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RANKL induces components of the extrinsic coagulation pathway in osteoclasts

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ABSTRACT

Prothrombin is converted to thrombin by factor Xa in the cell-associated prothrombinase complex. Prothrombin is present in calcified bone matrix and thrombin exerts effects on osteoblasts as well as on bone resorption by osteoclasts.

We investigated whether (1) osteoclasts display factor Xa-dependent prothrombinase activity and (2) osteoclasts express critical regulatory components upstream of the prothrombinase complex.

The osteoclast differentiation factor RANKL induced formation of multinucleated TRAP positive cells concomitant with induction of prothrombinase activity in cultures of RAW 264.7 cells and bone marrow osteoclast progenitors.

Expression analysis of extrinsic coagulation factors revealed that RANKL enhanced protein levels of factor Xa as well as of coagulation factor III (tissue factor). Inhibition assays indicated that factor Xa and tissue factor were involved in the control of prothrombinase activity in RANKL-differentiated osteoclasts, presumably at two stages (1) conversion of prothrombin to thrombin and (2) conversion of factor X to factor Xa, respectively.

Activation of the extrinsic coagulation pathway during osteoclast differentiation through induction of tissue factor and factor Xa by a RANKL-dependent pathway indicates a novel role for osteoclasts in converting prothrombin to thrombin.

1. Introduction

The bone resorbing osteoclast together with the bone building osteoblast are responsible for bone turnover and bone homeostasis. Osteoclasts are ontogenetically related to macrophages and two cytokines are necessary for the differentiation of monocytes/macrophages to osteoclasts. First, binding of M-CSF (macrophage colony stimulating factor) to the receptor c-fms, results in up-regulation of the expression of the receptor RANK, and secondly, binding of RANKL (receptor activator of nuclear factor κB ligand) to RANK leads to onset of osteoclast gene transcription, cell fusion, and ability to resorb bone through acidification and secretion of proteases into the osteoclast resorption lacuna [1].

Prothrombin (PT or coagulation factor II) is synthesized predominantly in the liver [2], and similar to several other serum proteins, PT can be detected in the calcified matrix of bone [3]. PT is converted to α-thrombin in a cell-dependent assembly known as the prothrombinase complex (reviewed in [2]). Subsequently, the serine protease α-thrombin converts fibrinogen to fibrin in the coagulation cascade resulting in blood clotting. Thrombin has been additionally reported to have several effects on bone. Thrombin is elevated in synovial fluids from rheumatoid arthritis patients, stimulates bone resorption *in vitro*, and has been proposed to be involved in fracture healing and periodontal diseases [4].

The cleavage of PT to thrombin occurs as PT binds to negatively charged phospholipids on cell surfaces of platelets [5], tumor cells [6], monocytes [7], macrophages [8], and endothelial cells. This facilitates the interaction of PT with cell-bound activated forms of the protease factor X (FXa) [9] and its co-factor factor V (FVa) [10], forming the prothrombinase complex. The conversion of factor X to Xa occurs either through the intrinsic coagulation pathway by FXII–FIXa, or through the extrinsic pathway by tissue factor-FVIIa (reviewed in [2]). PT, FX, and FV belong to both the extrinsic and intrinsic pathways, whereas tissue factor (TF) acts as the initiator of extrinsic coagulation [11]. TF is an integral membrane glycoprotein which forms a complex with FVII. Upon binding to TF, FVII is activated to FVIIa, and subsequently the TF–FVIIa complex activates FX to the prothrombin-cleaving protease FXa [12,13]. Moreover, in the amplification phase of the coagulation cascade, FV is activated by proteolytic cleavage by thrombin, thus further enhancing the prothrombinase reaction [10,14].

Abbreviations: PT, prothrombin; TF, tissue factor (coagulation factor III); FX, coagulation factor X; BMM, bone marrow macrophage; TRAP, tartrate-resistant acid phosphatase.

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It is of interest that macrophages are reported to express prothrombinase activity where prothrombin is the only exogenous factor required to generate active thrombin [8]. In addition, mouse peritoneal macrophages are able to synthesize components of the extrinsic coagulation pathway, e.g. tissue factor [15], factor VII [16], factor X [16,17], and factor V [15].

Therefore, in the context of the close ontogenetic relationship between macrophages and osteoclasts, the intention of the present study was to elucidate if osteoclasts derived by RANKL induction expressed prothrombinase activity and tissue factor. For this purpose RAW 264.7 promonomyelocytic leukemia cells, representing a homogenous cell population, and bone marrow macrophage cells, representing a physiological crude population of osteoclast precursors were used.

2. Material and methods

2.1. Ethical permission

The Stockholm South Animal Ethics Committee, Sweden, approved the study (D. no. S63-08 and S3-08).

2.2. RAW 264.7 cell cultures

The RAW 264.7 cell line (American Type Culture Collection, USA) was cultured in α-MEM (Sigma–Aldrich, USA), 10% heat-inactivated FBS, 2 mM L-Glutamine and 50 μg/ml Gentamicin (all from Gibco-Invitrogen, USA), in a humidified atmosphere at 37 °C with 5% CO_2. The cells were seeded at a density of 12,500 cells per well in 24-well plates, and stimulated with 3 ng/ml recombinant mouse RANKL (R&D Systems, UK). Control cultures were non-RANKL stimulated. Media were replaced at day 3 of differentiation.

2.3. Bone marrow macrophage (BMM) cultures

BMMs were isolated from male BALB/c mice at an age of 6–8 weeks according to Takeshita et al. [18]. BMMs were seeded out at a density of 25,000 cells/well in 24-well plates (Nunc, VWR, Denmark) in the previously mentioned media with 100 ng/ml of recombinant mouse M-CSF or 100 ng/ml of recombinant mouse M-CSF and 3 ng/ml of recombinant mouse RANKL (R&D Systems, UK). Media were replaced at day 3 of differentiation.

2.4. TRAP histochemical staining and enzyme activity assay

Cell cultures were fixed in 4% paraformaldehyde and stained for TRAP with Leukocyte Acid Phosphatase Kit (Sigma–Aldrich Chemie, Germany) including tartrate according to the manufacturers protocol. TRAP enzyme activity assay was performed as described by Hu et al. [19].

2.5. Preparation of cell lysates and protein quantification

Lysis buffer (75 μl), containing 0.15 M KCl, 0.1% Triton X-100, and Complete protease inhibitor cocktail (Roche, Germany), was added per well using 24-well plates. After lysis on ice for 40 min, total protein amount of each lysate was determined with the micro BCA protein assay kit (Pierce, USA) according to the standard protocol with Bovine Serum Albumin (BSA) as the reference protein.

2.6. Prothrombinase activity assay (S-2238)

Prothrombinase activity was measured with a chromogenic substrate for thrombin, S-2238 (Chromogenix Instrumentation Laboratory SPA, Italy). Cells were cultured as previously described and thereafter washed twice with 1 ml of α-MEM (Sigma–Aldrich, USA) without serum, followed by the addition of 150 μl medium containing 1 μg prothrombin (MCP-5010, Hematologic Technologies, USA) per well. The cells were incubated at 37 °C for 1 h followed by collection and centrifugation of the prothrombin containing media at 10,000*g* for 5 min. Hundred microlitre of the supernant were mixed with 50 μl of a buffer solution containing S-2238, giving the final concentrations; 1 mM S-2238, 50 mM Tris (pH 7.6), 0.15 M NaCl, and 5 mM $CaCl_2$. After 30 min of incubation at 37 °C, the absorbance was measured at 405 nm. The absorbance was correlated to the activity of a standard curve generated with different concentrations of bovine thrombin (T-9000, Sigma–Aldrich, USA).

2.7. Factor Xa inhibition assay (S-2222)

FXa activity was investigated using S-2222 (Chromogenix Instrumentation Laboratory SPA, Italy) as a competitive inhibitor for FXa. RAW cells were cultured as previously described for 4 days. The cells were washed twice with 1 ml of α-MEM without serum. Thereafter the cells were incubated for 5 min with 150 μl media containing 1–4 mM S-2222. After 5 min, 1 μg prothrombin was added per well. Subsequent steps were performed as described for the prothrombinase activity assay.

2.8. Tissue factor inhibition assay

RAW cells were cultured as previously described for 4 days. The cells were washed twice with 1 ml of α-MEM. Thereafter the cells were incubated at 37 °C in 150 μl media containing 20 μg/ml rabbit anti-mouse TF (FL-294, Santa Cruz Biotechnology, USA). After 30 min, 1 μg prothrombin was added per well. Subsequent steps were performed as described for the prothrombinase activity assay.

2.9. RT-qPCR of extrinsic coagulation factors

RNA was extracted with the RNeasy mini kit and DNAse treated with RNase-Free DNase (Qiagen, USA). Thereafter the RNA was reversely transcribed to cDNA with Superscript III (Invitrogen, USA). Real time PCR reactions included TaqMan Universal PCR Master Mix and were performed with Taqman probes for mouse FIII (Mm00438855_m1), FV (Mm01284448_m1), FVII (Mm00487333_m1), FX (Mm00484177_m1), cathepsin K (Mm00484036_m1) and GAPDH (4352932E) all from Applied Biosystems, USA. The PCR's were run on a 7500 Applied Biosystems machine with a thermal profile including 10 min start at 95 °C, followed by 40 cycle's amplification; 15 s at 95 °C and 60 s elongation at 60 °C. Expression data was normalized to GAPDH and calculated as fold change (FC) compared to control cells ($2^{-\Delta\Delta Ct}$).

2.10. Western blot

Protein (80 μg) were run under reducing conditions on a NuPAGE 10% Bis–Tris SDS Gel (Invitrogen, USA), followed by transferring to a Immobilon-Psq PVDF Transfer membrane (Millipore, USA). Subsequently, membranes were blocked for 1 h with TBS (20 mM Tris, 150 mM NaCl, pH 7.6)/0.05% Tween-20 (TBS/T) supplemented with 3% milk. Staining was performed overnight at 4 °C with rabbit anti-mouse TF (FL-294, Santa Cruz Biotechnology Inc., USA) diluted 1:100 in TBS/T 0.1% BS, followed by HRP-conjugated swine anti-rabbit IgG (DakoCytomation, Denmark) diluted 1:1000; mouse anti-actin (A4700, Sigma–Aldrich, USA) diluted 1:500, followed by HRP-conjugated rabbit anti-mouse IgG