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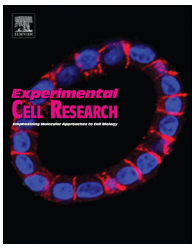

Research Article

Tumor necrosis factor stimulates osteoclastogenesis from human bone marrow cells under hypoxic conditions

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ABSTRACT

Bone homeostasis is maintained by the balance between osteoblastic bone formation and osteoclastic bone resorption. In this study, we used human bone marrow cells (BMCs) to investigate the role of hypoxic exposure on human osteoclast (OC) formation in the presence of tumor necrosis factor (TNF). Exposing the BMCs to 3%, 5%, or 10% O_2 in the presence of receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) generated tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells, consistent with OCs. The addition of TNF under hypoxic conditions generated significantly greater numbers of mature OCs with more nuclei than OCs generated under normoxic conditions. Longer initial hypoxic exposure increased the number of OC precursor cells and facilitated the differentiation of OC precursor cells into multinucleated OCs. Quantitative RT-PCR analysis revealed that RANKL and TNFR1 were expressed at higher levels in non-OC cells from BMCs under hypoxic conditions than under normoxic conditions. Furthermore, to confirm the involvement of TNF-induced signaling, we examined the effects of blocking antibodies against TNFR1 and TNFR2 on OC formation under hypoxic conditions. The TNFR1 antibody was observed to significantly suppress OC formation. These results suggest that hypoxic exposure plays an important role in TNF-induced osteoclastogenesis from human BMCs.

Introduction

Bone homeostasis is maintained by the balance between osteoblastic bone formation and osteoclastic bone resorption [1–3]. Osteoclasts (OCs) are mature bone-resorbing multinucleated cells that differentiate from monocyte/macrophage lineage cells under the tight regulation of osteoblast cells in the local bone environment [1,4–6]. Multinucleated OCs are characterized by the presence of tartrate-resistant acid phosphatase (TRAP) activity, expression of vitronectin receptors (VNR), and pit-forming activity

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on bone. They are formed in co-cultures of mouse osteoblasts and hematopoietic cells in the presence of osteotrophic factors such as 1–25-dihydroxyvitamin D3 [1,25$(OH)_2$D3], parathyroid hormone (PTH), and interleukin (IL)-11 [6–8]. Osteoblasts express receptor activator of NF-κB (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which are essential cytokines for OC differentiation [9,10]. M-CSF is constitutively expressed by osteoblasts, whereas RANKL expression is up-regulated by osteotrophic factors. OC precursors express RANK and the M-CSF receptor (CSF-1R), and differentiate into mature OCs in the presence of both cytokines [1,4,6]. Thus, osteoblast-lineage cells provide a suitable microenvironment for osteoclastogenesis [11].

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that stimulates bone resorption and is expressed in abundance at sites of inflammation and in the bone microenvironment [12]. A variety of cell types express TNF protein including monocytes/macrophages, OC precursors, and mature OCs themselves [13,14]. Merkel et al. demonstrated that TNF mediates the orthopedic osteolysis induced by implant-derived particles [15]. TNF also plays important roles in the bone loss associated with osteoporosis and periodontitis [16,17]. Several biological responses are induced by TNF acting via the TNFR1 and TNFR2 cell-surface receptors, which are also termed TNFR p55 and TNFR p75, respectively [18–20]. Both TNFR1 and TNFR2 initiate intracellular signals that stimulate the proteolytic breakdown of IκB, which is a cytoplasmic inhibitor of NF-κB protein [21,22]. The activated NF-κB then translocates from the cytoplasm to the nucleus, where it induces the transcription of several TNF-responsive genes. In addition, the binding of TNF to TNFR1 triggers programmed cell death in many cells. This process depends upon the presence of the "death domain" located in the cytoplasmic region of TNFR1, but not in TNFR2 [23,24]. Murine TNF binds to both murine TNFR1 and TNFR2 with high affinity, whereas human TNF binds to murine TNFR1 with higher affinity than to murine TNFR2 [18,19].

Hypoxia is a feature of skeletal conditions including rheumatoid arthritis [25], pathological fracture [26], primary bones tumors [27], and cancer metastases to bone [28], periodontitis and orthodontic tooth movement [29,30]. Measurement of oxygen partial pressure (pO_2) in bone marrow aspirates from normal volunteer donors yield a mean value of 6.6% [31]. The pO_2 is often considerably lower in environments such as inflamed tissues, infected tissues, tumors, wounds, fracture sites or sites of orthodontic tooth movement [32], Hypoxia stimulates both the formation and activation of OCs from feline [33], murine [28,34], and human [35,36] monocytes. In addition, we previously demonstrated that hypoxic stress enhances OC differentiation from murine bone marrow cells (BMCs) [37].

In the present study, we investigated human OC formation from human BMCs using a hypoxic culture system to confirm the effect of hypoxia on OC formation we observed using mouse BMCs. In addition, we examined the role of the pro-inflammatory cytokine TNF in human OC differentiation under relative hypoxic conditions. We also explored the contribution of hypoxia to TNF-induced osteoclastogenesis using human BMCs.

Materials and methods

Reagents

The following reagents were used: recombinant human (rh) M-CSF, TNF, neutralizing mouse anti-human TNF receptor 1 (TNFR1; Mab225) monoclonal antibody and anti-human TNF receptor 2 (TNFR2; Mab226) monoclonal antibody (R&D Systems, Minneapolis, MN, USA); and rh soluble RANKL (Sigma, St Louis, MO, USA).

Human OC culture

BMCs were obtained from approximately 40 patients who underwent total hip arthroplasty for osteoarthritis and the study was approved by the Ethic Committee of Nagoya City University. A specimen of 15 mL of bone marrow was aspirated from each patient during rasping of the femoral canal prior to implant placement. All of the subjects gave their written informed consent before participating in the study. BMCs were isolated by density gradient centrifugation with Lymphosepar (Ficoll-Conray solution, Immuno-Biological Laboratories, Fujioka, Japan) at 1800 rpm for 30 min at room temperature. Nucleated cells were collected from the interface between the plasma and the Lymphosepar solution. The cells were washed with phosphate-buffered saline (PBS), and resuspended in Stem Pro 34 medium, which uses serum replacement without fetal bovine serum (FBS) (Invitrogen Corp., NY, USA).

In vitro assay of TRAP-positive OC formation

In vitro osteoclastogenesis was assayed by seeding cells in three replicate wells of a 24-well plate at a density of 2×10^6 cells per well and incubating for 21 days in Stem Pro 34 containing RANKL (100 ng/mL) and M-CSF (50 ng/mL). Cultures were treated with TNF (10 ng/mL) at the indicated period of time in the presence of RANKL and M-CSF from day 0. Cells incubated under hypoxic conditions were cultured in a controlled atmosphere culture chamber (Bellco Glass, Vineland, NJ, USA). This airtight apparatus had a humidified atmosphere with inflow and outflow control valves. The apparatus was flushed for 15 min prior to incubating the cells with gas mixtures containing 5% CO_2 and 10%, 5% or 1% O_2 and N_2 as the balance of the atmosphere. The apparatus chamber was sealed to maintain the gas composition and was incubated at 37 °C. The medium was changed every 3 d by replacing 0.5 mL of used culture medium with an equal quantity of fresh medium containing the appropriate cytokines. After culturing for 21 d, cells were fixed and stained for TRAP as described previously [37,38]. The number of multinucleated TRAP-positive cells was counted using an Olympus inverted microscope at 40× magnification. Cells with three to 10 nuclei were counted as TRAP-positive small cells and cells with more than 10 nuclei were counted as TRAP-positive large cells.

Cell imaging analysis

OC differentiation was assessed using TRAP activity staining. BMCs were seeded in replicates of three wells in 24 well plates at a density of 2×10^6 cells per well and were cultured under normoxic or hypoxic (5% O_2) conditions for various periods of hypoxia. After 21 d in culture the cells were fixed and stained for TRAP activity. The nuclei were stained with Hoechst 33258 (Wako Pure Chemical Industries, Osaka, Japan) to obtain a total nuclear count. High-content cellular images were acquired at 20× magnification with an IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, UK). Small or large TRAP-positive cells were defined as described above. Otherwise, cells with a cell area less than 2000 μm^2 were counted as TRAP-positive small area cells and cells with a cell area more than 2000 μm^2 were counted as TRAP-positive large area cells.