Dexamethasone Enhances Osteoclast Formation Synergistically with Transforming Growth Factor-β by Stimulating the Priming of Osteoclast Progenitors for Differentiation into Osteoclasts*

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Long-term administration of glucocorticoids (GCs) causes osteoporosis with a rapid and severe bone loss and with a slow and prolonged bone disruption. Although the involvement of GCs in osteoblastic proliferation and differentiation has been studied extensively, their direct action on osteoclasts is still controversial and not conclusive. In this study, we investigated the direct participation of GCs in osteoclastogenesis. Dexamethasone (Dex) at <10⁻⁸ M stimulated, but at >10⁻⁷ M depressed, receptor activator of NF-κB ligand (RANKL)-induced osteoclast formation synergistically with transforming growth factor-β. The stimulatory action of Dex was restricted to the early phase of osteoclast differentiation and enhanced the priming of osteoclast progenitors (bone marrow-derived monocytes/macrophages) toward differentiation into cells of the osteoclast lineage. The osteoclast differentiation depending on RANKL requires the activation of NF-κB and AP-1, and the DNA binding of these transcription factors to their respective consensus cis-elements was enhanced by Dex, consistent with the stimulation of osteoclastogenesis. However, Dex did not affect the RANKL-induced signaling pathways such as the activation of IκB kinase followed by NF-κB nuclear translocation or the activation of JNK. On the other hand, Dex significantly decreased the endogenous production of interferon-β, and this cytokine depressed the RANKL-elicted DNA binding of NF-κB and AP-1, as well as osteoclast formation. Thus, the down-regulation of inhibitory cytokines such as interferon-β by Dex may allow the osteoclast progenitors to be freed from the suppression of osteoclastogenesis, resulting in an increased number of osteoclasts, as is observed in the early phase of GC-induced osteoporosis.

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)¹ cell lineage (1, 2). Although many systemic hormones and local cytokines participate in regulating osteoclast differentiation (3, 4), the receptor activator of NF-κB (RANK) ligand (RANKL) is the most critical molecule for osteoclastogenesis in cooperation with macrophage colony-stimulating factor (M-CSF) in the interaction between stromal cells and cells of the osteoclast lineage (5–7). Extensive studies indicate that the induction of osteoclast differentiation by RANKL requires the activation of NF-κB and JNK pathways via tumor necrosis factor receptor-associated factor (TRAF) family proteins from RANK, the RANKL receptor (8–10). We demonstrated recently (11) that the endogenous production of transforming growth factor (TGF-β) was also essential for osteoclastogenesis as a cofactor with RANKL and M-CSF. Besides these proteins, a variety of local cytokines participate in regulating osteoclast differentiation (3, 4). Most of the osteotropic factors regulating osteoclast differentiation also affect the immune system, suggesting an intimate relationship between these two systems. These immune mediators such as interleukins, tumor necrosis factor-α, and interferons (IFNs) can be categorized into different groups based on their stimulatory or inhibitory effects and their direct or indirect action on osteoclast differentiation (12–16). We and others reported more recently (17, 18) that the endogenous production of type-I IFNs such as IFN-β induced by RANKL in osteoclast progenitors intrinsically inhibited the differentiation of osteoclasts. Thus, osteoclast differentiation is governed by a delicate balance between the above stimulatory and inhibitory cytokines.

Glucocorticoids (GCs) have a multitude of effects on the immune response at several sites and are both anti-inflammatory and immunosuppressive when administered therapeutically (19–21). Although GCs are effective for the treatment of a wide variety of disorders ranging from autoimmune diseases to acute situations such as spinal cord injury, long-term therapy with GCs causes osteoporosis, resulting in severe bone loss that, at present, has become a big clinical problem (22–25). GCs receptor activator of NF-κB; RANKL, RANK ligand; M-CSF, macrophage colony-stimulating factor; TGF-β, transforming growth factor-β; IFN, interferon; GC, glucocorticoid; Dex, dexamethasone; TRAP, tartrate-resistant acid phosphatase; MNC, multinucleate cell; SOCS, suppressor of cytokine signaling; STAT, signal transducers and activators of transcription; ISGF-3, IFN-stimulated gene factor-3; PBS, phosphate-buffered saline; JNK, c-Jun NH₂-terminal kinase; TRAF, tumor necrosis factor receptor-associated factor; sRANKL, soluble RANKL; GST, glutathione S-transferase; GR, glucocorticoid receptor; MEM, minimum Eagle's medium; FBS, fetal bovine serum; RT, reverse transcription; p-ABSF, p-aminobenzensulfonyl fluoride; EMSA, electrophoretic mobility shift assay.

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¹ The abbreviations used are: M/MØ, monocyte/macrophage; RANK,
Direct Action of Dexamethasone on Osteoclasts

decrease calcium absorption in the gastrointestinal system and increase calcium excretion in the renal system, resulting in a high level of parathyroid hormone (26, 27). Therefore, GC-induced osteoporosis has been accepted for a long time to be caused by the secondary hyperparathyroidism (26). In addition, another possible mechanism for GC-induced osteoporosis is that GCs decrease gonadotropin production, which may result in increased bone resorption because of estrogen deficiency (28, 29). Considerable evidence, however, indicates that elevated levels of parathyroid hormone or subnormal vitamin D metabolite concentrations are not typical of patients receiving GC therapy, and there is no direct evidence indicating that GC-induced hypogonadism is responsible for the enhanced bone resorption, thus suggesting the existence of some other mechanism for GC-induced bone loss (30). On the other hand, many in vitro studies have indicated the direct action of GCs on osteoblasts (31–33). Recent studies (34, 35) show that GC acts directly on osteoblasts to up-regulate the expression of RANKL and M-CSF and that the steroid oppositely down-regulates osteoprotegerin, a decoy receptor of RANKL that prevents the transmission of the RANKL signal into cells of the osteoclast lineage. This regulation is likely to be a mechanism for induction of bone resorption by GCs. However, direct effects of GCs on osteoclasts are controversial and are not conclusive (36–38). The aim of this study was to evaluate precisely the direct action of GCs on osteoclast differentiation and to determine the point of GC action in the process of osteoclastogenesis.

In this study, we found that dexamethasone (Dex), at low concentrations (<10−8 M), enhanced RANKL-induced osteoclast formation synergistically with TGF-β by stimulating the priming of bone marrow-derived M/MØ as osteoclast progenitors for differentiation toward osteoclasts. Although numerous studies (39–42) have indicated the negative regulation of NF-κB and AP-1 activation by GCs as anti-inflammatory and immunosuppressive agents, the enhancement of osteoclastogenesis was accompanied by the additional activation by Dex of these transcription factors evoked by RANKL. However, Dex did not influence the signaling pathways from RANK by which these transcription factors are translocated into nucleus. On the other hand, Dex significantly depressed the endogenous production of a type-I IFN, IFN-β. IFN-β potently inhibited osteoclastogenesis, as demonstrated previously (17, 18), and the cytokine depressed the activation of NF-κB and AP-1 in osteoclast progenitors. Thus, down-regulation of the inhibitory cytokines such as IFN-β by Dex may cause release from the suppression of osteoclastogenesis, thus resulting in an increase in osteoclast number, as is observed in the early phase of GC-induced bone loss.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human M-CSF and recombinant mouse soluble RANKL (sRANKL) were kindly provided by Morinaga Milk Industries Co., Ltd. (Tokyo, Japan) and Snow Brand Milk Industry Co. (Tochigi, Japan), respectively. Recombinant human TGF-β1 and M-CSF were purchased from Genzyme/Technex (Cambridge, MA) and PBL Biomedical Laboratories (New Brunswick, NJ), respectively. Recombinant GST-conjugated c-Jun was from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA). Anti-glucocorticoid receptor antibodies were purchased from Santa Cruz Biotechnology, Inc. (Beverly, MA) and PBL Biomedical Laboratories (New Brunswick, NJ), respectively.

Quantiﬁcation of TRAP by Fluorescence Spectroscopy—Cellular TRAP activity was measured by fluorescence spectroscopy as described by Gallwitz et al. (43) with minor modifications. Isolated M/MØ-like hemopoietic cells were pre-cultured in α-MEM/10% FBS/M-CSF (20 ng/ml) and/or other cytokines or agents for 2 days and then further cultured in the presence of sRANKL (40 ng/ml) under various conditions for 4 days. After the culture period, the cells were washed with PBS and lysed by two cycles of freezing and thawing at 0.05% Triton X-100. After centrifugation, the supernatant was used for determination of TRAP activity. The cell lysate was incubated for 30 min at 37 °C in a reaction mixture consisting of 0.48 m sodium acetate/0.48 m acetic acid (pH 5.0), 20 mM tartaric acid, and 2 mM methylumbelliferyl phosphate as a substrate and then the reaction was terminated by adding glycine and EDTA (pH 10.5), each for a final concentration of 50 mM. The concentration of methylumbelliferone produced in the reaction mixture was determined by fluorometry with excitation at 366 nm and emission at 456 nm. Enzyme activity was expressed as nanomoles of methylumbelliferone hydrolyzed/min/mg protein. The concentration of proteins in the cell lysate was measured with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc.).

RT-PCR—Total RNA (1 μg) extracted from cultured cells was used as a template for cDNA synthesis. cDNA was prepared by use of a SuperScript II preamplification system (Invitrogen). Primers were synthesized on the basis of the reported mouse cDNA sequences for TRAP, c-fos, c-jun, GR, and PPARγ. The concentration of methylumbelliferone produced in the reaction mixture was determined by fluorometry with excitation at 366 nm and emission at 456 nm. Enzyme activity was expressed as nanomoles of methylumbelliferone hydrolyzed/min/mg protein. The concentration of proteins in the cell lysate was measured with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc.).

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forward, 5'-TCACCCACACTGTGCCCATCTAC-3'; β-actin reverse, 5'-
GAGTACTTGCCGTGAGGAGG-3'. Amplification was conducted for 22–32 cycles, each of 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, 0.2 μl dNTP, and 1 unit 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.

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 μM EDTA, 1 mM p-aminomethyl-benzensulfonyl fluoride (p-ABSF), 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin and sonicated for 15 s. The samples, containing equal amounts of protein, were subjected to 10% SDS-PAGE, and the proteins separated in the gel were subsequently electrotransferred onto a polyvinylidene difluoride membrane. After having been blocked with 5% skim milk, the membrane was incubated with anti-GR, anti-NF-κB p50, anti-NF-κB p65, anti-phospho-NF-κB p65, anti-IκB-α, or anti-phospho-IκB-α antibodies and subsequently with peroxidase-conjugated antimouse or anti-rabbit IgG antibody. Immunoreactive proteins were visualized with Western blot chemiluminescence reagents (Amersham Biosciences) following the manufacturer’s instructions.

Assay of JNK Activity—M/MØ-like hemopoietic cells were pretreated or not with Dex and/or TGF-β in the presence of M-CSF for 2 days prior to treatment with RANKL for 1 h. After the treatment, the cells were extracted in a lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM orthovanadate, 1 μg/ml leupeptin, and 1 mM p-ABSF. 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 5000 × 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 p-ABSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 100 μM [γ-32P]ATP (5 μCi) and 1 μg of recombinant GST-c-Jun and incubated at 30 °C for 30 min. The reaction was terminated by adding SDS sample buffer, and the mixture was then boiled. After the samples had been subjected to SDS-PAGE (12% gel), the phosphorylated GST-c-Jun in the gel was visualized by autoradiography at −80 °C.

Electrophoretic Mobility Shift Assay (EMSA)—After pretreatment or not with Dex and/or TGF-β in the presence of M-CSF for 2 days, the cells were treated with sRANKL for 1 h 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 p-ABSF, 2 μg/ml aprotinin, 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 5000 × 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 p-ABSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 2 μg/ml leupeptin. Then, the extracts were centrifuged at 14,000 × g for 30 min, and the supernatants were used for EMSA. Double-stranded oligonucleotides containing an NF-κB binding site (5'-AGTTGAGGGGACCTTCCAGGGC-3'), an AP-1 binding site (5'-CGTTCTGATGAGTCAACCTGGTCAGTTCCAGGGC-3'), or an IFN-stimulated response element (5'-GGATCCATGCC-TGGGGAAAGGGGAACCTGACTGAGCC-3'), element underlined (4'-32P)ATP by using T4 polynucleotide kinase (Promega, Madison, WI) according to the manufacturer’s instruction and combined and incubated for 30 min at room temperature with 1 μg of nuclear extract in binding buffer (10 mM Tris-HCl (pH 7.5), 4% glycerol, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, and 0.5 mM dithiothreitol). The specificity of the reaction was confirmed by competition with a 50-fold molar excess of nonlabeled oligonucleotides. The precipitated DNA complexes were resolved by PAGE in 0.5 × TBE buffer and visualized by autoradiography. In addition, other nucleic extracts were incubated with anti-NF-κB p50, anti-NF-κB p65, anti-c-Fos, anti-STAT-1, anti-STAT-2, or anti-ISFG-3 antibodies and subsequently with peroxidase-conjugated anti–mouse or anti–rabbit IgG antibody. Immunoreactive proteins were visualized with Western blot chemiluminescence reagents (Amersham Biosciences) following the manufacturer’s instructions.

RESULTS

Dexamethasone Stimulates Osteoclastogenesis from Bone Marrow M/MØ Cells—When bone marrow-derived M/MØ 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 (11). The addition of exogenous Dex increased the number of osteoclastic TRAP-positive MNCs formed in these cultures in the presence of TGF-β in a Dex dose-related manner, with the maximal increase at 10⁻³⁻¹⁻⁰⁻⁸ M (Fig. 1A); whereas the total number of nuclei was not changed (data not shown). However, at a higher concentration of 10⁻⁰⁻¹⁻⁰⁻⁷ M, Dex completely depressed the osteoclast formation. Thus, Dex revealed biphasic effects on osteoclastogenesis, one being stimulatory at lower concentrations (≤10⁻⁰⁸ M) and the other, inhibitory at higher doses (≥10⁻⁰⁷ M). A time-course

Fig. 1. Stimulatory effect of exogenous Dex on formation of osteoclasts from their precursors. A, osteoclast progenitors (M/MØ cells) were isolated after 3-day treatment of bone marrow cells with M-CSF (100 ng/ml), and the isolated M/MØ (2 × 10⁶ cells/well of 48-well plate) were then cultured with various concentrations of Dex in the presence of M-CSF (20 ng/ml) and sRANKL (40 ng/ml) for 5 days (open circles) or in the presence of M-CSF (20 ng/ml), sRANKL (40 ng/ml), and TGF-β1 (2 ng/ml) for 4 days (closed circles). B, isolated M/MØ cells were incubated with (closed symbols) or without (open symbols) Dex (10⁻⁹ M) in the presence of M-CSF and sRANKL (triangles) or M-CSF, sRANKL, and TGF-β (circles) for the indicated times. At the end of the culture period, the cells were stained for TRAP activity, and the number of TRAP-positive MNCs per well was counted. The experiments were performed 4 times, and the reproducibility was confirmed. Values are the means ± S.E. for four cultures in a representative experiment. C-F, photomicrographs of the TRAP-stained cells in cultures. The isolated M/MØ cells were incubated with (E and F) or without (C and D) Dex (10⁻⁷ M) in the presence of M-CSF and sRANKL (C and E) for 5 days or M-CSF, sRANKL, and TGF-β (D and F) for 4 days.
experiment showed that Dex accelerated the osteoclast formation from osteoclast progenitors in both the presence and absence of TGF-β (Fig. 1B). As shown in Fig. 1, C–F, when the M/MØ cells were treated for 4 days with Dex at 10−8 M in the presence of sRANKL, M-CSF, and TGF-β, TRAP-positive MNCs were fully generated in the culture dish. In the absence of TGF-β, Dex also enhanced the formation of osteoclastic TRAP-positive MNCs, but the stimulation was much less than in the presence of TGF-β. We next examined the effect of Dex at a stimulatory dose on mRNA levels of TRAP, cathepsin K, calcitonin receptor, integrins αv and β3, all of which are known to be abundantly expressed in the process of osteoclast differentiation, CD14, a marker molecule of macrophage differentiation, and β-actin. Numbers in parentheses indicate the number of PCR cycles. B, expression of GR in cells treated as described above was determined by RT-PCR and Western blotting (GR-IB). The cDNA was amplified for the number of PCR cycles indicated in parentheses. In both A and B the respective cDNAs were not saturated.

FIG. 2. RT-PCR analysis of the expression of various mRNAs involved in osteoclast differentiation and expression of glucocorticoid receptors. Isolated M/MØ cells were treated with M-CSF (20 ng/ml) + sRANKL (40 ng/ml; MR), M-CSF + TGF-β (2 ng/ml) + sRANKL (MTR), M-CSF + Dex (10−8 M) + sRANKL (MDexR), or M-CSF + TGF-β + Dex + sRANKL (MDexR) for 0, 2, or 4 days. A, primers used were designed for mouse genes of TRAP, cathepsin K, and integrins. B, expression of GR in cells treated as described above was determined by RT-PCR and Western blotting (GR-IB). The cDNA was amplified for the number of PCR cycles indicated in parentheses. In both A and B the respective cDNAs were not saturated.

FIG. 3. Point of Dex action in the process of osteoclast differentiation and the synergistic effect with TGF-β. A, isolated M/MØ cells were treated or not with Dex (10−8 M) for the entire 4 days or for the first 2 days or the last 2 days in the presence of M-CSF (20 ng/ml), TGF-β (2 ng/ml), and sRANKL (40 ng/ml). After the end of the culture period, the cells were stained for TRAP activity, and the number of TRAP-positive MNCs per well was then counted. B, isolated M/MØ cells were pretreated with M-CSF alone (M), M-CSF + TGF-β (MT), M-CSF + Dex (MDex), or M-CSF + TGF-β + Dex (MDex) for 2 days, and thereafter, the cells were further treated for an additional 4 days with sRANKL in the presence of M-CSF (open bars), M-CSF + TGF-β (diagonal bars), M-CSF + Dex (hatched bars), or M-CSF + TGF-β + Dex (closed bars). Then the cells were extracted, and the TRAP activity was measured. The experiments were performed three times, and the reproducibility was confirmed. Values are the means ± S.E. for four cultures in a representative experiment.

in the presence of sRANKL, M-CSF, and TGF-β (Fig. 3A). When the osteoclast progenitors were exposed to Dex for the entire 4 days, the osteoclast formation was significantly increased compared with that in the cultures without Dex. In addition, the Dex-exposure for 0–2 days resulted in osteoclast formation to the same extent as that in the cultures treated with Dex for the entire 4 days. However, when the cells were treated only for the last 2 days with Dex, the stimulation of osteoclast formation was not observed, suggesting that Dex acts on osteoclast progenitors at the early stage in the process of osteoclast differentiation.

In addition, when the isolated progenitors were pretreated only with M-CSF prior to treatment with M-CSF or the combination of M-CSF and TGF-β, or M-CSF, TGF-β, and Dex in the presence of sRANKL, the TRAP activity of the cells induced by sRANKL was stimulated by TGF-β and further enhanced by the combination of TGF-β and Dex (Fig. 3B, the first group including four bars starting at the left). However, the pretreatment with M-CSF and TGF-β caused the equivalent increase in the activity even in the absence TGF-β during the last 4-day treatment with sRANKL, suggesting the TGF-β-stimulated priming of the osteoclast progenitors toward differentiation into osteoclasts as described before (see Ref. 11 and Fig. 3B, the second group of bars from the left). In addition, pretreatment...
with M-CSF, TGF-β, and Dex resulted in the further enhancement of the activities compared with those induced by pretreatment with M-CSF and TGF-β (Fig. 3B, the fourth group from the left). However, with Dex alone in the presence of M-CSF during the pretreatment, those stimulations were less (Fig. 3B, the third group from the left). These obtained results indicate that Dex acts synergistically with TGF-β to enhance the priming of osteoclast progenitors toward osteoclast differentiation in the early stage of osteoclast differentiation.

Dexamethasone Stimulates Activation of NF-κB and AP-1 in Osteoclast Progenitors Synergistically with TGF-β—Activation of NF-κB and AP-1 has been indicated to be required for osteoclastogenesis induced by sRANKL (8–10). Thus, we next examined how Dex is involved in the activation of these transcription factors. We found that Dex up to $10^{-8}$ M in both the presence and absence of TGF-β stimulated the sRANKL-evoked binding of nuclear proteins in osteoclast progenitors to oligonucleotide sequences containing the NF-κB or AP-1 binding site in a dose-related manner and that the stimulation was synergistic with TGF-β (Fig. 4). When the probes and the nuclear extracts were incubated with anti-NF-κB p50 or anti-NF-κB p65 antibodies, or with anti-c-Jun or c-Fos antibodies, the complex was further shifted to a slower mobility, or its labeling intensity was decreased in the gel (Fig. 4). However, the stimulation of the binding decreased at $10^{-7}$ M. These dose-dependent and synergistic effects of Dex are consistent with those seen on osteoclast formation, suggesting a positive linkage between Dex-induced activation of NF-κB and AP-1 and accelerated osteoclastogenesis.

Dexamethasone Does Not Affect the Signaling Pathway of the RANKL/RANK System up to the Translocation of NF-κB and AP-1 into the Nucleus—The above results suggest a possible up-regulation by Dex of signalings from the association of RANKL and RANK up to the nuclear translocation of NF-κB and AP-1. Therefore, we first examined the effect of Dex on the expression of molecules related to the RANK signaling pathway such as RANK, TRAF2, TRAF6, c-Fos, Fra-1, and c-Jun. At the time (day 0) of isolating osteoclast progenitors after a 3-day preculture in M-CSF at a high concentration (100 ng/ml), the mRNAs of RANK, TRAF2, and TRAF6 were already expressed, and the levels of these mRNAs were not changed by the 2-day treatments with the combination of Dex and TGF-β in the presence of M-CSF (data not shown). On the other hand, the constitutive expression of c-fos mRNA in osteoclast progenitors was suppressed by the treatment with the combination of Dex and TGF-β to 15% of the level in the cultures treated only with M-CSF (Fig. 5A), the effect inconsistent with the up-regulation of RANKL-induced AP-1 binding to its cis-element by Dex and/or TGF-β. However, the levels of fra-1 and c-jun mRNA were not altered by Dex and/or TGF-β.

We next examined the influence of Dex on the activation of NF-κB by RANKL in the cytosol of osteoclast progenitors. The level of phosphorylated IκB-α, the degradation of IκB-α, and the phosphorylation rate of NF-κB evoked by sRANKL were not changed by the pretreatment with Dex and/or TGF-β (data not shown). In addition, the combination of Dex and TGF-β altered neither the level of NF-κB p50 and p65 proteins expressed in osteoclast progenitors (data not shown) nor the level of these transcription factors that had been translocated to the nucleus (Fig. 5B). On the other hand, activation of JNK kinase activity elicited by sRANKL was slightly decreased by the pretreatment with Dex and/or TGF-β (Fig. 5C), consistent with the decrease in the constitutive expression of c-fos mRNA (Fig. 5A). These findings indicate that although the combination of Dex and TGF-β synergistically stimulated the DNA binding of NF-κB and AP-1 to their binding consensus oligonucleotide sequences, resulting in stimulation of osteoclast formation, this...
combination did not impact the signaling pathway of the RANKL/RANK system up to the nuclear translocation of NF-κB, but rather depressed the activation of AP-1, suggesting some other mechanism for the stimulation of the DNA binding of these transcription factors and of osteoclastogenesis.

**Dexamethasone Suppresses Expression of Inhibitory Cytokine IFN-β in Osteoclast Progenitors**—We and other investigators (17, 18) demonstrated recently the inhibitory action of endogenous production of type-I IFNs such as IFN-β on osteoclast differentiation. Thus, we suspected that the endogenous expression of IFN-β in osteoclast progenitors might be involved in the stimulation of osteoclastogenesis and the activation of the DNA binding of NF-κB and AP-1. Therefore, we examined the effect of Dex and/or TGF-β on the expression of IFN-β mRNA in osteoclast progenitors. Dex or TGF-β strongly decreased the amount of IFN-β mRNA expressed in the cells, and the combination of these factors further reduced the level (Fig. 6A), whereas the mRNA levels of SOCS-1 and -3, signaling suppressors of IFNs, and those of STAT-1, STAT-2, and ISGF-3γ, were not altered (data not shown), suggesting that the signaling of IFN-β in the osteoclast progenitors was decreased by the action of Dex and/or TGF-β. In fact, when osteoclast progenitors were incubated with Dex and/or TGF-β without adding exogenous IFN-β, the constitutive binding activity of ISGF-3, composed of STAT-1, STAT-2, and ISGF-3γ (determined by a supershift experiment), to oligonucleotides containing IRSE was attenuated by the treatment with these factors alone or in combination (Fig. 6B). In addition, when osteoclast progenitors in the presence of M-CSF, TGF-β, and Dex, under which conditions the endogenous production of IFN-β was less, were exposed to exogenous IFN-β, the DNA binding activity of NF-κB evoked by sRANKL was depressed by the exposure in a time-dependent manner (Fig. 6C). Binding activity of AP-1 was also significantly reduced by the exposure to exogenous IFN-β. These results indicate that Dex and/or TGF-β down-regulates the endogenous production of IFN-β, thereby releasing the cells from the differentiation-inhibiting action of inhibitory cytokines such as IFN-β.

**DISCUSSION**

In this study, we demonstrated that Dex at <10⁻⁸ M enhanced, but at >10⁻⁷ M depressed, RANKL-induced osteoclast formation synergistically with TGF-β by acting at the early stage of osteoclast differentiation when bone marrow-derived osteoclast progenitors are primed toward osteoclasts. Consistent with the enhancement of osteoclastogenesis, Dex with TGF-β additionally stimulated DNA binding of NF-κB and AP-1 elicited by sRANKL to consensus oligonucleotide sequence containing their respective binding sites. However, Dex did not activate the signaling pathways from the RANKL/RANK association to the activation of JNK or the nuclear translocation of NF-κB. On the other hand, Dex significantly decreased the endogenous production of IFN-β, which depressed the DNA binding of NF-κB and AP-1 in osteoclast progenitors, as well as osteoclastogenesis, as we reported previously (17); thereby the osteoclast differentiation could progressively proceed, resulting in an increased number of osteoclasts, as is observed in the early phase of GC-induced bone loss.

With GC treatment for various diseases, the loss of bone is biphasic with a rapid initial phase of ∼12% during the first few months, followed by a slow phase of about 2–5% per year (25). Consequently, the GC-induced bone loss is categorized as a low turnover type of osteoporosis that is defined by impaired recruitment and function of osteoblasts and osteoclasts. The inhibition of osteoblastogenesis and the apoptosis of osteoblasts and osteocytes induced by GC are currently considered to be the primary cause for the bone loss (33, 36, 45, 46). However, the involvement of osteoclasts in the early phase of great bone loss has not satisfactorily been elucidated and is still controversial, as inhibitory (38, 47) and stimulatory (36, 37) effects of GC on osteoclastic differentiation and bone-resorbing activity have been reported. Weinstein et al. (48) demonstrated recently that Dex decreased the number of osteoclast progenitors in bone marrow but showed, inversely, that the total number of osteoclasts on the bone surface was increased by the promotion of osteoclast survival by Dex via the prevention of their apo-
ptosis. However, many in vitro studies using cultures of bone marrow cells, in which osteoclast differentiation-supporting stromal or osteoblastic cells were present, showed the enhancement of osteoclast formation by GCs in the presence of osteotropic factors such as 1,25-dihydroxyvitamin D3 (36, 49), the stimulatory effect being consistent with our findings presented here. In our culture system, however, the stimulation occurred even in the absence of the osteoclast-supporting cells such as osteoblasts. In addition, Dex has been demonstrated in cultures of human and murine osteoblasts to increase the expression of RANKL and M-CSF, which are essential for osteoclastogenesis, and to decrease the production of osteoprotegerin, which is a decoy receptor of RANK that interferes with RANKL/RANK signalings (34, 35, 50, 51). This indirect action of GC via osteoblastic cells is one of the mechanisms for GC-induced osteoclastogenesis. In the present study, we demonstrated the biphasic effects of Dex on osteoclastogenesis. It should be particularly noted that Dex at lower concentrations directly enhanced the osteoclast formation and that the action was restricted to the early stage of the osteoclast differentiation when the priming for the osteoclast phenotype occurs. This restricted action is consistent with earlier results indicating the stimulatory effect of Dex on osteoclast formation in cultures of human monocytes (52). However, Dex did not affect the late stage of osteoclast differentiation and, furthermore, did not prevent the apoptotic death of the formed osteoclastic MNCs (data not shown), as was demonstrated previously (48). Taken together, the available data indicate that the action of Dex depends on the dose, the microenvironment of bone (e.g. composition of cell components), and the cell differentiation stage. However, Dex intrinsically stimulates osteoclastogenesis, resulting in a temporary increase in osteoclast number and bone resorption during GC treatment, until the proliferation and function of osteoblastic cells are fully down-regulated by Dex and subsequently the signals controlling osteoclastogenesis through osteoblasts are no longer generated.

From numerous studies elucidating the mechanisms for anti-inflammatory and anti-proliferative effects of GCs on the immune system, the antagonism between GR and pro-inflammatory transcription factors such as NF-κB and AP-1 has been demonstrated extensively for inhibition of the expression of pro-inflammatory cytokines dependent on the activation of NF-κB and AP-1 (39–42, 53). This inhibition by GCs is exerted by blockade of the transactivation of genes through NF-κB and AP-1 (54). The blockade by Dex is currently viewed as being because of the following possible mechanisms. The first is GC-activated GR binding of NF-κB or AP-1, forming a GR/transcription factor complex that does not bind to DNA (54, 55). In the second one the ligand-activated GR does not bind to DNA but associates with the transcription factor bound at its DNA binding site, leading to inhibition of downstream transcriptional activities (56, 57). The third mechanism states that GC-activated GR, by binding to nuclear coactivators such as cAMP-response element-binding protein-binding protein, competes with the transcription factors for the nuclear coactivators, thereby breaking the link between the transcription factors and the pre-initiation complex with DNA polymerase II (58, 59). However, in this study we demonstrated the up-regulation by Dex (at lower concentrations) of NF-κB and AP-1 binding to their DNA binding site sequences evoked by RANKL. To our knowledge, this is the first report indicating the agonism between GC and these inflammatory transcription factors. However, we observed that the GR did not form a complex with NF-κB and AP-1 in the nuclei of osteoclast progenitors, as judged from the data of EMSA supershift experiments with anti-GR antibody (data not shown), thus suggesting a GR-independent mechanism. In addition, Dex did not affect the signaling cascade transduced from RANKL/RANK such as the IkB kinase activation followed by the nuclear translocation of NF-κB, but rather slightly decreased the JNK activation, in the osteoclast progenitors. These results suggest some other possible mechanism for the stimulation of the DNA binding activity of these transcription factors.

The inhibition of osteoclastogenesis by type-I IFN was reported recently by our group and others (17, 18). Takayanagi et al. (18) showed that the production of this cytokine induced by RANKL was dependent on c-Fos. However, as shown in this study, even in the absence of RANKL signaling, the osteoclast progenitors constitutively expressed IFN-β, and the decreased expression of this cytokine caused by Dex and/or TGF-β paralleled the reduced expression of the constitutive c-Fos. We also found that exogenous IFN-β abrogated the enhancement of DNA binding activity of NF-κB and AP-1 caused by the combination of Dex and TGF-β. Furthermore, the constitutive expression of SOCSs, suppressors of IFN signaling, was not affected by Dex and/or TGF-β, implying that the negative signaling by IFN-β for osteoclastogenesis is negated by the reduced expression of this cytokine. Such a down-regulation of type-I IFN expression by GCs has been demonstrated in cultures of human fibroblasts and peripheral blood mononuclear cells (60, 61), and GCs have been reported more recently (62) to inhibit IFN-γ signaling mainly because of the decreased STAT-1 expression in macrophages dependent on lymphocytes coexisting in peripheral blood mononuclear cells. In addition, similar to GC-induced osteoporosis, mice deficient in one of the IFN-α/β receptor components (IFNAR1/- or - mice) exhibit severe osteopenia resulting from loss of bone mass accompanied by enhanced osteoclastogenesis even without any inflammatory stimuli (18). Taken together, these results indicate that the down-regulation of the IFN-β expression by Dex and/or TGF-β is yet another mechanism for the Dex-enhanced osteoclast formation. However, accumulating evidence has indicated a positive transcriptional synergy between NF-κB and Janus kinase/STAT in mediating IFN signaling in a variety of cells (63–65). Our findings presented here cannot account for such a positive interaction between these transcription factors, and the precise mechanism at the molecular level remains to be clarified.

In conclusion, although Dex did not affect the nuclear translocation of NF-κB or the activation of JNK induced by RANKL, the steroid in collaboration with TGF-β enhanced the DNA binding activities of these transcription factors and subsequently accelerated the formation of osteoclasts. The stimulation by Dex of osteoclastogenesis is at least associated with the down-regulation of production of IFN-β, allowing osteoclast progenitors to be freed from the differentiation-depressing effect of this inhibitory cytokine and to proceed toward the phenotype of mature osteoclasts. Our present results provide a novel cellular mechanism for rapid and severe bone loss observed at the early phase of GC-induced osteoporosis caused by long-term therapy of GCs.

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