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A role for the immunomodulatory molecules CD200 and CD200R in regulating bone formation

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Abstract

Altered osteoprotogerin (OPG) and OPG ligand (RANKL) ratios are known to regulate bone metabolism. We investigated whether CD200:CD200R interaction would alter OPG:RANKL ratios, and thus modulate bone differentiation in cultures derived from neonatal calvariae, a source of osteoblast precursors (OBp), or bone marrow-derived myeloid cells as a source of osteoclast precursors (OCp). We characterized cells in cultures using real-time PCR to measure expression of a number of mRNAs characteristic of cells differentiating towards the osteoblast or osteoclast lineage, and enumerated bone nodule formation and osteoclasts directly. CD200Fc or anti-CD200 mAbs were included as modulating agents. In addition, calvariae from transgenic mice overexpressing CD200 under control of a doxycycline-inducible promoter were used as a source of OBp endogenously overexpressing CD200. Our data show that increased endogenous expression of CD200 on OBp, or addition of CD200Fc into cultures, led to increased OPG:RANKL ratios and increased bone nodule growth, while anti-CD200 abolished this effect.

Keywords: Bone development; CD200; Cytokines; CD200R

1. Introduction

The receptor activator of nuclear factor-kappaB ligand (RANKL) is a transmembrane ligand expressed in osteoblasts and bone marrow stromal cells and produced by T cells [1–4]. Following binding to RANK, a receptor vital for osteoclast (OC) differentiation, activation and survival, RANKL induces osteoclastogenesis in a pathway which synergizes with signals derived from M-CSF (CD98) [5,6]. OPG, also produced by osteoblasts (OB) and marrow stromal cells, lacks a transmembrane domain and acts as a decoy receptor for RANKL, thus regulating bone metabolism [7]. The crucial role played by RANKL/OPG in regulating bone metabolism is supported by the findings of extremes of skeletal phenotypes (osteoporosis versus osteopetrosis) in mice with altered expression of these molecules [6]; by reports that polymorphisms in the osteoprotegerin gene in human are associated with osteoporotic fractures [8]; and by increased osteolysis in humans in association with elevated RANKL:OPG ratios [9]. Secretion of OPG and RANKL from OB and stromal cells is regulated by numerous hormones and cytokines, often in a reciprocal manner [10].

Inflammatory (and anti-inflammatory) cytokines also regulate bone development in a complex fashion. TNFα cooperates with RANKL in the generation of OC [11], and inhibits OB differentiation and osteocalcin/bone nodule formation [12] at a stage downstream of signals provided by insulin-like growth factor I (IGF-I) or the osteogenic bone morphogenic proteins (BMPs-2, -4, and -6). TGFβ1 inhibits BMP-2 induced OB development [13], and particularly at low $TGF\beta_1$ concentrations, increases RANKL:OPG ratios [14]. Other proinflammatory cytokines (including IL-1 and -6) modulate bone turnover [15,16]. The interaction between cytokines and OPG, RANK, and RANKL in regulating bone growth/loss, particularly in respect to malignancy, was recently reviewed by Steeve et al. [17].

Abbreviations: MLC, mixed leukocyte culture; RANKL, ligand for osteoprotegerin (also called OPGL); OBp, osteoblast progenitor cells (from 7-day cultures of calvariae); BMc, bone marrow-derived myeloid cells; vitD3, 1alpha 25(OH)2 vitamin D-3

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A study by Kim et al. [18] identified and characterized a novel OC-associated receptor (OSCAR) as a member of the leukocyte receptor complex (LRC)-encoded family expressed specifically in OCs. OSCAR expression was detected in preosteoclasts or mature OC, while its ligand (OSCAR-L) was expressed primarily in OB. A soluble form of OSCAR added to OB inhibited the formation of OCs from bone marrow precursor cells in the presence of bone-resorbing factors, leading this group to conclude that OSCAR was another important bone-specific regulator of OC differentiation.

Our laboratory has reported at length on the immunomodulatory effects induced by interactions of the novel receptor:ligand pair, CD200:CD200R, known to be expressed by cells of the monocyte/myeloid lineage [19–21]. CD200 itself is ubiquitously expressed, while the multiple isoforms of the receptor, CD200R, which bind CD200 [21], show a more tissue-restricted expression. We have already documented that activation of CD200R1, expressing ITIM motifs in the intracellular cytoplasmic domain, can directly suppress release of inflammatory cytokines from activated macrophages [19–21]. Since these same cytokines regulate bone metabolism, we anticipated that CD200:CD200R interactions would regulate bone metabolism indirectly. However, we also now report studies investigating evidence for a direct effect of CD200:CD200R interactions on bone metabolism in vitro. These studies used cultured bone marrow-derived myeloid cells as a source of OC precursors, OCp and cultured primary mouse calvarial cells as a source of OB precursors (OBp). Manipulation of bone development used a soluble form of CD200 (CD200Fc) or anti-CD200 monoclonal antibodies [19], or cells from transgenic mice overexpressing CD200 in the presence of doxycycline [22]. We show that enhanced expression of CD200 suppresses osteoclast development, and is permissive for continued osteoblastogenesis.

2. Materials and methods

2.1. Mice

Breeding pairs of male and female C57BL/6 mice were purchased from the Jackson Laboratories, Bar Harbour, Maine. Newborn mice (<24 h post-birth) were used as donors of calvariae. Transgenic mice expressing CD200 under control of a doxycycline promoter were produced as described elsewhere [22].

2.2. Cells and cell cultures

RAW264.7 cells were obtained from the American Type Culture Collection (ATCC). Primary calvarial cells were isolated from a pool of calvariae from newborn mice, and adult bone marrow from a pool of five mice. Tissue was digested with collagenase and dispase (Sigma, Mississauga, Ont., Canada) for 45 min at 37 °C, washed with α-MEM x2, and cultured in α-MEM medium with 10% fetal calf serum and 1×10^{-8} M dexamethasone (DEX) for 7 days. Cultured calvariae or bone marrow were used as sources of OBp or OCp, respectively. Following trypsinization of cultures cells were stained for FACS analysis with PE-conjugated antibodies detecting F4/80, DEC205, MAC-1, MAC-3, CD45, and Sca-1, or with rat monoclonal antibodies specific for mouse CD200, CD200R1 or CD200R2, followed by FITC–anti-rat Ig [27]. CD200 transgenic mice received doxycycline (DOX, 1mg/ml) in the drinking water for 7 days before sacrifice, and cultures were maintained in the presence of 3 μg/ml DOX.

OCp (1.5×10^3) or OBp were recultured in osteologic slides (Millenium Biologix, Kingston, Canada). Culture medium at this stage contained DEX, ascorbate (75 μg/ml) and 3 ng/ml mouse M-CSF (Sigma). In some experiments cells were incubated in the presence of exogenous CD200Fc (2 μg/ml) or rat IgG2a anti-mouse CD200 (5 μg/ml). In these studies cultures with normal rat IgG2a were used as controls. The mouse CD200Fc was prepared using an IgG2a Fc construct [19], while the rat anti-mouse CD200 is of IgG2a isotype (unpublished). Seventy percent of medium replacement was performed at 3-day intervals. Bone nodules present after a total of 17 days of culture were readily mineralized following addition of β-glycerol phosphate (10 mM) for the remaining 7 days of culture, with enumeration by von Kossa staining under 30× magnification [23]. OB and OC were evaluated in cultures based on staining for alkaline phosphatase activity using Napthol-AS-MX Phosphate and FAST RED TR (Sigma) in 100 ml of 1 M Tris–HCl and 0.1 M $MgCl_2$ [24], and TRAP staining (below), respectively.

In some studies culture supernatants of cells were assayed for cytokine protein analysis by ELISA, and mRNA was harvested from cells in TRizol solution for real-time PCR analysis.

2.3. Real-time PCR

Primer pairs were designed in all cases to detect ∼100 bp amplicons for the genes of interest (see below: RpL13A, ribosomal protein L13a; CypA, cyclophlinA (peptidylprolyl isomerase A)). Gene expression in real-time PCR was normalized to a composite of the geometric mean expression of the three housekeeping genes (GAPDH, RpL13a, and CypA), to account for the >100-fold variability in expression even in housekeeping genes (see figures) [25].

OPG	F	GCT GAG TGT TTT GGT GGA CAG TT
OPG	R	GCT GGA AGG TTT GCT CTT GTG
RANKL	F	CAT CGG GAA GCG TAC CTA CAG
RANKL	R	GCT CCC TCC TTT CAT CAG GTT
RANK	F	TCT GCA GCT CTT CCA TGA CAC T
RANK	R	CGA TGA GAC TGG GCA GGT AAG
TRAP	F	TGC ACA GAT TGC ATA CTC TAA GAT CTC
TRAP	R	TGG CCA CAG TTA TGT TTG TAC GT
BSP	F	AGG CAG AGA ACG CCA CAC TTT
BSP	R	TTG GAG TGC CGC TAA CTC AAA
Cbfa1	F	AGGCTCTGGCGTTTAAATGGT
Cbfa1	R	GTGCCCTCTGTTGTAAATACTGCTT
GAPDH	F	TGC CAA GTA TGA TGA CAT CAA GAA G
GAPDH	R	TGA AGT CGC AGG AGA CAA CCT
CypA	F	TCG AGC TCT GAG CAC TGG AG
CypA	R	GTA AAG TCA CCA CCC TGG CAC
RpL13a	F	TCC CTC CAC CCT ATG ACA AGA
RpL13a	R	GCC CCA GGT AAG CAA ACT TTC
CD200	F	GGG CAT AGC AGC AGT AGC G
CD200	R	TGT GTG CAG CGC CTT TCT T