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Soluble glucocorticoid-induced tumor necrosis factor receptor stimulates osteoclastogenesis by down-regulation of osteoprotegerin in bone marrow stromal cells

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Abstract

Soluble glucocorticoid-induced tumor necrosis factor receptor (sGITR) is a potent stimulator of osteoclastogenesis. The mechanism by which it induces osteoclastogenesis was studied by culturing bone-marrow-derived macrophages (BMM) with conditioned medium from mouse bone marrow stromal cells. GITR and GITR ligand (GITRL) were expressed on the surface of bone marrow stromal cells, and sGITR-induced osteoclastogenesis was inhibited by anti-GITRL Ab, indicating that stimulatory effect of osteoclastogenesis by sGITR involved signaling via GITRL. Bone marrow stromal cells up-regulated cyclooxygenase-2 (COX-2) and produced prostaglandin E_2 (PGE_2) early in their response to sGITR, and the stimulation of osteoclastogenesis was markedly inhibited by NS398, a COX-2 inhibitor. Later, sGITR markedly reduced the steady-state level of osteoprotegerin (OPG) mRNA and increased receptor activator of nuclear factor-κB ligand (RANKL) mRNA. NS398 blocked the sGITR-induced reduction of OPG mRNA but did not significantly affect the sGITR-induced rise in RANKL mRNA. This suggests that down-regulation of OPG by PGE_2 is involved in sGITR-induced osteoclast (OC) formation in the presence of conditioned medium from mouse bone marrow stromal cells.

Keywords: sGITR; GITRL; Osteoclastogenesis; Bone marrow stromal cells; OPG

Introduction

Bone metabolism is regulated by a balance between bone resorption by osteoclasts (OC) and bone formation by osteoblasts. OCs are multinucleated giant cells that originate from hematopoietic stem cells of the macrophage/monocyte lineage. Bone marrow stromal cells or osteoblasts also produce essential factors for osteoclastogenesis. Osteoclastogenesis is dependent upon exposure to receptor activator of nuclear factor-κB ligand (RANKL), a transmembrane glycoprotein expressed on the surface of stromal cells in bone [1], and is regulated by this factor. OC precursor cells that express receptor activator of nuclear factor-κB (RANK), a member of the tumor necrosis factor receptor (TNFR) super-family, interact with stromal cells and differentiate into mature OCs in the presence of macrophage-colony stimulating factor (M-CSF), which promotes their survival and proliferation. The stromal cells also secrete osteoprotegerin (OPG), a decoy receptor that inhibits OC formation by blocking the interaction between RANKL and RANK [2]. OPG-deficient mice develop severe osteoporosis and undergo increased osteoclastogenesis, resulting in lack of trabecular bone [3,4]. These results show that OPG is an important physiological regulator of OC formation. OCs are generated in response to bone-resorbing factors that regulate the expression of RANKL and OPG by stromal cells. There is an increase in RANKL mRNA, and a decrease in OPG mRNA, when osteoblasts or stromal cells are treated with parathyroid hormone or prostaglandin E_2 (PGE_2) [5–7].

Glucocorticoid-induced tumor necrosis factor receptor (GITR), a member of the TNFR family, was cloned from glucocorticoid-induced murine T cells [8], and its human form was cloned later using an EST data base [9]. The expression and function of GITR have not been studied extensively. GITR and its ligand are expressed in macrophages [10,11] and regulatory T

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cells [12], and it has been proposed that GITR is involved in the control of regulatory T cells [12,13] and in macrophage activation [10,14–16] associated with inflammatory processes [17]. The N-terminal domain of GITR shares a motif with some members of the TNFR family [8]. Although there is generally little homology between the cytoplasmic domains of TNFR family members themselves, the cytoplasmic domain of GITR has homology with those of 4-1BB, CD27, and OX40 [9,18].

We have examined the mechanism by which OC formation is stimulated by incubating BMM with conditioned medium from bone marrow stromal cells stimulated by sGITR. GITR ligand (GITRL) is expressed on the surface of bone marrow stromal cells, and stimulation by sGITR occurs via an action on the GITRL of bone marrow stromal cells.

Materials and methods

Cells and reagents

ST2, a murine bone marrow stromal cell line, was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in 6-well plates or in 10 cm dishes. Mouse stromal cells were isolated by the method of Tropel et al. [19]. Bone marrow was isolated from 4-week-old C57BL/6J mice by flushing their femurs and tibias with α-MEM, 10% fetal bovine serum (FBS), and penicillin/streptomycin. The cells were washed and plated at a density of 2×10^6 cells/cm^2. After 3 days, non-adherent cells were removed by several washes, and adherent cells further cultured in complete medium for 4 days. The adherent cells were retrieved by trypsinization and immuno-depleted using biotinylated antibody (Ab) against CD11b and streptavidin-coated micro-beads (Miltenyl Biotech.), according to the manufacturer's instructions. The CD11b-negative cells were subsequently seeded on fibronectin-coated Petri dishes in complete medium supplemented with fibroblast growth factor (10 ng/ml) (R&D Inc., Minneapolis, MN). Fibroblastoid cells were cultured in complete medium without growth factor and used as primary bone marrow stromal cells. Soluble GITR, murine GITR-Fc fusion protein expressed in a human embryonic kidney cell line (HEK 293), anti-GITR Ab, and anti-GITRL Ab were purchased from KOMED (Seoul, Korea). Anti-OPG Ab for neutralization and biotinylated anti-OPG Ab were from R&D Inc.

Assay of in vitro osteoclastogenesis

Bone marrow cells were isolated from 4-week-old C57BL/6J mice as described [20]; the bone ends were cut, and the marrow cavity was flushed out from one end of the bone with α-MEM using a sterile 21-gauge needle. The resulting bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain single cells. These were washed twice and resuspended in α-MEM containing 10% FBS, and the suspension was incubated in plates together with M-CSF (100 ng/ml) for 16 h. The non-adherent cells were then harvested and cultured for 2 more days, by which time large numbers of adherent monocyte/macrophage-like cells had formed on the bottom of the culture dishes. A few non-adherent cells and adherent stromal cells were removed by rinsing the dishes with PBS and by subsequent incubation for 5 min in 0.25% trypsin/0.05% EDTA, respectively. The adherent BMM were harvested by vigorous pipetting and confirmed by FACS analysis to be positive for CD11b and F4/80 and negative for CD3 and CD45R (data not shown). The absence of contaminating stromal cells was established by the lack of growth when M-CSF was omitted (data not shown). The isolated BMM were seeded at a density of 3×10^4 cells/well in 48-well plates in 100 μl of medium containing M-CSF (20 ng/ml) and RANKL (40 ng/ml), and a further 100 μl of conditioned medium harvested from a culture of bone marrow stromal cells was added. To remove the low MW fraction (below 10,000 Da), the conditioned medium from ST2 or primary bone marrow cells was filtered through a PM10 membrane. The high MW fraction was resuspended in PBS, incubated with goat IgG or anti-OPG Ab in the presence or absence of anti-GITR Ab, and then added to the BMM cells. After 3 days, the cells were fixed in 10% formalin for 10 min and stained for tartrate-resistant acid phosphatase (TRAP) as described [20]. TRAP-positive multinucleated cells (MNC) containing three or more nuclei were scored.

Flow cytometry

Samples of 10^6 cells were incubated on ice in PFS buffer (phosphate-based saline, 2.5% FBS, and 0.1% sodium azide) with anti-GITR Ab or anti-GITRL Ab (rat IgG: isotype control) for 30 min to detect GITR and GITRL respectively. The cells were washed three times with PFS, incubated on ice for 30 min with fluorescence isothiocyanate (FITC)-conjugated goat anti-rat IgG, washed as above, and analyzed by flow cytometry with a FACS Calibur (Becton Dickinson).

Isolation of RNA and RT-PCR

Expression of GITR, GITRL, cyclooxygenase (COX)-2, RANKL, OPG, and GAPDH mRNA was assessed by RT-PCR analysis. RNA was isolated from murine primary bone marrow stromal cells and ST2 cells using TRI reagent (Sigma Chemical Co.). The total RNA was used for cDNA synthesis by reverse transcriptase with a cDNA synthesis kit (Invitrogen, San Diego, CA). The cDNAs were amplified by PCR for 35 cycles (GITRL, RANKL), 30 cycles (GITR), 26 cycles (OPG, COX-2), and 25 cycles (GAPDH), with the following specific PCR primers: GITR, 5′-GCATATGTGTCACACCTGAGTACC-3′ (forward) and 5′-CTGGACTGTGGTTAGGAAGAAAAT-3′ (reverse); GITRL, 5′-CAAGTCCT-CAAAGGGCAGAG-3′ (forward) and 5′-AGCTTCCCATCAGATGTCGT-3′ (reverse); mouse RANKL, 5′-CAGCACTCACTGCTTTTATAGAATCC-3′ (forward) and 5′-AGCTGAAGATAGTCTGTAGGTACGC-3′ (reverse); mouse OPG 5′-ATGCAACACATGACAACGTG-3′ (forward) and 5′-GGAACCT-CATGGTCTTCCTC-3′ (reverse); mouse COX-2, 5′-TCAGCCAGGCAG-CAAATCCTTG-3′ (forward) and 5′-TAGTCTCTCCTATGAGTATGAGTC-3′ (reverse); and mouse GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). Each cycle consisted of 30 s of de-naturation at 94°C, 30 s of annealing at 60°C, and 30 s of extension at 72°C. GAPDH was used as an internal control. The sizes of the PCR products for mouse GITR, GITRL, RANKL, OPG, COX-2, and GAPDH were 338, 242, 464, 534, 943, and 452 bp, respectively.

PGE2 production

PGE_2 levels in the supernatants of ST2 cells stimulated with sGITR were measured with an enzyme immunoassay kit from Amersham Pharmacia Biotech (Piscataway, NJ).

Statistical analysis

All values are expressed as means ± SEM. Student's t test was used to evaluate differences between samples of interest and their respective controls. Multiple measurement ANOVA was used to analyze the anti-OPG Ab-treated groups. P values of less than 0.05 were considered statistically significant.

Results

Soluble GITR transmits a signal through GITRL in bone marrow stromal cells

We showed previously that sGITR stimulates osteoclastogenesis by acting directly on OC precursors via a signal through GITRL [21]. We found that sGITR could stimulate RANKL-induced OC formation not only in OC precursors but also in whole bone marrow cells. The stimulatory effect of sGITR was higher in whole bone marrow cells than in OC precursors, indicating that stromal cells present in bone marrow could be