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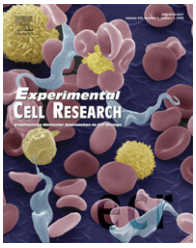

Research Article

Osteoclast size heterogeneity in rat long bones is associated with differences in adhesive ligand specificity

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ABSTRACT

Prothrombin (PT) is an RGD-containing bone-residing precursor to the serine protease thrombin (TH), which acts as an agonist for a variety of cellular responses in osteoblasts and osteoclasts. We show here that PT, TH, osteopontin (OPN) and fibronectin (FN) promoted adhesion of isolated neonatal rat long bone osteoclasts. However, the cells that adhered to PT and TH were smaller in size, rounded and contained 3–4 nuclei, in comparison to the cells adhering to OPN and FN, which were larger with extended cytoplasmic processes and 6–7 nuclei. Attachment of the larger osteoclasts to OPN and FN was inhibited by antibodies towards β_3 and β_1 integrin subunits, respectively. Whereas an RGD-containing peptide inhibited adhesion of the smaller osteoclasts to PT and TH, this was not seen with the β_3 or β_1 antibodies. In contrast, the β_1 antibody augmented osteoclast adhesion to PT and TH in an RGD-dependent manner. Small osteoclasts were less efficient in resorbing mineralized bovine bone slices, as well as expressed lower mRNA levels of MMP-9 and the cathepsins K and L compared to large osteoclasts. The small osteoclast adhering to PT and TH may represent either an immature, less functional precursor to the large osteoclast or alternatively constitute a distinct osteoclast population with a specific role in bone.

Introduction

Thrombin (TH), a multifunctional serine protease with a central role in thrombosis and hemostasis, is a cleavage product of prothrombin (PT), and is responsible for the conversion of fibrinogen to fibrin important in platelet aggregation and wound healing [1–4]. Several cellular responses to TH are mediated by protease-activated receptors (PAR) [5,6]. In bone, PAR-1 is expressed by osteoblasts and macrophages, but not by osteoclasts and their precursors [7,8]. TH stimulates bone resorption in organ culture [9–11], and is thought to mediate the resorptive response primarily through PAR-1 by enhancing the synthesis and release of factors such as prostaglandin E_2 and interleukin-6 from osteoblasts [12–15]. Another protease-activated receptor, PAR-2, was recently demonstrated to be expressed by osteoblasts [16] and also by RAW264.7 macrophages [17]. Unlike PAR-1, activation of PAR-2 inhibits osteoclast differentiation by acting on cells of the osteoblast lineage to modulate the actions of parathyroid hormone, $1,25(OH)_2$ vitamin D_3, and interleukin-11 [17]. Moreover, the TH receptors PAR-3 and -4 were shown to be expressed by human SaOS-2 osteosarcoma cells and primary murine osteoblasts, respectively [18].

Bone resorption is a complex process initiated by attachment of osteoclasts to the bone surface, followed by the polarization of

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the cell leading to development of a sealing zone, formation of a ruffled border, and the subsequent extrusion of protons and proteolytic enzymes into the extracellular resorption zone [19]. Interactions between cell surface receptors such as integrins and CD44 with bone matrix proteins have been implicated in the adhesion of osteoclasts to bone and activation of resorption [20–24]. The vitronectin receptor, $\alpha_v\beta_3$, which is abundantly expressed by osteoclasts, plays an essential role in osteoclast polarization, activation and capacity to degrade bone [21,22]. Deletion of the β_3 integrin subunit leads to failure of osteoclasts to reorganize their cytoskeleton upon ligand binding [25], resulting in the impairment of cell polarization and subsequent bone resorption. Since β_3 deficient osteoclasts still attach to bone [25], other osteoclast integrins besides the $\alpha_v\beta_3$ integrin must also be implicated in osteoclast–bone interactions *in vivo*.

Little is known about the direct effects of PT and TH on osteoclasts and their precursors. The facts that both PT and TH possess a functional RGD sequence [26,27], that PT is associated with the bone matrix [28], and that TH cleavage exposes cryptic adhesive sites in bone matrix proteins, such as osteopontin (OPN) [29–31] prompted us to investigate whether these proteins could also serve as adhesive factors for osteoclasts. Different β_3-containing integrins have been shown to interact with PT and/or TH. For instance, PT binds the major integrin $\alpha_{IIb}\beta_3$ on the platelet surface and this interaction was shown to enhance the prothrombinase-mediated conversion of PT to TH [32]. Both PT and TH can interact with the $\alpha_v\beta_3$ integrin on vascular endothelial and smooth muscle cells [33,34]. Moreover, Chiang et al. [35] demonstrated that TH-enhanced adhesion of the osteosarcoma cell line ROS 17/2.8 to both human umbilical vein endothelial cells and the extracellular matrix was associated with the up-regulation of β_3 integrins.

The present study demonstrates that PT and TH promoted integrin-dependent adhesion of a population of small osteoclasts, derived from long bones of newborn rats, which were distinguishable from conventional large osteoclasts that attached to OPN or fibronectin (FN). The small osteoclasts exhibited a lower capacity for degradation of bone matrix and a lower expression of MMP-9 and cathepsin K compared to the large osteoclasts, thus suggesting differences in the origin or maturational stage of these osteoclast populations.

Materials and methods

All use of experimental animals complied with the national guidelines in Sweden and the experimental procedures were reviewed and approved by the Southern Stockholm Regional Board for Animal Welfare.

Materials

All cell culture reagents were purchased from Invitrogen (UK). The 96-well plates used was of the brand Nunc MaxiSorp (Denmark). Bovine serum albumin (BSA, A-2153), bovine thrombin (TH, T-9000) and the leukocyte acid phosphatase kit (387-A) was supplied by Sigma and mouse prothrombin (PT, cat# MCP-5010) was from Haematologic Technologies Inc. (USA). Bovine milk osteopontin (OPN) was purified according to [36] with modifications according to [37]. Human fibronectin (FN) and the SuperScript reverse transcriptase system were purchased from Invitrogen (USA).

Peptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were synthesized by Innovagen AB (Sweden). Micro BCA ™ Protein Assay Kit (23235) was purchased from Pierce (USA). Mature human cathepsin K (CK) was a gift from SmithKline Beecham Pharmaceuticals (UK), the cathepsin substrate Cbz-Phe-Arg-AMC was purchased from Sigma and the cysteine protease inhibitor E64 and Complete protease inhibitor cocktail came from Roche.

The antibodies hamster anti-rat integrin β_1 (555002) and mouse anti-rat integrin β_3 (554950) were from Pharmingen, BD Biosciences (USA). Complete protease inhibitor cocktail was supplied by Roche Diagnostics GmbH (Germany). Sheep anti-murine prothrombin antibody (PAMFII-S) was purchased from Haematologic Technologies (USA) and 10 nm gold particles conjugated with donkey anti-sheep IgG (EM. DAS10) came from BB International (UK).

CTX-I/CrossLaps® for culture kit was bought from Nordic Biosciences Diagnostics A/S (Denmark), the iQ SYBR Green Supermix from Bio-Rad Laboratories (USA) and the RNeasy Mini Kit from Qiagen (USA).

Methods

Osteoclast isolation and adhesion assay

96-well plates were pre-coated with 50 μl of the following proteins dissolved in phosphate-buffered saline (PBS) overnight at 4 °C: mouse prothrombin (50 μg/ml), bovine thrombin (50 μg/ml), bovine osteopontin (5 μg/ml) and human fibronectin (10 μg/ml). Wells were blocked with heat-denatured BSA (10 mg/ml) in PBS at 37 °C for 1 h, and then washed 3 times with 100 μl adhesion medium (alpha-MEM medium supplemented with 1 mg/ml BSA and 100 mM HEPES, pH 7.4).

Osteoclasts were mechanically harvested from long bones (femora, humeri, tibiae, fibulae) of 2- to 5-day old Sprague–Dawley (SD) rat pups as described before [38]. Briefly, long bones were dissected and cleaned from soft tissue. The long bones corresponding to 1 animal were minced in 1 ml adhesion medium (see above) using a scalpel blade, after which bone fragments and medium were transferred to tubes and shaked gently by a vortex mixer (at lowest setting) for 1 min. The bone fragments were allowed to sediment (approx. 10 s) and 100 μl of the supernatant containing osteoclasts was seeded into each pre-coated well. Cells were allowed to attach for 20 min at 37 °C in a humidified 5% CO_2 atmosphere. The wells were then washed 4 times with fresh adhesion medium. Cells were further incubated in a CO_2-incubator for 45 min to allow spreading followed by fixation with 4% paraformaldehyde. The cells were stained for tartrate-resistant acid phosphatase (TRAP) using leukocyte acid phosphatase kit according to the manufacturer's instructions. Multi-nucleated TRAP-positive cells with at least 2 nuclei were scored as osteoclasts. TRAP-positive cells with <5 nuclei were defined as small osteoclasts and cells with ≥5 nuclei as large osteoclasts. The experiments, performed in duplicates, were performed on at least three different occasions.

To characterize the receptors that mediate osteoclast binding to the different immobilized ligands, the cell suspensions