Regulation of Receptor Activator of NF-κB Ligand-induced Osteoclastogenesis by Endogenous Interferon-β (INF-β) and Suppressors of Cytokine Signaling (SOCS)

THE POSSIBLE COUNTERACTING ROLE OF SOCSs IN IFN-β-INHIBITED OSTEOCLAST FORMATION*  

Received for publication, April 19, 2002, and in revised form, May 14, 2002
Published, JBC Papers in Press, May 22, 2002, DOI 10.1074/jbc.M203836200

Toshikichi Hayashi‡§†, Toshio Kaneda‡‡∥, Yoshiaki Toyama§, Masayoshi Kumegawa‡‡, and Yoshiyuki Hakeda***

From the ‡Department of Oral Anatomy, Meikai University School of Dentistry, Sakado, Saitama 350-0283, the ∥Department of Orthopaedic Surgery, Keio University School of Medicine, Shinanomachi, Tokyo 160-8582, and ¶Department of Pathophysiology, Faculty of Pharmaceutical Sciences, Hoshi University, Ebara, Tokyo 142-8501, Japan

Bone resorption and the immune system are correlated with each other, and both are controlled by a variety of common cytokines produced in the bone micro-environments. Among these immune mediators, the involvement of type I interferons (IFNs) in osteoclastic bone resorption remains unknown. In this study, we investigated the participation of IFN-β and suppressors of cytokine signaling (SOCS)-1 and -3 in osteoclastogenesis. Addition of exogenous IFN-β to osteoclast progenitors (bone-derived monocytes/macrophages) inhibited their differentiation toward osteoclasts induced by the receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor with/without transforming growth factor-β, where inhibition was associated with down-regulation of the gene expressions of molecules related to osteoclast differentiation. In addition, RANKL induced the expression of IFN-β; furthermore, neutralizing antibody against type I IFNs accelerated the osteoclast formation, indicating type I IFNs as potential intrinsic inhibitors. On the other hand, RANKL also induced the expression of SOCS-1 and -3, suppressors of the IFN signaling. Pretreatment with RANKL for a sufficient time for the induction of SOCSs attenuated phosphorylation of STAT-1 in response to IFN-β in osteoclast progenitors, causing a decrease in the binding activity of nuclear extracts toward the interferon-stimulated response element. mRNA levels of STAT-1, STAT-2, and IFN-stimulated gene factor-3, comprising IFN-stimulated gene factor-3, were not altered by RANKL. Thus, although the inhibitory cytokine such as IFN-β is produced in response to RANKL, the inhibition of osteoclastogenesis may be rescued by the induction of signaling suppressors such as SOCSs.

Osteoclasts, the cells primarily responsible for bone resorption, are of hemopoietic stem cell origin. Precursors of osteoclasts have been demonstrated to share common properties with those of the monocyte/macrophage (M/MØ) cell lineage (1, 2). Many systemic hormones and local cytokines participate in regulating osteoclast differentiation (3, 4). Receptor activator of NF-κB (RANK) ligand (RANKL) has been identified as the most important and critical molecule for osteoclast development in cooperation with macrophage colony-stimulating factor (M-CSF), and the RANKL/RANK system produces an essential signal for osteoclast differentiation in the interaction between stromal cells and cells of the osteoclast lineage (5–7). More recently, we demonstrated that endogenous production of transforming growth factor (TGF-β) was essential for osteoclastogenesis as a cofactor with RANKL and M-CSF (8). Most of the osteotropic factors regulating osteoclast differentiation also affect the immune system as well, suggesting a strong relationship between the two systems. Such immune mediators can be divided into the following two groups regarding their effects on osteoclast differentiation: one consists of stimulatory cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-11, IL-17, and tumor necrosis factor-α; and the other consists of inhibitory cytokines including IL-4, IL-10, IL-12, IL-13, IL-18, and interferon-γ (INF-γ) (9–19). Furthermore, the cytokines belonging to each group can also be separated into two subclasses based on their direct or indirect action via stromal cells on cells of the osteoclast lineage.

Among the above inhibitory cytokines, one well-known immune mediator is the T-cell product IFN-γ, a type II IFN (20). IFN-γ has been demonstrated from in vivo and in vitro observations to inhibit strongly osteoclast differentiation and function (21). On the other hand, involvement of type I IFNs (IFNa/β) in osteoclastogenesis remains to be investigated, although the inhibitory effect has been reported recently (22). Type I IFNs are produced by a variety of cell types in response to viral, bacterial, and protozoan infections, representing a critical link between innate and acquired immunity through their multiple effects on natural killer cells and T-cells (23–25). In addition, evidence indicating their actions on other cell types such as monocytes, macrophages, and dendritic cells is accumulating (26–30). Osteoclasts, macrophages, and dendritic

* This work was supported by Grants-in-aid for Scientific Research 12671780 and 1247038 from the Japanese Ministry of Education, Science, Sports, and Culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† ‡ Both authors contributed equally to this work.
§ To whom correspondence should be addressed: Dept. of Oral Anatomy, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel.: 81-492-79-2769; Fax: 81-492-71-3523; E-mail: y-hakeda@dent.meikai.ac.jp.

1 The abbreviations used are: M/MØ, monocyte/macrophage; RANK, receptor activator of NF-κB; RANKL, RANK ligand; sRANKL, soluble RANKL; M-CSF, macrophage colony-stimulating factor; TGF-β, transforming growth factor; IL, interleukin; IFN, interferon; SOCS, suppressor of cytokine signaling; ISGF-3, IFN-stimulated gene factor-3; TRAP, tartrate-resistant acid phosphatase; MNCs, multinucleate cells; ISRE, interferon-stimulated response element; IFNAR, IFN-α receptor; JAK, Janus kinases; STAT, signal transducers and activators of transcription; LPS, lipopolysaccharide; PBS, phosphate-buffered saline.
that the expression of IFN-β osteoclast progenitors constitutively expressed type I IFN and binding to NF-

nuclei of these cells were stained with propidium iodide (50 μMNCs) with more than three nuclei were counted under a microscope. The total number of cells and numbers of TRAP-positive multinucleate cells (10% formalin and stained for tartrate-resistant acid phosphatase

10% minimum Eagle

mum Eagle

B and AP-1 (32, 33). The expression of IFN-β in macrophages also depends on the activation of the transcription factor followed by the binding to NF-κB and AP-1 sites in the promoter of IFN-β gene (34–36). Taken together, the available data suggest that type I IFNs may potentially influence osteoclastogenesis in an autocrine manner.

In this study, we found that bone marrow-derived M/M0 as osteoclast progenitors constitutively expressed type I IFN and that the expression of IFN-β was particularly up-regulated by RANKL. IFN-β intrinsically inhibited the differentiation of osteoclasts. However, RANKL simultaneously induced the expression of suppressors of cytokine signaling (SOCS)-1 and SOCS-3 (37), which were earlier shown to block the signaling of IFNs by inhibiting phosphorylation of STATs by Janus kinases (JAK) (38), thus causing a decrease in IFN-dependent transcription factor complex (IFN-stimulated gene factor-3 (ISGF-3)) formation (39). Thus, although the inhibitory cytokines such as type I IFNs are produced in response to RANKL, the inhibition of osteoclastogenesis may be rescued by inducing the production of signaling suppressors such as SOCSs. The results presented here indicate the intimate cross-talk between inhibitory and stimulatory signalings in osteoclast formation.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Recombinant human M-CSF and recombinant mouse soluble RANKL (sRANKL) were kindly provided by Morinaga Milk Industry Co. (Tokyo, Japan) and Snow Brand Milk Industry Co. (Tochigi, Japan). Recombinant human TGF-β1 was obtained from Genzyme/Technicon (Cambridge, MA). Recombinant mouse IFN-β was purchased from PBL Biomedical Laboratories (New Brunswick, NJ). The IFN-β was tittered with the use of the cytopathic effect inhibition assay (40). In this antiviral assay, 1 unit/ml IFN-β was the quantity necessary to produce a cytopathic effect of 50%. Sheep neutralizing antiserum against mouse IFN-α/β was purchased from BIOSOURCE International (Camarillo, CA). Anti-STAT-1 and -STAT-2 antibodies were from Santa Cruz Biotechnologies, Inc. (San Diego, CA). Anti-phospho-STAT-1 antibody was obtained from Cell Signaling Technology (Beverly, MA).

**Isolation of M/M0-like Hemopoietic Cells from Mouse Bone Marrow Cells—**Femora and tibiae were obtained from 4- to 5-week-old ICR mice (Shizuoka Laboratories Animal Center, Shizuoka, Japan), and the connective soft tissues were removed from the bones. Bone marrow cells were flushed out from the bone marrow cavity, suspended in α-minimum Eagle’s medium (ICN Biomedicals, Aurora, OH), supplemented with 10% fetal bovine serum (Intergen, Purchase, NY), M-CSF (100 ng/ml), and 100 units/ml of penicillin, and cultured in Petri dishes in a humidified atmosphere of 5% CO₂. During the 3 days in culture, the dishes with PBS and by subsequent incubation for 5 min in 0.25% trypsin, 0.05% EDTA, respectively, the M/M0-like hemopoietic cells were harvested by PBS vigorously pipetting.

**Osteoclastogenesis from M/M0-like Hemopoietic Cells—**Isolated M/M0-like hemopoietic cells were seeded at an initial density of 2.5 × 10⁵/cm² and cultured in α-minimum Eagle’s medium, 10% fetal bovine serum, M-CSF (20 ng/ml) with or without sRANKL (50 ng/ml) and/or other cytokines or agents. The culture medium was exchanged every 2 days. After a culture period of the desired length, the cells were fixed in 10% formalin and stained for tartrate-resistant acid phosphatase (TRAP) activity with a leucocyte acid phosphatase kit (Sigma). The total number of cells and numbers of TRAP-positive multinucleate cells (MNCs) were counted in 3 nuclei containing 3 were analyzed by 1.5% agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining.

**Western Blot Analysis—**After various treatments, the cells were washed with PBS, scraped into a solution consisting of 10 mM sodium phosphate (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM aprotinin, 0.1% S3 reverse, 5′-CTTCCACCCACAGGGCTTCTC-3′; IFN-β reverse, 5′-CCACCACAAATCACTTCTC-3′; IFN-α reverse, 5′-CCACGACCTTTCAAGTG-3′; SOCS-1 forward, 5′-AGGTCATGAGCA-3′; SOCS-3 forward, 5′-GATCTGGAAGGGAGGAACTGCAGT-3′; SOCS-3 forward, 5′-GATGGAAGCTCACACAGAGTT-3′; STAT-1 forward, 5′-GCCATGTCACCAAGCCCAAAGTT-3′; STAT-1 reverse, 5′-TGCTGGGAAGGGAAGAGAGT-3′; STAT-2 forward, 5′-TTTGCTGCGGTCGCTCA-3′; STAT-2 reverse, 5′-TGGGTCCTGGGCTGTTCTT-3′; ISGF-3p forward, 5′-GACCCCTCTACACCAAC-3′; ISGF-3p reverse, 5′-AGTTGGAACGACGGAGT-3′; STAT-1 forward, 5′-TCAAC ACTGGTCCTGGGTACAC-3′; β-actin forward, 5′-GAGTACTGGC GCTAGGAGGAGC-3′. Amplification was conducted for 22–32 cycles, each for 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min in a 25-μl reaction mixture containing 0.5 μl of each cDNA, 25 pmol of each primer, 0.5 mM dNTP, and 1 units of Taq DNA polymerase (Qiagen, Valencia, CA). After amplification, 15 μl of each reaction mixture was analyzed by 1.5% agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining.

**Electrophoretic Mobility Shift Assay—**After M/M0-like hemopoietic cells had been incubated with or without sRANKL for 8 h, the cells were treated with IFN-β for the indicated times and then washed twice with ice-cold PBS, incubated for 10 min on ice in 1 ml of ice-cold buffer A (10 mM Hepes (pH 7.4), 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM aminothiobenzensulfonyl fluorid, 2 μg/ml actinomycin D, 2 μg/ml pepstatin, 2 μg/ml leupeptin), and scraped into the buffer. The cell lysates were incubated further for 10 min on ice and then transferred to tubes. The nuclei obtained by centrifugation for 1 min at 5,000 × g were extracted by a 30-min incubation in ice-cold buffer C, consisting of 50 mM Hepes (pH 7.5), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 20% glycerol, 1 mM dithiothreitol, 1 mM aminothiobenzensulfonyl fluorid, 2 μg/ml actinomycin D, 2 μg/ml pepstatin, and 2 μg/ml leupeptin. The extracts were then centrifuged at 14,000 × g for 30 min, and the supernatants were used for the electrophoretic mobility shift assay.

The cells were incubated with or without sRANKL for 8 h, the cells were treated with IFN-β for the indicated times and then washed twice with ice-cold PBS, incubated for 10 min in ice 1 ml of ice-cold buffer A (10 mM Hepes (pH 7.4), 10 mM KCl, 0.1% EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM aminothiobenzensulfonyl fluorid, 2 μg/ml actinomycin D, 2 μg/ml pepstatin, 2 μg/ml leupeptin), and scraped into the buffer. The cell lysates were incubated further for 10 min on ice and then transferred to tubes. The nuclei obtained by centrifugation for 1 min at 5,000 × g were extracted by a 30-min incubation in ice-cold buffer C, consisting of 50 mM Hepes (pH 7.5), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 20% glycerol, 1 mM dithiothreitol, 1 mM aminothiobenzensulfonyl fluorid, 2 μg/ml actinomycin D, 2 μg/ml pepstatin, and 2 μg/ml leupeptin. The extracts were then centrifuged at 14,000 × g for 30 min, and the supernatants were used for the electrophoretic mobility shift assay.

The cells were incubated with or without sRANKL for 8 h, the cells were treated with IFN-β for the indicated times and then washed twice with ice-cold PBS, incubated for 10 min on ice 1 ml of ice-cold buffer A (10 mM Hepes (pH 7.4), 10 mM KCl, 0.1% EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM aminothiobenzensulfonyl fluorid, 2 μg/ml actinomycin D, 2 μg/ml pepstatin, 2 μg/ml leupeptin), and scraped into the buffer. The cell lysates were incubated further for 10 min on ice and then transferred to tubes. The nuclei obtained by centrifugation for 1 min at 5,000 × g were extracted by a 30-min incubation in ice-cold buffer C, consisting of 50 mM Hepes (pH 7.5), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 20% glycerol, 1 mM dithiothreitol, 1 mM aminothiobenzensulfonyl fluorid, 2 μg/ml actinomycin D, 2 μg/ml pepstatin, and 2 μg/ml leupeptin. The extracts were then centrifuged at 14,000 × g for 30 min, and the supernatants were used for the electrophoretic mobility shift assay.
EDTA, 0.5 mM dithiothreitol) for 30 min at room temperature. The specificity of the reaction was confirmed by competition with a 50-fold molar excess of nonlabeled oligonucleotides. The protein-DNA complexes were resolved by 7.2% PAGE in 0.5 M H11003 TBE buffer and visualized by autoradiography. In addition, the nuclear extracts were incubated with anti-STAT-1 and anti-STAT2 for 30 min on ice after binding to the oligonucleotides and then were subjected to PAGE.

RESULTS

Exogenous IFN-β Suppresses Osteoclastogenesis Induced by M-CSF and RANKL—When bone marrow-derived M/MØ cells were incubated with M-CSF and sRANKL, and particularly when TGF-β was also present, most of the cells differentiated into cells of the osteoclast lineage and became mature osteoclasts, as described previously (8). Addition of exogenous IFN-β reduced the number of osteoclastic TRAP-positive MNCs formed in these cultures in a dose-related manner (Fig. 1), whereas the total nuclear number was not changed (data not shown). The fusion index of TRAP-positive MNCs, defined as the percent of cells participating in the fusion, was suppressed by IFN-β in parallel with the inhibition of the formation of TRAP-positive MNCs. Semiquantitative PCR analysis revealed that treatment with M-CSF and sRANKL for 6 days or with M-CSF, TGF-β, and sRANKL for 4 days induced an increase in the levels of mRNA for TRAP, cathepsin K, calcitonin receptor, and integrin αv and β3, all of which are abundantly expressed in osteoclasts and considered as marker molecules for osteoclast differentiation (Fig. 2). IFN-β decreased the induced levels of mRNA for these molecules (Fig. 2). These results indicate that exogenous IFN-β strongly suppressed the process of osteoclast differentiation from the progenitors, bone marrow-derived M/MØ cells.

Inhibition of Osteoclastogenesis by Endogenous Type I IFNs—Type I IFNs were demonstrated earlier to be produced by a variety of cell types including M/MØ cells (27, 30), suggesting the potential ability of osteoclast progenitors to produce type I IFNs in osteoclast progenitors. Thus, we examined the
expression of type I IFNs in osteoclast progenitors in response to sRANKL. The messenger RNA level of IFN-α did not change consistently (data not shown). However, the level of IFN-β mRNA significantly increased as early as 1 h after exposure to sRANKL (Fig. 3A). The increase reached a maximum after 5 h of treatment, and the increase was not affected by adding cycloheximide (Fig. 3B), indicating that sRANKL strongly induced the expression of IFN-β in osteoclast progenitors and that the induction was not mediated by any other RANKL-induced proteins but occurred via the direct action of RANKL. Thus, we next examined how the endogenous type I IFNs influenced osteoclastogenesis from osteoclast precursors. When anti-IFN-β neutralizing antibody was added to cultures of bone marrow-derived M/MØ cells in the presence of M-CSF and sRANKL with or without TGF-β, formation of osteoclastic TRAP-positive MNCs in the cultures was accelerated compared with that in the absence of the antibody (Fig. 4A). The acceleration depended on the dose of the neutralizing antibodies (Fig. 4, B and C), suggesting that the osteoclast progenitors endogenously produced type I IFNs depending on the signal of RANKL and that these cytokines inhibited the osteoclastogenesis induced by RANKL.

Induction of SOCS-1 and -3 by sRANKL in Osteoclastogenesis—These above results highlight the discrepancy between the induction of osteoclast differentiation and the production of the inhibitory cytokines such as type I IFNs, both of which were mediated by RANKL signaling, thus leading to a possibility that there may be a mechanism for negating the inhibitory action of endogenous IFN-α/β on osteoclastogenesis. So we next focused on SOCSs as negative regulators of the action of IFNα/β. Eight isoforms of SOCS were earlier identified as a family of intracellular proteins controlling the magnitude and/or duration of signals propagated by diverse cytokine receptors by suppressing their signal transduction process (37). We examined the effect of sRANKL on the expression of SOCSs-1 and -3 mRNAs in osteoclast progenitors by semiquantitative RT-PCR. Messenger RNA levels of SOCSs in bone marrow-derived M/MØ cells as osteoclast progenitors were time-dependently increased by treatment with sRANKL. The magnitude of the induction of SOCSs-3 mRNA was greater than that of SOCSs-1, and the increase was observed as early as 1 h after adding sRANKL to the cultures, reached a maximum at 5 h, and continued until 22 h, although the levels gradually decreased after 8 h of treatment (Fig. 5A). RANKL-induced SOCSs-1 expression was delayed slightly compared with that of SOCSs-3. In addition, the inhibitory effect of sRANKL was not reduced by the addition of cycloheximide (Fig. 5B), indicating the direct induction by sRANKL.

RANKL-induced SOCSs Counteract Inhibitory Effects of IFNα/β on Osteoclastogenesis—The signal of type I IFNs is transmitted through the IFN-α/β receptor (IFNAR) expressed on the plasma membrane of target cells into an intracellular transcription complex (ISGF-3) composed of STAT-1, STAT-2.
and ISGF-3γ, and SOCSs inhibit the signaling (39). Thus, we examined whether the RANKL-induced SOCSs would reduce the IFNα/β signaling in osteoclast progenitors. IFN-β stimulated the binding of nuclear proteins to an oligonucleotide sequence containing the interferon-stimulated response element (ISRE), and the increase was significant as early as 20 min after the addition of IFN-β (Fig. 6A). When the probe and the nuclear extracts were incubated with anti-STAT-1 and anti-STAT-2 antibodies, the complex was further shifted to the upper position and decreased, respectively, in the gel (Fig. 6B). However, when the progenitors were pretreated for 8 h with sRANKL prior to the treatment with IFN-β, the IFN-β-induced complex with the nuclear extracts and ISRE probe was significantly reduced, suggesting a possible mechanism for suppression of the IFN-β signaling. The duration of the pretreatment was enough for induction of SOCS expression by RANKL as demonstrated in Fig. 5. As SOCS-1 and SOCS-3 have been demonstrated to inhibit the phosphorylation of STAT-1 and STAT-2 by JAKs, which is essential for formation of the ISGF-3 complex (39), we examined the effect of sRANKL on the phosphorylation of STAT-1 in osteoclast progenitors. IFN-β increased the level of phosphorylated STAT-1 in the progenitors, and the increase was significant as early as 15 min after exposure to IFN-β, with a maximal stimulation at 45 min (Fig. 7). The time-dependent profile was consistent with that of formation of the complex between nuclear extracts and ISRE probe. Pretreatment with sRANKL for 8 h reduced the levels of the phosphorylated STAT-1, whereas the total protein level of STAT-1 was not changed (Fig. 7). Furthermore, the levels of STAT-2 and ISGF-3γ mRNAs were also unaltered by exposure to sRANKL (Fig. 8). These results suggest that the IFN-β signaling was reduced by the blocking of the phosphorylation of STATs due to the increase in SOCSs induced by RANKL. Thus, RANKL-induced SOCSs appeared to counteract the inhibitory effects of IFNα/β on osteoclastogenesis.

**DISCUSSION**

In this study, we demonstrated that IFN-β endogenously produced in osteoclast progenitors potentially depressed the differentiation toward mature osteoclasts induced by RANKL and M-CSF. However, expression of SOCSs, particularly that of SOCS-3, was simultaneously induced by RANKL. When the progenitors were pretreated with RANKL for a period of time sufficient to induce SOCS expression, the signaling of IFN-β was significantly suppressed, and the suppression was mediated by the inhibition of phosphorylation of STAT-1, which resulted in impairment of the ISGF-3 complex formation responsible for the signal transduction of type I IFNs. Thus, the isolated osteoclast progenitors expressed the immune mediators and their suppressors, in the balance of which the osteoclast differentiation could progressively proceed.

The expression of type I IFNs is stimulated by a variety of...
extracellular stimuli such as viral and bacterial infections and cytokines involved in host defense. Various hematopoietic cells including cells belonging to the monocyte and macrophage lineage express type I IFNs according to immune system, and their proliferation and differentiation are widely regulated by type I IFNs (26–29, 43). A potent inducer of type I IFN production in macrophages is bacterial lipopolysaccharide (LPS, 44–46). LPS has been demonstrated to induce the expression of IFN-β in macrophages, mediating the induction of inducible nitric-oxide synthase (45, 46). The LPS action is mediated through a Toll-like receptor-dependent signal pathway finally by activation of NF-κB and AP-1 (47–49), suggesting each or both transcription factor-dependent type I IFN expression. Similarly to the above case of macrophages, Takayanagi et al. (22) have most recently demonstrated the induction of IFN-β by RANKL through the c-Fos pathway in osteoclast precursors, although they did not define the contribution of NF-κB to the induction. Their observations are consistent with our findings presented in this study. In fact, the promoter of the IFN-β gene contains AP-1- and NF-κB-binding sites, which may mediate the RANKL-induced IFN-β expression (22, 34–36).

Studies on the involvement of IFNs in osteoclastogenesis have been restricted to the action of IFN-γ produced by T-cells, indicating the direct and inhibitory effect on osteoclast recruitment and function (21, 50–54). Mice lacking IFN-γ receptor showed a notable exacerbation of osteoclast formation and severe bone destruction when LPS was locally microinjected into calvariae of the knockout mice (21). On the other hand, the involvement of type I IFNs in osteoclastogenesis has so far been less studied. However, mice lacking IFNAR1, a receptor of IFN-α/β, or IFN-β showed a notable increase in osteoclast formation and a reduction in trabecular bone mass, representing osteopenia (22). Consistently, we also demonstrated in this study the direct inhibition by endogenous IFN-β of osteoclast differentiation in vitro. It should be noted that the bone mass in IFN-γ receptor knockout homozygotes was the same level as that of the heterozygotes and that the notable destruction of bone mass in the mutant mice was T-cell-dependent (21). On the other hand, the reduction in bone mass in IFNAR1−/− or IFN-β−/− mice was observed without any stimuli (22). Taken together, these results suggest that type I IFNs are involved in the physiological process of osteoclastogenesis and IFN-γ may be involved pathologically in the process.

RANKL mediates an essential signal to osteoclast progenitors for their differentiation into mature and functional osteoclasts (7). As shown in this study, however, RANKL simultaneously induces the production of inhibitory cytokines such as type I IFNs in the osteoclast progenitors. Such endogenous actions of IFNs may represent a feedback regulation of osteoclastogenesis as demonstrated in a recent study (22). If so, the differentiation from the progenitors would be blocked by the endogenous inhibitory cytokines. Therefore, we assume that there would be a mechanism to avoid such possible feedback inhibition in process of osteoclast differentiation. Signals of type I IFNs are mediated through JAK/STAT systems following the binding of the ligands to their receptors (IFNARs) (55). Several mechanisms of inhibition have been characterized that negatively regulate the JAK/STAT activation pathway. These include the family of protein inhibitors of activated STATs, of which there are four isoforms that bind to activated STATs and inhibit DNA binding (56). However, as presented here, phosphorylation of STAT-1 was inhibited by pretreatment with RANKL, meaning a blockade of activation of STATs, simultaneously with a reduction in the binding of nuclear extracts to ISRE probes. These results imply a protein inhibitors of activated STATs-independent mechanism. SOCS, also called JAK-binding protein and cytokine-inducible SH2 protein, was originally discovered as the product of an immediate early gene induced by stimulation with IL-3 and erythropoietin; so far eight isoforms have been cloned (37). Among these SOCSs, SOCS-1 and SOCS-3 have been extensively studied for their actions and are considered to be potent negative feedback regulators of a number of cytokines and growth factors whose signals are mediated by the JAK/STAT system (38).

These SOCSs block the phosphorylation of STATs by inhibiting the kinase activity of JAKs, causing a blockade of STAT activation (57, 58). In this study, we showed that RANKL induced the expression of SOCS-1 and SOCS-3 in osteoclast progenitors. Pretreatment with RANKL for a period sufficient for the induction of SOCSs attenuated phosphorylation of STAT-1 in response to IFN-β in the osteoclast progenitors. In parallel with the inhibition of STAT-1 phosphorylation, the pretreatment with RANKL reduced the binding of nuclear extracts to ISRE sites. Although we have not ruled out the possibility that dephosphorylation of STAT1 by SH2 domain-containing tyrosine phosphatase 1 is involved in the inhibition of JAK/STAT signaling (59), the RANKL-induced SOCS expression would be one of the mechanisms for the blockade of the signaling in osteoclastogenesis. To our knowledge, this is the first report indicating the involvement of SOCSs in osteoclast recruitment. However, we should note that the inhibition of IFN signals by the suppressors was not complete, because the neutralization by anti-type 1 antibody still had an effect on the formation of osteoclasts.

Expression of SOCS genes is controlled at the transcriptional level, with the STAT family of transcription factors being the major mechanism of SOCS induction (60, 61), representing a classical negative feedback mechanism. On the other hand, the JAK/STAT-independent SOCS gene activation has also been demonstrated. For instance, LPS and CpG-DNA stimulated the expression of the SOCS genes in macrophages, and thereby STAT-1 activation in response to IFN-γ was suppressed (62, 63). Similarly, IL-1β induction of SOCS-3 has been demonstrated to mediate growth hormone resistance in primary rat liver hepatocytes (64). We found that the RANKL induction of SOCS gene expression was caused by the direct action of RANKL and not mediated by newly synthesized protein in osteoclastogenesis. The signal of RANKL in osteoclast progenitors was mediated by the activation of NF-κB and AP-1 as occurred with LPS and IL-1β, and the promoter region of SOCS-3 gene contained NF-κB and AP-1 binding sites in addition to STAT-response elements (60). Taken together, the data suggest that the induction of SOCS expression by RANKL in osteoclast progenitors presented here may result from an NF-κB- and/or AP-1-dependent mechanism and not from a STAT-dependent one.

In conclusion, although the inhibitory cytokine such as type I IFNs is produced in response to RANKL, the potential inhibition of osteoclastogenesis may be rescued by the induction of signaling suppressors such as SOCSs, thus allowing the differentiation of osteoclasts to proceed. These results presented here indicate the intimate cross-talk between inhibitory and stimulatory cytokines in osteoclast formation.

Acknowledgements—We thank Drs. K. Yamaguchi (Snow Brand Milk Industry Co.) and M. Yamada (Morinaga Milk Industry Co.) for their generous gifts of recombinant sRANKL and recombinant M-CSF, respectively.

REFERENCES

1. Felix, R., Cecchini, M. G., Hofstetter, W., Elford, P. R., Stutzer, A., and Fleisch, H. (1990) J. Bone Miner. Res. 5, 781–789.
2. Kodama, H., Yamasaki, A., Nose, M., Niiwa, S., Ohgane, Y., Abe, M., Kumegawa, M., and Suda, T. (1991) J. Exp. Med. 173, 269–272.
3. Chambers, T. J. (1985) J. Clin. Pathol. 38, 241–252.
4. Suda, T., Udagawa, N., Nakamura, I., Miyaura, C., and Takahashi, N. (1995) J. Clin. Pathol. 38, 241–252.
Roles of Endogenous IFN-β and SOCSs in Osteoclastogenesis

Bone (New York) 17, 87–91

5. Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Kuhn, J. T., Colahan, T., and Wold, P. M. (2000) J. Immunol. 165, 165–176

6. Yasuda, C., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Suchi, M., Tsuchiya, N., Murata, S., Uchida, E., and Hoshino, S. (2000) J. Biol. Chem. 275, 25430–25435

7. Kim, S. W., and Chambers, T. J. (2000) J. Immunol. 165, 5424–5430

8. Han, T. L., Zhao, T., Liu, Y., and Wang, C. (2000) J. Immunol. 165, 3847–3852

9. Weiden, M., Tanaka, N., Xiao, M., Yao, J., Han, B., and Hoshino, S. (2000) J. Biol. Chem. 275, 26422–26427

10. Kawai, T., Takeda, K., and Akira, S. (2000) J. Immunol. 165, 5441–5448

11. Carano, A. W., and Klagsbrun, M. (2000) J. Biol. Chem. 275, 25439–25444