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Soluble glucocorticoid-induced tumor necrosis factor receptor (sGITR) stimulates osteoclast differentiation in response to receptor activator of NF-κB ligand (RANKL) in osteoclast cells

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Abstract

We found that treatment of osteoclast (OC) precursors with soluble glucocorticoid-induced tumor necrosis factor receptor (sGITR) promoted osteoclastogenesis in the presence of macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor-κB ligand (RANKL). Low levels of GITR and its ligand were expressed on the surface of OC precursor cells after incubation with RANKL. Stimulation of osteoclastogenesis by sGITR was blocked by neutralization with anti-GITR ligand antibody (Ab), whereas endogenous GITR did not affect osteoclastogenesis, indicating that enhancement of osteoclastogenesis by sGITR involves signaling via GITR ligand. The addition of sGITR decreased the level of interferon (IFN)-β, and blockade of endogenous IFN-β did not affect osteoclastogenesis stimulated by sGITR. We conclude that sGITR enhances osteoclastogenesis by acting on OC precursor cells to lower the level of IFN-β.

Keywords: GITR; GITR ligand; IFN-β; Osteoclast; Osteoclastogenesis

Introduction

Osteoclasts (OCs), which derive from hematopoietic stem cells, have an important role in remodeling physiological bone and also participate in the bone destruction associated with chronic inflammatory disease [1]. Osteoclastogenesis requires two molecules produced by bone-marrow mesenchymal cells, macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor-κB (NF-κB) ligand (RANKL) [2]. Binding of M-CSF to its receptor on OC precursors generates signals for their survival and proliferation. RANKL, a member of the tumor necrosis factor (TNF) cytokine super-family, exists in both soluble and membrane-bound form, and is crucial for the differentiation and fusion of precursors into mature OCs. Most factors known to stimulate OC formation, such as TNF-α, interleukin (IL)-1, IL-6, and other protein mediators, as well as non-protein mediators such as prostaglandins, neurotransmitters, and nitric oxide (NO), are all associated with the local inflammatory response and eventual tissue destruction. They interact with stromal cells/osteoblast cells rather than with OC progenitors and induce the release of OC-stimulating factors; however, this is controversial [3].

Glucocorticoid-induced tumor necrosis factor receptor (GITR), a member of the tumor necrosis factor receptor (TNFR) family, was cloned following induction of murine T cells by dexamethasone [4], but as yet little is known of its expression and function. It is present in macrophages [5] and regulatory T cells [6], and there are indications that it is involved in the control of regulatory T cells [6,7] and in the activation of macrophages [5,8–10] associated with inflammatory processes [11]. GITR shares a common motif with the TNFR family in its N-terminal extra-cellular domain [4]. Its cytoplasmic domain has homology with those of 4-1BB, CD27, and OX-40 [12–14], pointing to the existence of a new subfamily within the TNFR super-family. The promoter

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region of GITR contains several binding sites responsible for T-cell activation [15]. Recently, GITR ligand was cloned, and its interaction with GITR was found to inhibit suppression by regulatory T cells. It was also expressed in dendritic cells, macrophages, and B cells, although at a low level [16,17].

In this report, we show that GITR ligand is expressed on the surface of OCs after they are incubated with RANKL, and that soluble GITR (sGITR) enhances osteoclastogenesis via an action on GITR ligand on OC precursor cells. We also show that the stimulatory effect of sGITR on osteoclastogenesis is at least, in part, attributed to the decrease in the level of interferon (IFN)-β by sGITR.

Materials and methods

Osteoclast formation

Bone marrow cells were isolated from 4- to 6-week-old C57BL/6N mice. Femora and tibiae were removed aseptically and dissected free of adherent soft tissue. The bone ends were cut, and the marrow cavity was flushed out with α-MEM from one end of the bone with a sterile 21-gauge needle. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain a single-cell suspension, and the cells were washed twice, re-suspended in α-MEM containing 10% fetal bovine serum (FBS), and plated at 3×10^5 cells/well. Additional medium containing M-CSF (100 ng/ml) (R&D Systems, MN) and RANKL (100 ng/ml) (R&D Systems), with or without various concentrations of soluble GITR, GITR-Fc (KOMED, Seoul, Korea), was added, and the medium was replaced on day 3. As a negative control, we used human CD30-Fc protein (R&D Systems). The cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). We scored the number of TRAP-positive multinucleated cells (MNC) containing three or more nuclei.

OC precursor cells free of stromal cells were prepared from the bone marrow cells as follows: the bone marrow cells were incubated for 24 h in α-MEM containing 10% FBS in the presence of M-CSF (100 ng/ml), and non-adherent cells were harvested and layered on a Ficoll-Hypaque gradient (Sigma, St. Louis, MO); the cells at the interface were collected, washed, and re-suspended in α-MEM containing 10% FBS. They were then plated in 48-well plates (1.5×10^5 cells/well). The absence of contaminating stromal cells was confirmed by culturing aliquots in the absence of M-CSF and observing absence of growth (data not shown). To exclude the possible presence of lymphocytes in the cultures, OC precursor cells free of stromal cells were incubated in culture plates; after 2 h, the culture plates were washed three times with PBS to remove non-adherent and loosely adherent cells. This procedure depleted the cultures of lymphocytes [18]. The adherent cells were analyzed by FACS and found to be negative for CD3 and CD45R, and positive for CD11b and F4/80 (data not shown); they were then incubated with M-CSF and RANKL with or without sGITR. The following antibodies were used: anti-GITR antibody (Ab) (KOMED), anti-GITR ligand Ab (KOMED), and anti-IFN-β Ab (PBL Biomedical, Rockford, IL).

OC formation was evaluated by quantifying TRAP-positive MNCs. Murine OC cells contain a high concentration of TRAP, which serves as an OC marker [19]. After incubation, the cells were washed in PBS, fixed in 10% formalin for 10 min, and stained for acid phosphatase (Sigma Diagnostics Acid Phosphatase Kit, 387-A) according to the manufacturer's instructions. TRAP-positive MNCs were counted with a light microscope. OC was further characterized by assessing their ability to form resorption pits on dentine slices, as described previously [20]. OC cells were generated with M-CSF and RANKL for 5 days. Then, after treatment with trypsin/EDTA, the cells were harvested. The cells obtained (2000 cells) were seeded on dentine slices and incubated for 1 day with M-CSF and RANKL in the presence or absence of sGITR (2 ng/ml). The cells on some dentine slices were stained for TRAP activity. The remaining slices were cleaned by ultrasonication in 1 M NH_4OH to remove adherent cells and stained with Mayer's hematoxylin (Sigma) according to the manufacturer's directions to visualize resorption pits.

Flow cytometry

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

RNA isolation and RT-PCR

Expression of GITR, GITR ligand, RANK, IFN-β, and GAPDH mRNA was assessed by RT-PCR analysis. RNA was isolated with TRI reagent (Sigma) from cells incubated with M-CSF and RANKL in the presence or absence of sGITR. Total RNA was used for cDNA synthesis using the reverse transcriptase supplied with the cDNA synthesis kit (Invitrogen, San Diego, CA). cDNAs were amplified by PCR for 35 cycles (GITR ligand), 30 cycles (GITR, IFN-β, and RANK), and 25 cycles (GAPDH) with the following specific PCR primers: GITR, 5′-GCATATGTGTCACACCTGAGTACC-3′ (forward) and 5′-CTGGACTGTGGTTAGGAAGAAAAT-3′ (reverse); GITR ligand, 5′-CAAGTCCTCAAAGGGCAGAG-3′ (forward) and 5′-AGCTTCCCATCAGATGTCGT-3′ (reverse); mouse RANK, 5′-CTTCGACTGGTTCACTGCTCCTAAT-