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Megakaryocytes modulate osteoblast synthesis of type-l collagen, osteoprotegerin, and RANKL

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Abstract

We have previously reported evidence that megakaryocytes may play a role in bone remodeling, possibly by interactions with cells at the bone surface. To investigate the direct effects of megakaryocytes on osteoblasts, maturing megakaryocytes (CD61 positive cells) were isolated and added to cultures of human osteoblasts. Osteoblasts alone and osteoblasts treated with CD61-negative (non-megakaryocytic) cells were used as control cultures. After 48 h in culture, megakaryocytes were removed and osteoblasts immunolocalized for type-1 collagen, osteoprotegerin (OPG), and RANKL expression. Similar cultures were used for RNA extraction with mRNA for Col 1A1, OPG, and RANKL in osteoblasts measured quantitatively by RT-PCR.

Osteoblasts cultured alone showed high levels of expression of collagen with 74% (±7) of cells staining positively. When cultured with megakaryocytes, the number of positively staining cells remained similar but the intensity of expression was increased 1.54-fold ($P < 0.02$). OPG was expressed by 32% (±6.3) of osteoblasts increasing to 51% (±5.5) when cultured in the presence of megakaryocytes ($P < 0.01$) with a 1.63-fold increase in intensity of expression ($P < 0.01$). In contrast, osteoblasts cultured with megakaryocytes showed suppression of RANKL expression; 35.6% (±5.8) of osteoblasts cultured alone stained positively decreasing to 24.3% (±5.3) with a 1.6-fold diminished intensity of expression ($P < 0.02$). Osteoblasts co-cultured with CD61-negative cells showed no differences in collagen, OPG, or RANKL expression levels compared to osteoblasts cultured alone. mRNA data supported these findings with a 3.1-fold increase in Col 1A1 expression in megakaryocyte-treated cultures compared to controls ($P < 0.02$). Low-level OPG mRNA expression increased 8.14-fold in osteoblasts cultured in the presence of megakaryocytes ($P < 0.01$), while RANKL expression was suppressed 3.3-fold ($P < 0.02$).

These results demonstrate that in vitro, megakaryocytes have direct effects on osteoblastic production of factors affecting both bone formation and resorption. These data provide further evidence that megakaryocytes may play an important role in bone remodeling.

Keywords: Megakaryocytopoiesis; Immunocytochemistry; Bone formation; CD34+ cells; CD61+ cells

Introduction

Little is known about the interactions of bone cells and megakaryocytes within the bone marrow. The aim of this investigation was to determine the effect of megakaryocytes on osteoblast protein and mRNA synthesis of type-1 collagen, OPG, and RANKL, three important factors in bone remodeling, and thus investigate further mechanisms by which megakaryocytes might exert effects on bone remodeling.

Osteoblasts are responsible for the production of type l collagen and other bone matrix proteins. The amount of collagen synthesized is dependent on the rate at which osteoblasts differentiate which, in turn, is regulated by a variety of systemic hormones, growth factors, and locally produced factors.

Receptor activator of NF kappa B ligand (RANKL), a membrane-bound molecule, is a member of the tumor necrosis factor (TNF) ligand family and has been shown to be crucial for osteoclast formation [14]. Two receptors for

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RANKL have been identified, RANK, a membrane-bound signaling receptor expressed on the cell surface of osteoclast progenitors, and OPG, a secreted cytokine receptor. OPG, a member of the tumor necrosis factor receptor (TNF-R) super-family [24,27], acts as a decoy receptor by blocking the interaction of RANKL with its functional receptor RANK [1], thereby inhibiting osteoclastogenesis. Thus, it is now well established that these two factors act in concert to regulate bone remodeling.

Within the bone marrow compartment, stem cells differentiate along mesenchymal and hematopoietic lineages, regulated by local and systemic factors. Megakaryocyte progenitors produce numerous colony-forming units-megakaryocyte (CFU-MK) which differentiate and undergo endomitosis with the generation of large cells with lobulated nuclei. During the final stages of maturation, a complex demarcation system is developed, culminating in the release of platelets into the circulation [13,16]. In a previous in vivo study, we reported an increase in the megakaryocyte population in the bone marrow of post-menopausal women treated with oestrogen compared to age-matched untreated women [9]. In addition, in separate in vitro studies, we have shown that megakaryocytopoiesis is modulated by oestrogen and that megakaryocytes synthesize OPG and RANKL [5,6]. In addition to their known function of platelet release, megakaryocytes express a number of factors, some of which are involved in the regulation of bone remodeling [3,15,17,20,26]. This study describes the effects of megakaryocytes on osteoblastic production of factors modulating both bone formation and resorption.

Materials and methods

Cell culture

Megakaryocytes were generated from CD34+ cells isolated from human cord blood by magnetic bead technology (MACS). Umbilical cord blood was obtained from natural and cesarean births from 0 to 19 h after delivery prior to CD34 selection. Cord blood was anti-coagulated with sodium heparin in PBS, centrifuged at 800 × *g* (10 min), the plasma removed and discarded, and red cells removed by lysis in 5–10 volumes of ammonium chloride red cell lysis buffer (incubated 10 min at room temperature). Cells were centrifuged as previously and washed once in PBS. The remaining cells were selected for CD34 using the MACS system (Miltenyi Biotec, Bisley, UK) according to the manufacturer's instruction. Briefly, cells were counted, suspended in MAC buffer, incubated in MAC blocking reagent, and MAC CD34 antibody (clone QBEND/10, isotype mouse IgG, 15 min at 4°C). Following washing and centrifugation, cells were re-suspended in MACS buffer and microbeads conjugated to an anti-hapten antibody and passed through a positive selection column surrounded by a magnetic field. CD34 + ve cells were collected by eluting from the column. Cells were then cultured for 6 days in liquid medium to generate CD61+ megakaryocytes [Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% human serum, 2 mM glutamine, 2 mg/ml sodium pyruvate, 1% non-essential amino acids, 1% minimum essential vitamin solution, 0.1 mM β-mercaptoethanol, stem cell factor (40 ng/ml; Insight Biotechnology, Middlesex, UK), thrombopoietin 20 ng/ml (20 ng/ml; Insight Biotechnology), IL-3, and IL-6 (10 ng/ml; Insight Biotechnology)]. CD61+ megakaryocytes and CD61− cells were isolated according to the manufacturer's instructions using the MACS system (Miltenyi Biotec), incorporating large cell separation columns and MACS colloidal super-paramagnetic microbeads conjugated with mouse monoclonal anti-human CD61antibodies (isotype mouse IgG, clone Y2/51). Cells were washed and added to osteoblast cultures as below. Four cord blood collections were used for separate experiments.

Primary human osteoblasts were isolated from bone samples from accessory digits of two young donors (female 3 months and male 2 months) undergoing routine surgery. Cells were isolated by sequential enzymatic digestion as described previously [21]. Briefly, the bone was cleaned and finely minced prior to digestion in trypsin and dispase. Osteoblasts were released by two collagenase digestions and then grown to confluence in HAMS F12/DMEM supplemented with 10% heat-inactivated FBS (Life Technologies) penicillin/streptomycin (Life Technologies), and ascorbic acid (100 mM, Wako, Alpha Labs, Eastleigh, UK). Cultures were incubated at 37°C in a humidified chamber with 5% CO_2. At confluence, the cells were seeded into 8-well chamber slides (Nalge Nunc International) at 10^4 cells/well in McCoys 5A medium supplemented with 10% human serum. After 2 h settling time, CD61+ cells (at a ratio of 1 to 10 osteoblasts) were added to the wells and cultured for 48 h. Control cultures either had medium alone or medium containing CD61− cells (at a ratio of 1 to 10 osteoblasts) added to the osteoblast cultures.

To investigate if the effects of megakaryocytes on osteoblasts were due to cell–cell interaction or megakaryocyte-secreted factors, osteoblasts were cultured for 48 h on thermanox coverslips (Nalge Nunc International) in 24-well plates either alone or with CD61+ megakaryocytes or with CD61+ megakaryocytes within inserts with a cell-impermeable membrane (BD Biosciences, Oxford, UK) separating the osteoblasts from the megakaryocytes. The thermanox coverslips were immunolocalized as described below.

Immunolocalization

At the end of the incubation period, the medium was removed and cells rinsed in phosphate-buffered saline (PBS) pH 7.4, fixed with 4% paraformaldehyde for 5 min