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Modulation of TNF-α Gene Expression by IFN-γ and Pamidronate in Murine Macrophages: Regulation by STAT1-Dependent Pathways

Kae Takagi,* Masatoshi Takagi, †Siva Kanangat,* ‡ Kenneth J. Warrington, † Hidenobu Shigemitsu,* ‡ and Arnold E. Postlethwaite2*†

Aminobisphosphonates are drugs used in the treatment of hypercalcemia, Paget’s disease, osteoporosis, and malignancy. Some patients treated with aminobisphosphonates have a transient febrile reaction that may be caused by an increased serum concentration of proinflammatory cytokines. Aminobisphosphonates induce the production of certain proinflammatory cytokines in vitro, especially in cells of monocytic lineage. A unique feature of aminobisphosphonates is that they bind the Vγ2Vδ2 class of T cells, which are found only in primates, and stimulate cytokine production. The effects of aminobisphosphonates on other cells, including macrophages, are incompletely understood. We show in this study that treatment of murine macrophages with pamidronate, a second generation aminobisphosphonate, induces TNF-α production. Furthermore, pretreatment of murine macrophages with pamidronate before stimulation with IFN-γ significantly augments IFN-γ-dependent production of TNF-α. This pamidronate-mediated augmentation of TNF-α production results in sustained phosphorylation of the tyrosine residue at position 701 of STAT1 after IFN-γ treatment. Our data suggest that this sustained phosphorylation results from inhibition of protein tyrosine phosphatase activity. We also show that pamidronate treatment increases TNF-α production in vivo in mice. Pamidronate-augmented TNF-α production by macrophages might be a useful strategy for cytokine-based anticancer therapy. The Journal of Immunology, 2005, 174: 1801–1810.

B isphosphonates are compounds with a chemical structure similar to that of inorganic pyrophosphate. Those that have two amino chains covalently bound to the central carbon atom are called aminobisphosphonates (e.g., risedronate, pamidronate, ibandronate, olpadronate, and alendronate) (1). Aminobisphosphonates are strikingly effective against clinical disorders associated with bone resorption, including tumor-associated osteolysis and hypercalcemia, Paget’s disease, and osteoporosis (2). However, treatment with aminobisphosphonate results in a transient febrile reaction in some patients (3, 4). The mechanism underlying this reaction is not fully understood, but increased production of the proinflammatory cytokines IL-1, IL-6, IFN-γ, and TNF-α is thought to play a role (3–8), and bisphosphonate treatment of peripheral mononuclear cells or macrophages in vitro has been reported to increase the production of the inflammatory cytokines IL-1, IL-6, TNF-α, and IFN-γ (7, 9–11).

IFN-γ, the main cytokine through which T cells activate macrophages, is produced by activated T and NK cells. The complex genetic programs elicited by IFN-γ in the immune system account for the diverse activities of IFN-γ in host defense and in the immunopathogenesis of some diseases (12). Most effects of IFN-γ can be attributed to several gene products that are regulated by the JAK-STAT pathway (13–15), a signaling pathway that has been implicated in the mediation of biologic responses induced by many cytokines (16, 17). A recent report showed that the diverse gene expression patterns mediated by STAT1-dependent pathways, STAT1-independent mechanisms, and the balance between these two pathways play an important role in the biological response to IFN-γ (18).

TNF-α plays important roles in host defense against infections and tumors and in mediating inflammatory and immune responses. It triggers the local expression of chemokines and cytokines and promotes the adhesion, extravasation, attraction, and activation of leukocytes at the site of infection (19, 20). Most TNF-α is produced by activated macrophages after completion of a series of reactions: precursor differentiation (recruitment and differentiation of immature blood-derived mononuclear phagocytes into competent, cytokine-responsive macrophages by activators at sites of inflammation), priming (induction, by certain cytokine signals, of a receptive or primed state in which the inflammatory macrophages are not yet cytotoxic), and the trigger reaction (the ability of primed macrophages to develop full functional activity in response to trigger signals). The cytokine priming and trigger signals form the basis of a regulatory system that sets the threshold and determines the onset of macrophage effector activity (21). IFN-γ activates macrophages and induces them to produce a variety of cytokines, including TNF-α (22–28). This signal pathway has been believed to use the JAK-STAT pathway (25). Production of TNF-α is also induced by stimulation of macrophages with LPS, which induces the activation of multiple forms of NF-κB (29–31). However, LPS also activates the JAK-STAT pathway (32). It is not completely understood how the JAK-STAT pathway and

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Received for publication July 20, 2004. Accepted for publication November 8, 2004.

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This work was supported by a Merit Review Grant funded by the U.S. Department of Veterans Affairs.

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NF-κB family members interact to regulate TNF-α gene expression. Recent reports suggest that bisphosphonates have important effects on macrophages (11, 33). One of these effects is thought to be activation of the NF-κB pathway to increase production of TNF-α by macrophages (33). However, the molecular mechanism by which bisphosphonates modulate the cytokine network is not fully understood.

The purpose of the present study was to investigate the mechanism by which pamidronate acts on murine macrophages and augments IFN-γ-dependent TNF-α production by these cells. We show that treatment of murine macrophages with pamidronate induces TNF-α production. Furthermore, pretreatment of murine macrophage with pamidronate before stimulation with IFN-γ significantly augments IFN-γ-dependent production of TNF-α. This enhancement of IFN-γ-mediated TNF-α production by pamidronate is the result of prolonged STAT1 phosphorylation as a result of inhibition of protein tyrosine phosphatases (PTPs).³

Certain types of malignant tumors are sensitive to TNF-α (34, 35), and low dose TNF-α enhances the cytotoxic effects of chemotherapy agents on tumor cells (36, 37). Activation of macrophages by aminobisphosphonates might therefore be useful in cytokine-mediated cancer therapy.

Materials and Methods

Cells and cell culture

Murine peritoneal macrophages were isolated by using a previously described protocol (38) after i.p. injection of thioglycolate (BD Biosciences) into 6- to 12-wk-old C57BL/6J mice. After culture of the macrophages in plastic flasks for 2 h, floating cells were removed by extensive washing, and attached cells were maintained in RPMI 1640 medium (Invitrogen Life Technologies) containing 9% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. More than 90% of the cultured cells were macrophages as determined by flow cytometric analysis of CD11b-positive cells. Cells of the 293T and RAW 264.7 lines were obtained from the American Type Culture Collection and grown in DMEM (Invitrogen Life Technologies) containing 9% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. All cultures were incubated at 37°C in 5% CO₂ and a humidified environment. Human monocytes from healthy volunteers were isolated by using a positive selection protocol to enrich for CD14⁺ monocytes according to the manufacturer’s instructions (Miltenyi Biotec) and incubated with RPMI 1640 containing 9% FBS.

Reagents

We used commercially available pamidronate (Novartis Pharmaceuticals), murine IFN-γ (R&D Systems), and human IFN-γ (BD Biosciences). Clo-\textdagger\textdaggerdash dronate was a gift from Dr. G. Palmieri (University of Tennessee Health Science Center). The JAK inhibitor 2-(1,1-dimethyl-ethyl)-9-fluoro-3,6-di-hydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one (39) and the NF-κB inhibitor SN50 were obtained from Calbiochem (40). Sodium orthovanadate, inhibitor SN50 were obtained from Calbiochem (40). Sodium orthovanadate, inhibitor SN50 were obtained from Calbiochem (40). Sodium orthovanadate, inhibitor SN50 were obtained from Calbiochem (40). Sodium orthovanadate, inhibitor SN50 were obtained from Calbiochem (40). Sodium orthovanadate, inhibitor SN50 were obtained from Calbiochem (40). Sodium orthovanadate, inhibitor SN50 were obtained from Calbiochem (40).

ELISA

Supernatants from cultured cells were harvested 18 h after culture with PBS and murine or human IFN-γ, pamidronate alone, pamidronate with murine or human IFN-γ, clodronate alone, or clodronate with murine IFN-γ. The TNF-α concentration in supernatants was determined by ELISA (R&D Systems). The detection limits of the assay were between 5 pg/ml and 300 pg/ml. Each result is expressed as the mean value of three or four independent experiments performed in duplicate.

Semiquantitative RT-PCR analysis to measure TNF-α mRNA concentration

Total RNA was isolated from 10⁶ murine peritoneal macrophages using TRizol (Invitrogen Life Technologies), and 1 µg of total RNA was subjected to RT using poly(dT) oligomer and SuperScript II (Invitrogen Life Technologies) to obtain cDNA. The cDNA was amplified by 18 and 2 cycles of PCR using the following primer pairs: TNF-α: sense, 5'-GGGCGAATTCACTTGGAGTATT-3', and antisense, 5'-ATTTCTG AGACAGAGCCACCTGC-3'; and GAPDH: sense, 5'-AAATCTCACC ATCCATTCCAGAG-3', and antisense, 5'-AAACCTGTCGTTGACCAAAATC-3'. Amplified PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide, and photographed under UV light.

Preparation of cell lysates

Cells were prepared from 10⁶ cells by washing the cells with PBS and incubating them in 150 mM NaCl containing 0.1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4), and protease inhibitors for 30 min on ice. Lysate was cleared by centrifugation at 13,000 × g for 15 min at 4°C.

Western blot analysis

The total protein concentration in the lysate was measured using the DC protein assay kit (Bio-Rad). Samples containing 30 µg of protein were boiled in sample buffer, and the denatured proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Blots were probed with the following primary Abs: anti-phospho-STAT1 Y701, anti-STAT1, anti-phospho-p38 T180/Y182, anti-p38, and anti-phospho-protein kinase RNA dependent (anti-phospho-PKR) T446/451 (Cell Signaling Technology); anti-β-actin (I19), anti-JAK2 (N17), and anti-PKR (K17; Santa Cruz Biotechnology). Primary Abs were detected using HRP-conjugated anti-rabbit or anti-mouse secondary Abs (Amersham Biosciences) by ECL (Amersham Biosciences). Phospho-STAT1 expression was quantified using NIH image 1.63.

Flow cytometry

Macrophages were pretreated with PBS or pamidronate (20 µM) for 2 h, then incubated with PBS, murine IFN-γ (10 pg/ml), or pamidronate and murine IFN-γ for 4 h with a Golgi plug (BD Biosciences) to block cytokine secretion. After washing with PBS, macrophages were incubated with anti-mouse CD11b-allophycocyanin (BD Biosciences) or rat IgG2b-allophycocyanin phytohemagglutinin (BD Biosciences) for 20 min at 4°C. These cells were washed again in PBS, then fixed by incubation in 4% paraformaldehyde for 30 min at 4°C and washed with PBS containing 0.5% BSA. Aliquots of 2 × 10⁷ cells/100 µl were placed in each assay tube to which we added 100 µl of Cytofix/Cytoperm buffer (BD Biosciences). Samples were incubated for 20 min at room temperature, rinsed with Perm/Wash buffer (BD Biosciences), then incubated in PBS containing 5% BSA for 10 min at room temperature. Anti-phospho-STAT1 Y701 Ab (Cell Signaling Technology) or rabbit IgG isotype control (Sigma-Aldrich) was added to the assay tubes, and incubation was continued for 30 min at room temperature. After rinsing with 0.5% BSA in PBS, secondary Ab (FITC-conjugated anti-rabbit IgG; Jackson ImmunoResearch Laboratories) was added, and samples were incubated for 30 min at room temperature. After rinsing in 0.5% BSA in PBS, we added PE-conjugated anti-TNF-α Ab or PE-conjugated mouse IgG isotype control (BD Biosciences) to each tube and incubated the samples for 20 min at room temperature. Cells were washed with PBS again before being subjected to flow cytometry on a FACSCalibur instrument (BD Biosciences).

Immunoprecipitation

Cell lysates were prepared from mouse peritoneal macrophages by incubation of the cells in lysis buffer (150 mM NaCl containing 1.0% Nonidet P-40, 0.5% Tween 20, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4), and protease inhibitors) for 30 min on ice. Lysate was cleared by centrifugation at 13,000 × g for 15 min at 4°C, then precleared by incubation with rabbit IgG and protein A/G agarose for 60 min at 4°C with rotation. Precleared lysates were immunoprecipitated with 5 µl of anti-STAT1 Ab and 25 µl of protein A/G agarose (Santa Cruz Biotechnology) for 120 min at 4°C with rotation. After extensive washing with lysis buffer, immunoprecipitates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Src homology domain 2-containing phosphatase 1 (SHP2) (PTPN11) was detected with anti-SHP2 Ab (ab9214; Abcam), TC-PTP (PTPN2) was detected with mouse Ab 3E2 (gift from Dr. M. L. Tremblay, McGill University, Montréal, Canada).

Phosphatase assay

Cell lysates were prepared from RAW 264.7 cells by incubation in lysis buffer (25 mM Tris-HCl (pH 7.4), 10 mM 2-ME, 2 mM EDTA, 1.0% Triton X-100, and protease inhibitors) with sonication. Lysate was cleared by centrifugation at 13,000 × g for 15 min at 4°C, then subjected to phosphatase assay, for which 50 µg of cell lysate was mixed with 100 µl

³Abbreviations used in this paper: PTP, protein tyrosine phosphatase; ChIP, chromatin immunoprecipitation; PKR, protein kinase RNA dependent; pNPP, p-nitrophenylphosphate; SHP2, Src homology domain 2-containing phosphatase 1; TC-PTP, T cell PTP.
of p-nitrophenyl phosphate (pNPP) liquid substrate system (Sigma-Aldrich) with pamidronate or sodium orthovanadate and incubated at room temperature. Absorbance at 405 nm was measured using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad).

Luciferase assay

The luciferase assay was conducted as previously described (41). Briefly, 293T cells were seeded in 96-well plates at 50% confluent density. Cells were transfected with a firefly luciferase reporter plasmid (2×M67SJE Luc plasmid; gift from Dr. Y. Eugene Chin, Brown University School of Medicine, Providence, RI) or NF-κB luciferase reporter plasmid (gift from Dr. K. Tago, St. Jude Children’s Research Hospital, Memphis, TN) using Li-potectamine 2000 transfection reagent (Invitrogen Life Technologies). The 2×M67SJE Luc plasmid was used to measure STAT1 activation. The Renilla luciferase reporter plasmid pRL-SV40 (Promega) was used as an internal control. After 24 h, cells were treated with 20 μM pamidronate or PBS for 4 h, then human IFN-γ (50 μg/ml) or PBS was added, and incubation was continued for 6 h. Firefly and Renilla luciferase activities were measured using the dual luciferase reporter assay system (Promega) and a MicroLumat Plus 96V luminometer (Berthold Technologies).

Chromatin immunoprecipitation (ChIP) assay

Cells were washed once with PBS after removal of culture medium, then incubated in 6 ml of 1% formaldehyde in PBS for 10 min at room temperature to cross-link proteins to DNA. The cross-linking reaction was quenched by adding 2 ml of 1 M glycine in PBS and continuing the incubation at room temperature for 10 min. Cells were washed twice with cold PBS, collected into tubes, and lysed with 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.05% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 150 mM NaCl, and protease inhibitors, then sonicated (three times, 20 s each time) on ice. Cell lysates were precleared with 1 μg of protein G-agarose (Santa Cruz Biotechnology) coated with salmon sperm DNA (Invitrogen Life Technologies) for 1 h at 4°C with rotation. Cross-linked DNA-protein complexes were immunoprecipitated by anti-phospho-STAT1 Y701 Ab and protein A/G-agarose coated with salmon sperm DNA. After extensive washing in RIPA buffer, immunoprecipitated phospho-STAT1 DNA complexes were dissociated by heating at 65°C overnight with decross-link buffer (50 mM Tris-HCl (pH 7.0), 5 mM EDTA, 10 mM DTT, and 1% SDS). DNA was extracted by phenol/chloroform purification, followed by ethanol precipitation with glycogen. Precipitated DNA was dissolved in 20 μl of Tris-EDTA buffer and subjected to PCR.

Measurement of serum TNF-α concentration

Sixteen C57BL/6J mice, 6–12 wk of age, were given a single i.p. injection of 0.1 ml of PBS alone, PBS containing pamidronate (0.1 ml of 200 μg/ml/mouse), PBS containing murine IFN-γ (0.1 ml of 1000 pg/ml/mouse), or PBS containing pamidronate and murine IFN-γ at the same concentrations as given individually. Serum concentrations of TNF-α were determined by ELISA (kit from R&D Systems) 2 h later.

Statistical analysis

We used the Mann-Whitney U test for statistical analysis.

Results

Effect of pamidronate and IFN-γ on release of TNF-α by mouse macrophages in vitro

To determine whether IFN-γ induction of TNF-α production is modulated by pamidronate, we measured, by ELISA, TNF-α concentrations in supernatants from cultures of mouse peritoneal macrophages stimulated with IFN-γ after pretreatment with pamidronate or without pamidronate pretreatment. As previously reported, IFN-γ activated mouse peritoneal macrophages in vitro and induced TNF-α production in a dose-dependent manner (Fig. 1A). TNF-α was not abundantly secreted at a low (10 pg/ml) IFN-γ concentration (mean TNF-α concentration, 31 ± 42 pg/ml), but incubation of macrophages with 100 pg/ml IFN-γ markedly stimulated secretion of TNF-α (mean TNF-α concentration, 1053 ± 389 pg/ml; p < 0.001). Pretreatment of macrophages with 20 μM pamidronate before exposure to low concentrations of IFN-γ significantly augmented TNF-α production compared with that obtained by IFN-γ treatment alone (p < 0.001; Fig. 1A). Increased secretion of TNF-α was detected even at 1 pg/ml IFN-γ when macrophages were pretreated with pamidronate (Fig. 1A). Similar augmentation was observed when cells were pretreated with up to 20 μM pamidronate before stimulation with 10 pg/ml IFN-γ. However, 100 μM pamidronate, with or without IFN-γ, induced massive cell death, and we were unable to detect any residual TNF-α. Pamidronate itself (20 μM) weakly induced TNF-α production (70 ± 49.9 pg/ml in the absence of IFN-γ (Fig. 1B)). Cells treated with clodronate, which is not an aminobisphosphonate, showed no increase in TNF-α production; indeed, clodronate seemed to suppress IFN-γ-mediated TNF-α production in a dose-dependent manner (Fig. 1C). Pamidronate also induced augmentation of TNF-α production by human macrophages cultured in the presence of low concentrations of IFN-γ (p < 0.001; Fig. 1D).

Modulation of IFN-γ-mediated expression of TNF-α mRNA by pamidronate

TNF-α production is regulated at transcriptional and translational levels (19). To determine whether increased production of TNF-α is associated with an increase in TNF-α mRNA expression, we evaluated TNF-α mRNA expression by semiquantitative RT-PCR. TNF-α mRNA was not detected in macrophages treated with PBS (Fig. 2, lane 1, 18- and 22-cycle products). TNF-α mRNA was barely detected in macrophages stimulated with pamidronate alone (lane 2, 18- and 22-cycle products). As expected, IFN-γ treatment induced an increase in the expression of TNF-α mRNA (lane 3, 18- and 22-cycle products). However, greater induction of TNF-α mRNA occurred in macrophages treated with IFN-γ and pamidronate (lane 4, 18- and 22-cycle products). Pamidronate treatment did not affect the expression of GAPDH mRNA. These results indicate that enhanced production of TNF-α protein by pamidronate is associated with a concomitant increase in the amount of TNF-α mRNA transcribed.

Pamidronate affects mainly the TNF-α pathway that is mediated by the JAK-STAT pathway

Because the murine TNF-α promoter contains response elements for IFN-γ (the γ-activated sequence or GAS element) and NF-κB, which plays a key role in LPS-mediated TNF-α production (31), IFN-γ signaling may involve the NF-κB pathway as well as the JAK-STAT pathway. To determine which pathway is preferentially modulated by pamidronate, we treated cells with a JAK inhibitor or NF-κB inhibitor (SN50) before pretreatment with pamidronate and stimulation with IFN-γ. JAK-mediated phosphorylation of STAT1 at Y701 was partly inhibited at an intermediate concentration of TNF-α mRNA by semiquantitative RT-PCR.
STAT1 phosphorylation status and STAT1 expression

Previous reports have suggested that increased transcription of TNF-α mRNA by IFN-γ uses the JAK-STAT1 pathway (25, 42). IFN-γ stimulation increases the expression of STAT1, and phosphorylation of Y701 in STAT1 by JAK2 leads to rapid dissociation of the receptor-STAT1 complex and translocation of STAT1 into the nucleus as a homodimer (14, 16, 17). This process may be necessary for an IFN-γ-mediated increase in TNF-α mRNA transcription. To determine whether pamidronate induces an IFN-γ-mediated increase in the expression or phosphorylation of STAT1, we compared the amounts of STAT1 and STAT1 phosphorylated at Y701 in pamidronate-treated and IFN-γ-stimulated murine macrophages using anti-STAT1 and anti-phospho-Y701-STAT1 Abs in Western blot analyses. As expected, STAT1 expression was increased by IFN-γ stimulation (Fig. 4A). Pamidronate treatment had no effect on STAT1 expression or STAT1-Y701 phosphorylation, but IFN-γ treatment resulted in an increase in STAT1-Y701 phosphorylation, and treatment with pamidronate and IFN-γ augmented the phosphorylation of STAT1. The expression of JAK2 was unchanged by these stimulations (Fig. 4A). Next we examined STAT1 expression and Y701 phosphorylation in macrophages at

![FIGURE 1.](image)

**FIGURE 1.** Effects of IFN-γ and pamidronate on TNF-α secretion by murine (A–C) and human (D) macrophages. Mean concentrations of TNF-α in culture supernatants were measured by ELISA in three or four independent experiments. A, Dose dependency of TNF-α production on IFN-γ. The TNF-α concentration was measured 16 h after IFN-γ stimulation following pretreatment with pamidronate at a final concentration of 20 μM (■) or without pamidronate (PBS only; □) for 2 h. *, Statistically significant difference (p < 0.05) in TNF-α levels between samples treated with IFN-γ alone and those treated with IFN-γ and pamidronate. B, Dose dependency of TNF-α production on pamidronate. The TNF-α concentration was measured 16 h after stimulation with IFN-γ at a final concentration of 10 pg/ml (■) or without IFN-γ stimulation (PBS only; □). *, Statistically significant difference (p < 0.05) in TNF-α levels between samples treated with IFN-γ alone and those treated with IFN-γ and pamidronate. C, Dose dependency of TNF-α production on clodronate. The TNF-α concentration was measured 16 h after treatment with IFN-γ at a final concentration of 10 pg/ml. D, TNF-α production by human macrophages treated with PBS, pamidronate (Pam; final concentration, 20 μM), human IFN-γ (hIFN-γ; final concentration, 10 pg/ml), or 20 μM pamidronate 2 h before stimulation with 10 pg/ml human IFN-γ (Pam + hIFN-γ).

![FIGURE 2.](image)

**FIGURE 2.** Expression of TNF-α mRNA by cultured murine macrophages treated with pamidronate, IFN-γ, or pamidronate and IFN-γ. The amount of TNF-α mRNA was measured by semiquantitative RT-PCR assay (18 and 22 cycles) in macrophages treated with PBS (lane 1), pamidronate (final concentration, 20 μM; lane 2), murine IFN-γ (final concentration, 10 pg/ml; lane 3), or pamidronate and murine IFN-γ (lane 4). Pamidronate treatment lasted 2 h; IFN-γ treatment lasted 16 h. GAPDH was used as a control. Representative data from three independent experiments are shown.
different times after IFN-γ stimulation alone or with pamidronate treatment (Fig. 4B). Increased STAT1 expression was detected 30 min after IFN-γ stimulation, and this increase was sustained for up to 3 h after stimulation. This result was unaffected by pretreatment with pamidronate. Phosphorylation of STAT1 was detected 30 min after addition of IFN-γ and reached a peak at 1 h, after which it gradually declined. However, pretreatment with pamidronate prolonged STAT1 phosphorylation for at least 3 h after stimulation (Fig. 4B).

To confirm the Western blot findings and to evaluate TNF-α production by phospho-STAT1-positive cells, we labeled macrophages with anti-phospho-Y701-STAT1 and anti-TNF-α Abs and subjected them to flow cytometry 4 h after IFN-γ treatment. Before treatment with IFN-γ or pamidronate, 0.5% of the cells were positive for STAT1 phosphorylation. After treatment with pamidronate alone, 9% of the cells were positive for STAT1 phosphorylation. After treatment with IFN-γ alone, 48% of the cells were positive for STAT1 phosphorylation, and after treatment with pamidronate and IFN-γ, 83% of the cells were positive for STAT1 phosphorylation (Fig. 4C). We also evaluated intracellular TNF-α by double-labeling the cells with anti-TNF-α and anti-phospho-STAT1 Abs. Before treatment with IFN-γ or pamidronate, no cells were positive for TNF-α; 2% of the cells were positive for TNF-α and phospho-STAT1 treatment with pamidronate alone, and 14% were positive for TNF-α and phospho-STAT1 treatment with IFN-γ alone. In contrast, 21% of the cells were positive for TNF-α and phospho-STAT1 treatment with IFN-γ and pamidronate (Fig. 4D). These Western blot and flow cytometry findings suggest that prolonged phosphorylation of STAT1 may be related to increased TNF-α production.

**Prolonged phosphorylation of STAT1 results from inhibition of tyrosine phosphatase activity**

To determine whether prolonged phosphorylation of STAT1 results from pamidronate-mediated increases in JAK2 kinase activity, inhibition of tyrosine phosphatase activity, or both of these
mechanisms, we performed pulse-chase experiments using the protein kinase inhibitor staurosporine, which also inhibits JAK2 (43), and measured the residual amount of phosphorylated STAT1 at several time points (30, 35, 40, and 50 min) after treatment of murine macrophages with IFN-γ/H9253 alone or IFN-γ/H9253 and pamidronate (Fig. 5A). We detected a similar amount of phosphorylated STAT1 in macrophages treated with IFN-γ/H9253 alone or IFN-γ/H9253 and pamidronate 30 min after IFN-γ stimulation. However, in macrophages stimulated with IFN-γ/H9253 alone, phospho-STAT1 was dephosphorylated within 20 min after addition of staurosporine (50 min after IFN-γ treatment). In contrast, dephosphorylation of STAT1 occurred at a much slower rate after addition of staurosporine to cells treated with pamidronate. Because the expression of JAK2 was unchanged by addition of pamidronate (Fig. 4A), and similar amounts of phosphorylated STAT1 were detected in PBS-treated and pamidronate-treated cells 30 min after IFN-γ stimulation, we speculated that pamidronate inhibits tyrosine phosphatase. To determine whether pamidronate does inhibit protein phosphatase activity, we conducted an in vitro phosphatase assay using pNPP as a substrate. Sodium orthovanadate, a known PTP inhibitor, inhibited protein phosphatase activity in the cell lysate. However, pamidronate also inhibited protein phosphatase activity in a dose-dependent manner (Fig. 5B). These results suggest that pamidronate prolongs the phosphorylation status of STAT1 Y701 after IFN-γ treatment by inhibiting phosphatase activity. Another possibility is that impaired dephosphorylation of STAT1 results from pamidronate-mediated interference with the ability of the phosphatase to bind to phosphorylated STAT1. To investigate this possibility,
we measured the amounts of SHP2 (PTPN11) and TC-PTP (PTPN2), which is known to dephosphorylate STAT1 Y701, bound to STAT1 by immunoprecipitating STAT1 from cell lysates. There was no significant difference between the amounts of STAT1 bound to SHP2 (PTPN11) and to TC-PTP (PTPN2), even after treatment with pamidronate or IFN-γ (Fig. 5C). IFN-γ also activates RNA-dependent PKR, and it has a crucial role in the translational regulation of TNF-α production (44). TNF-α mRNA stabilization is regulated by p38 MAPK, which also controls the stability of several proinflammatory mRNAs (45). We therefore investigated activation of p38 MAPK or PKR after stimulation with pamidronate, IFN-γ, or pamidronate and IFN-γ. We found no p38 MAPK phosphorylation after any of these treatments. Phosphorylated PKR levels were elevated after IFN-γ stimulation, as expected, but treatment with pamidronate did not augment phosphorylated PKR levels (Fig. 5D). These experiments excluded the involvement of p38 MAPK and PKR pathways.

STAT1 reporter assay and ChIP assay
To determine whether sustained phosphorylation of STAT1 contributes to an increase in transcription of TNF-α mRNA, we used the luciferase assay to assay STAT1-dependent transcription after stimulation with pamidronate, IFN-γ, or pamidronate and IFN-γ. We found no p38 MAPK phosphorylation after any of these treatments. Phosphorylated PKR levels were elevated after IFN-γ stimulation, as expected, but treatment with pamidronate did not augment phosphorylated PKR levels (Fig. 5D). These experiments excluded the involvement of p38 MAPK and PKR pathways.

FIGURE 5. A, Inhibition of phosphatase activity by pamidronate. Murine macrophages were incubated with or without pamidronate for 2 h before IFN-γ stimulation. Stimulation with IFN-γ for 30 min was followed by a staurosporine chase (0.25 μM) for another 5–20 min. Cell extracts were analyzed by Western blot with anti-phospho-STAT1, generic anti-STAT1, and anti-β-actin Abs. The bar graph in the lower panel shows the relative densitometric values of the phospho-STAT1 bands. Similar results were obtained in two repeat studies. B, In vitro phosphatase assay. Purified cell lysates were mixed with the substrate pNPP, and their absorbance at 405 nm was measured at various time points as indicated in the graph. The reaction was performed without pamidronate (control), in the presence of pamidronate at several concentrations (6.5, 12.5, and 25 μM), or in the presence of 12.5 μM sodium orthovanadate. C, Western blots showing PTPN2- and PTPN11-bound STAT1. Cells were treated with PBS or pamidronate (Pam; final concentration, 20 μM) for 2 h before stimulation with IFN-γ (final concentration, 10 pg/ml) for 4 h. Whole cell lysates were immunoprecipitated with anti-STAT1 Ab, and STAT1 bound to PTPN2 or PTPN11 was detected by Western blot with anti-PTPN2 and anti-PTPN11 Abs. The total amount of immunoprecipitated STAT1 in the lysate was detected by anti-STAT1 Ab (lower panel). D, Western blots showing activation of p38 MAPK and PKR. Cells were treated with PBS or pamidronate (Pam; final concentration, 20 μM) for 2 h before stimulation with IFN-γ (final concentration, 10 pg/ml) for 4 h. Cells were also treated with anisomycin (5 μg/ml) for 30 min as a positive control for p38 MAPK phosphorylation. Whole cell lysates were analyzed by Western blot with anti-phospho-p38 MAPK T180/Y182 and anti-phospho-PKR T446/451 Abs; stripped blots were reprobed with a generic anti-p38 MAPK or anti-PKR Ab.
PAMIDRONATE-DEPENDENT TNF-α PRODUCTION

FIGURE 6. STAT1-dependent transcription of TNF-α. A, STAT1 or NF-κB-dependent activation of transcription as measured by luciferase assay. We transiently transfected 293T cells with 2×M673JE Luc or NF-κB reporter plasmid and a PRL-SV40 control plasmid. Twenty-four hours after transfection, cultures were incubated with PBS or pamidronate (Pam; final concentration, 20 μM) for 4 h, then with or without human IFN-γ (hIFN-γ; final concentration, 50 pg/ml) for 6 h. We prepared whole cell extracts and measured luciferase activity in them according to the manufacturer’s instructions. The graph shows mean values from three independent experiments. The firefly luciferase activity was calculated relative to Renilla luciferase activity, and the relative increases in firefly luciferase activity were standardized to that observed after PBS treatment alone (= 1). ■, STAT1 activation; □, NF-κB activation. B, Murine macrophages were incubated with PBS or pamidronate (Pam; final concentration, 20 μM) for 2 h, then treated with or without IFN-γ (final concentration, 10 pg/ml) for 4 h. Genomic TNF-α promoter DNA sites to which phospho-STAT1 was bound were identified by ChIP assay. We included a positive control of 0.1% input of genomic DNA PCR product (Posi). Representative data from two independent experiments are shown.

transfecting 293T cells with a STAT1 reporter plasmid. Pamidronate treatment alone activated the STAT1 reporter plasmid (mean, 2.8-fold relative increase in activation; p < 0.1), as did treatment with IFN-γ alone (mean, 3.0-fold increase; p < 0.1); the combination of IFN-γ and pamidronate increased STAT1 reporter activity (mean, 5.0-fold increase; p < 0.05). However, no NF-κB reporter activity was detected under these conditions (Fig. 6A). To determine whether this increased STAT1 reporter activity contributes to TNF-α promoter activity in murine macrophages in culture, we used the ChIP assay. Incubation of cells with IFN-γ alone for 4 h induced an increase in the binding of STAT1 to the TNF-α promoter, whereas pretreatment with pamidronate alone did not; the combination of pamidronate with IFN-γ enhanced the binding of phospho-STAT1 to the TNF-α promoter (Fig. 6B).

Elevation of serum TNF-α concentration after injection of mice with pamidronate and IFN-γ in vivo

Our in vitro experiments prompted us to determine whether pamidronate enhances IFN-γ-induced TNF-α production in vivo. Groups of four mice were given i.p. PBS alone, pamidronate (20 μg) alone, IFN-γ (100 pg) alone, or pamidronate (20 μg) and IFN-γ (100 pg). Two hours later, we measured the serum TNF-α concentration by ELISA. In PBS-injected mice, the TNF-α concentration was below the detection limit of the assay (i.e., <5 pg/ml). The mean serum TNF-α concentration was 77.2 ± 19.3 pg/ml in mice injected with pamidronate alone, 55.7 ± 24.3 pg/ml in mice injected with IFN-γ alone, and 433.8 ± 294.1 pg/ml in mice injected with pamidronate and IFN-γ (Fig. 6B). The serum TNF-α concentration in mice treated with pamidronate and IFN-γ was statistically significantly higher (p < 0.001) than that in mice treated with pamidronate or IFN-γ alone.

Discussion

LPS and IL-2 act synergistically with IFN-γ, activate macrophages, and cause enhanced production of TNF-α (24, 46–49). We have shown that treatment of human or murine macrophages with pamidronate augments the IFN-γ-stimulated production of TNF-α, and that in murine macrophages, this pamidronate-modulated augmentation of TNF-α production is regulated at the transcriptional level. Additional findings strongly suggested that pamidronate modulates the JAK-STAT pathway, but not the NF-κB pathway, because the NF-κB inhibitor SN50 was unable to suppress pamidronate-mediated augmentation of TNF-α production by IFN-γ and pamidronate-dependent TNF-α production, but a JAK inhibitor preferentially suppressed IFN-γ-dependent TNF-α production, pamidronate-mediated augmentation of TNF-α production by IFN-γ, and pamidronate-dependent TNF-α production (Fig. 3). In addition, we observed prolonged phosphorylation of STAT1 at Y701 after pamidronate pretreatment and IFN-γ stimulation, and we showed that this prolonged phosphorylation of STAT1 results from inhibition of tyrosine phosphatase activity. We then demonstrated STAT1-dependent transcription of TNF-α, and finally, we confirmed in vivo our in vitro findings by showing an elevation of the serum TNF-α concentration in mice injected with pamidronate and IFN-γ.

Treatment of macrophages with pamidronate alone weakly induced TNF-α production. This induction also involved an increase in TNF-α mRNA expression and increased binding of STAT1 to the TNF-α promoter, as shown by the luciferase assay. However, we did not detect phosphorylation of STAT1 at Y701 by Western blot analysis (Fig. 7). It is difficult to explain the increased TNF-α production resulting from treatment with pamidronate alone using our hypothesis that sustained phosphorylation of STAT1 results in augmentation of TNF-α production, but this discrepancy may simply be explained by different sensitivities of Western blot detection and flow cytometric detection, given that we observed weak STAT1 phosphorylation by flow cytometry. Furthermore, because a JAK inhibitor suppressed TNF-α secretion induced by treatment with pamidronate alone, it appears that pamidronate itself modifies
PTPs, PTPN2 and PTPN11, are involved in this regulation (41). Phosphorylation status of STAT1-Y701 also depends on the balance between protein-tyrosine kinases and PTPs (51, 52). The phosphorylation is controlled by the dynamic equilibrium of the receptor complex; sequential phosphorylation of JAK1, JAK2, and IFN-γ receptor 1 (IFNGR1); and subsequent recruitment of STAT1. STAT1 is phosphorylated by activated JAK2 kinase and translocated to the nucleus, where it participates in the regulation of TNF-α transcription. Pamidronate acts as a PTP inhibitor and thereby allows sustained phosphorylation of STAT1. Previous studies suggested that pamidronate activates the NF-κB pathway. This also might result in enhanced TNF-α transactivation.

FIGURE 8. Our proposed model of the interactions among IFN-γ, the JAK-STAT1 pathway, TNF-α transcription, and pamidronate in mouse macrophages. Engagement of IFN-γ with its receptor leads to aggregation of the receptor complex; sequential phosphorylation of JAK1, JAK2, and IFN-γ receptor 1 (IFNGR1); and subsequent recruitment of STAT1. STAT1 is phosphorylated by activated JAK2 kinase and translocated to the nucleus, where it participates in the regulation of TNF-α transcription. Pamidronate acts as a PTP inhibitor and thereby allows sustained phosphorylation of STAT1. Previous studies suggested that pamidronate activates the NF-κB pathway. This also might result in enhanced TNF-α transactivation.

the STAT pathway. However, solely on the basis of our experiment with the NF-κB inhibitor SN50, we cannot exclude involvement of the NF-κB pathway, as suggested in a previous report (33); examination of NF-κB knockout mice will provide additional insight into this mechanism. Another explanation for the discrepancy is that pamidronate treatment induces IFN-γ secretion by macrophages, the secreted IFN-γ stimulates the IFN receptor, and the macrophages then produce TNF-α. However, we detected no IFN-γ secretion by pamidronate-stimulated macrophages by ELISA (data not shown). Although we used purified macrophages, we cannot exclude the possibility that our cell preparations contained residual mononuclear cells. Therefore, pamidronate-mediated TNF-α production may result from modulation of the cytokine network by pamidronate. LPS-mediated signaling is also known to be involved in the STAT1 pathway (32). IFN-γ-dependent TNF-α production probably uses more than one pathway.

Usually, many cytokine signals are transiently activated by several mechanisms after a ligand interacts with its receptor (e.g., internalization of receptor, degradation of the signal-mediating protein, phosphorylation or dephosphorylation of the signal-mediating protein, and nuclear translocation of the signal-mediating transcription factor). Indeed, induction of activation of the JAK-STAT1 pathway by IFN-γ is regulated in this way (50). Our experiments confirmed that phosphorylation of Y701 in STAT1 occurred transiently after IFN-γ stimulation, as previously suggested. PTP plays an important role in the signal transduction pathways that control cellular growth, differentiation, and activity by dephosphorylating phosphorylated tyrosine residues. The extent of tyrosine phosphorylation is controlled by the dynamic equilibrium between protein-tyrosine kinases and PTPs (51, 52). The phosphorylation status of STAT1-Y701 also depends on the balance between JAK2 activity and tyrosine phosphatase activity. Two PTPs, PTPN2 and PTPN11, are involved in this regulation (41, 43). It is speculated that dephosphorylation of STAT1-Y701 depends on these tyrosine phosphatase activities. Our data show that treatment of macrophages with pamidronate before IFN-γ stimulation elevates phosphorylation of STAT1-Y701. Bisphosphonates, which are organic pyrophosphonate analogues, may influence biochemical pathways involved in phosphate metabolism, including protein phosphorylation and dephosphorylation. Indeed, several groups have found that alendronate and other bisphosphonates, including pamidronate, can inhibit several PTPs (53–57). The inhibitory effect of these compounds appears to result from oxidation of the active site cysteine residue in PTP. The results of our pulse-chase experiment and in vitro phosphatase assay suggested that pamidronate-treated macrophages have impaired phosphatase activity. These observations strongly suggest that pamidronate acts as a STAT1 tyrosine phosphatase inhibitor. We speculate that if pamidronate inhibits PTPs, it may be a target for PTPN2 or PTPN11; the action of these enzymes on pamidronate would result in sustained phosphorylation of STAT1. Sustainably activated STAT1 may then bind to the TNF-α promoter for longer than usual, as shown by our ChIP assay. This mechanism (Fig. 8) may be invoked in the enhanced production of TNF-α we observed after IFN-γ stimulation after pamidronate treatment.

Previous studies have shown increased serum concentrations of TNF-α and IL-6 after injection of bisphosphonates in patients with increased bone turnover or malignancy (6, 7), but it has not been clear why bisphosphonates have this effect. In primates, activation of the Vγ2Vδ2 class of T cells may be one of the crucial outcomes of increased production of proinflammatory cytokines (58, 59). The results of our in vitro studies strongly suggest that macrophages are involved in increased production of proinflammatory cytokines. Furthermore, our in vivo study showed that pamidronate-treated mice exhibit an increased serum concentration of TNF-α and that combined treatment with pamidronate and IFN-γ enhances TNF-α production to a significantly greater extent than that induced by treatment with IFN-γ alone. Our in vitro findings have allowed us to elucidate one mechanism of pamidronate-induced cytokine secretion. As mentioned above, pamidronate has been used to treat various diseases, including malignancies. Treatment of malignant tumors with pamidronate commonly results in induction of apoptosis, inhibition of tumor cell adhesion and invasion, and antiangiogenic effects (60). We now propose that pamidronate-mediated augmentation of TNF-α production by macrophages could also contribute to the antitumor effect of aminobisphosphonates.

Acknowledgments

The 2xM675JE luciferase plasmid was a gift from Dr. Y. Eugene Chin, the NF-κB luciferase plasmid was a gift from Dr. Kenji Tago, and the 3E2 anti-TC-PTP Ab was a gift from Dr. Michael L. Tremblay. We thank Patricia Wheller and Usha Nair for technical assistance, and Dr. Kenji Tago for helpful discussions.

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