL200, clodronate 200 nmol/L; IB60, ibandronate 60 nmol/L.
Effects of BPs on macrophage cell viability

To determine the duration these BPs’ action on cell viability, we performed a dose- and time-dependent cytotoxicity assay. The cell viability of the untreated control group was designated as 100%, indicating no cytotoxicity. All other values were expressed as a percentage of control cell viability, with LPS-induced cytotoxicity serving as a positive control for cell death. Cell death was dose dependent for both drugs. After 24 hours (Fig. 3, A), increasing concentrations of clodronate (100, 200, and 400 nmol/L) resulted in decreased cell viability values of 88.2%, 73.42%, and 68.85%, respectively, of the control. For ibandronate (30, 60, and 120 nmol/L), cell death was significantly higher, with viability values of 55.18%, 50.73%, and 45.49% of the control. After 48 hours’ exposure (Fig. 3, B), the cell viability for clodronate continued to decrease to 85.19%, 68.73%, and 65.77%, respectively, of the control. In contrast, the cell viability in the ibandronate-treated cultures slightly decreased to 69.18%, 61.30%, and 49.81%, respectively, of the control. After 72 hours’ (Fig. 3, C), the viability of the cells treated with ibandronate at 120 nmol/L and LPS were the only groups significantly different from the control. Owing to its increased potency, lower doses of ibandronate were more cytotoxic than the highest administered dose of clodronate.

DISCUSSION

Bisphosphonates continue to be an important drug class in the treatment of cancer and osteoporosis. It is possible that their effects may be due to modulation of a variety of chemical mediators and inflammatory cytokines, such as IL-1α, IL-β, IL-6,\textsuperscript{34} and tumor necrosis factor (TNF) α in immune cells (monocytes and macrophages), chondrocytes, osteoclasts, and osteoblasts.\textsuperscript{35-41} Ibandronate is a potent and long-acting BP, highly effective in the treatment of osteoporosis and pain associated with metastatic bone disease. As with other members in this class, it is rapidly cleared from the general circulation and accumulates in bone after repeated doses.\textsuperscript{42-44} Ibandronate is not as potent as zoledronic acid and has not been as widely associated with BRONJ; however, it is being more widely prescribed within the general population. To increase patient compliance, the development of high-dose intravenous formulations for monthly dosing may potentially result in a new cohort of patients at risk for the development of BRONJ, although most BRONJ cases have been associated with invasive dental procedures or periodontal disease. Because of the lack of efficacy at successfully treating BRONJ, one strategy for its prevention is the recommendation that patients seek dental treatment before the start of BP use. Because the length of time that these individuals are exposed to these formulations of medications increases, the potential for risk should be assessed.\textsuperscript{21,45}

To gain more insight into the additional mechanisms by which BPs may modulate inflammation, we set out to determine whether this action was achieved via regulation of SOCS3 levels. The present study represents the first report describing the effects of BPs on the STAT3-SOCS3 pathway. We found that BP treatment rapidly decreases the SOCS3 protein level in macrophages. That the STAT3 phosphorylation and STAT3 protein levels also transiently decreased suggests the ability of these compounds to manipulate other STAT3-mediated downstream events, such as cell survival and transcription. The concentrations used were based on the ability of these compounds to inhibit farnesyl diphosphate synthase, a key enzyme of the mevalonate pathway.\textsuperscript{17,46} Inhibition of this pathway prevents isoprenylation of small GTPases, an important step in the formation of membrane ruffling and vesicular trafficking in osteoclasts.\textsuperscript{47} Trafficking of the enzymes responsible for bone resorption allows them to be targeted and concentrated to the ruffled border which provides a sealed acidified interface between the cell and bone surface. Furthermore, STAT3-mediated regulation of GTPases has been implicated in cytoskeletal integrity, cell proliferation, and cellular migration.\textsuperscript{48}
Consistent with reports of its effects on tumor cells and other cell types, a dose-dependent decrease in macrophage cell viability was also observed with ibandronate with concentrations within the range of those observed in humans after an intravenous bolus. According to the Roche label, ibandronate (Boniva) is administered as 3 mL of a 1 mg/mL intravenous dose with serum concentrations ranging from 20 to 2,000 ng/mL compared with the maximal dose of 120 nmol/L (43.1 ng/mL) administered in the present study. We also compared ibandronate’s effects in macrophages with those of a non–nitrogen-containing BP (clodronate) to illustrate that the effects on SOCS3 were not unique to members of the nitrogen-containing class. Thus, these data suggest an additional cellular mechanism in innate immune cells for these BPs that may be distinct from their inhibition of the mevalonate pathway.

Although the 2 most-studied avenues of research investigating BRONJ focus on BP effects on angiogenesis and osteoclast function, it is now clear that this class of medications also affects other cell types. Most recently, their effects on fibroblasts, oral epithelial cells, and osteoblasts have been highlighted.49-51 For decades, clodronate derivatives have been used experimentally to deplete resident macrophages in a variety of tissues to emphasize their role in local innate and adaptive immune responses.14,52-56 Clearly, these cells are susceptible to the presence of BPs such that their functions as effectors of the immune system and potential osteoclast precursors could be significantly perturbed. Macrophage recruitment and activation play a significant role in several initiators implicated for BRONJ, such as bacterial infection and inflammation, which may occur after invasive dental procedures. Thus, definitive evidence of oral trauma is a common comorbidity, but its presence may not be necessary for jaw osteonecrosis to occur. Bone destruction can occur during inflammatory responses after macrophage release of reactive oxygen species and matrix metalloproteinases at the site of injury. Because BP use may not be solely responsible for the development of BRONJ, clinical assessments of additional risk factors, such as smoking and obesity are also being considered in an effort to identify those patients who may be predisposed to the disease.

During an immune response, the production of inflammatory mediators activates multiple macrophage cells divided by the mean absorbance of the control cells. The results shown are the mean ± SEM of experiments done in triplicate and are representative of 3 separate experiments. LPS, Lipopolysaccharide; other abbreviations as in Fig. 1. **P < .05.

Fig. 3. Dose-dependent bisphosphonate-induced cytotoxicity. Cell cultures were treated with increasing concentrations of clodronate or ibandronate for 24 (A), 48 (B), and 72 (C) hours. After the initial incubation, cell viability was assessed by MTT assay. Cell survival rates were expressed as percentages of the mean absorbance of the bisphosphonate-treated
receptors that signal through the STAT/SOCS3 pathway. STAT3 phosphorylation can occur in response to receptor binding of a diverse number of ligands, including molecules such as leptin, interferon-γ, IL-6, and IL-10.57-60 Specifically, phosphorylation of the amino acid tyrosine at position 705 causes STAT3 dimerization, translocation into the nucleus and facilitation of DNA binding.59,61,62 In addition, STAT3 binding to DNA after receptor activation can induce the transcription of proteins, such as SOCS3, whose purpose is to control the immune response by negatively regulating inflammatory cytokine production. SOCS3 prevents additional signaling through STAT3 by inactivating JAK activity as well as targeting proteins for ubiquitin-mediated degradation.63,64 The inhibitory effects of both clodronate and ibandronate on STAT3/SOCS3 in macrophages demonstrated in the present study support previous reports that these medications regulate multiple signaling pathways during inflammation and disease.65-68 Furthermore, the rapid onset of their ability to decrease STAT3 in our experiments suggests possible regulation of STAT3/SOCS3 degradation. The observed effect on STAT3 in particular is significant also because of its identification as an important regulator of cell survival, angiogenesis, and tumor metastasis and as a potential target of new anticancer therapies.69-73

The exact mechanism of SOCS3 regulation of bone homeostasis has yet to be fully elucidated. However, its function as a mediator of cytokine-induced effects on osteoclast differentiation and function has been well established. SOCS3 was shown to differentially modulate IL-6 and IL-10 signaling in both human and mouse macrophages.74,75 The absence of SOCS3 has been implicated in switching IL-6-induced proinflammatory responses to those more characteristic of the antiinflammatory cytokine IL-10. Moreover, a decrease or absence of SOCS3 correlated with decreased bone loss in 2 separate models of inflammatory bone destruction.30,32 In the model of diabetes-associated periodontal inflammation, transfection of an adenoviral vector carrying a dominant negative SOCS3 gene significantly attenuated tartrate-resistant acid phosphatase (TRAP) expression and bone-resorptive activity, although higher levels of SOCS3 expression were associated with RANKL.4 T-cell–mediated bone loss and increased dendritic cell (DC)–mediated osteoclastogenesis.32 In that study, the authors concluded that SOCS3 played a critical role in modulating cytokine signaling during DC interactions with T cells and that the development of SOCS3 inhibitors could be a strategy to abrogate inflammatory alveolar bone loss. In the inflammatory arthritis model, LPS-induced bone destruction in SOCS3 knockouts was suppressed after the administration of IL-6 compared with bone destruction in wild-type mice, although the bone density and mass for the 2 groups during normal physiologic conditions were not significantly different.37 This result is consistent with the finding that in SOCS3−/− macrophages, the IL-6 response could be converted to one characteristic of the antiinflammatory cytokine IL-10. Furthermore, these investigations suggest that during an inflammatory response, the function of SOCS3 is to serve as a switch to regulate bone homeostasis, depending on the cytokine milieu.

It is still unclear whether SOCS3 effects on inflammatory cytokine production are more important than its effects on osteoclastogenesis. The treatment of macrophages with SOCS3 small interfering RNA (siRNA) is capable of decreasing the LPS-induced production of TNF-α and IL-6.76 In our experiments, macrophage cell viability decreased in a dose-dependent manner after treatment with ibandronate and clodronate. One interpretation of these data implies that down-regulation of SOCS3 by ibandronate and other BPs leads to a depressed inflammatory response due to enhanced immune cell death. In this manner, decreased levels of SOCS3 resulting from BP use could promote susceptibility to bacterial infection and subsequent osteonecrosis by diminishing the immune response. From the extant data, it is clear that SOCS3 can modulate TLR signaling via multiple cytokine pathways, one pathway being transforming growth factor (TGF) β1. This finding is significant, because TGF-β is the most abundant cytokine in bone and is an important regulator of RANKL-mediated osteoclast formation.77 SOCS3 has been shown to sequester SMAD3, thus inhibiting TGF signaling.76 Furthermore, TGF-β1 itself can induce SOCS3 expression in mononuclear osteoclast precursors, leading to suppression of osteoclast apoptosis with subsequent survival and enhanced resorption.31,78,79

These results have provided additional insight into the mechanisms by which BPs achieve their therapeutic effects. Macrophages share a common lineage and other properties with osteoclasts, which favors them as preferential targets of these agents. Because regulation of SOCS3 expression in murine macrophages mimics that observed in human cells,24 we used a well characterized murine macrophage cell line to examine the effects of these agents on the STAT3-SOCS3 axis. We have shown that BPs representing 2 distinct chemical subclasses can decrease SOCS3 expression in macrophages. This is a significant finding because dental procedures that may cause inflammation and infection as well as increased macrophage activity have been identified as risk factors associated with the development of BRONJ. Furthermore, that BPs regulate this signaling element supports their role for controlling immune cell apoptosis and cytokine signaling in addi-
tion to their effects on bone homeostasis. Although it is not clear which process is more affected based on these experiments, we have identified a relationship that warrants continued investigation.

REFERENCES


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