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Osteoblastic cell secretome: A novel role for progranulin during risedronate treatment

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ABSTRACT

It is well established that osteoblasts, the key cells involved in bone formation during development and in adult life, secrete a number of glycoproteins harboring autocrine and paracrine functions. Thus, investigating the osteoblastic secretome could yield important information for the pathophysiology of bone. In the present study, we characterized for the first time the secretome of human Hobit osteoblastic cells. We discovered that the secretome comprised 89 protein species including the powerful growth factor progranulin. Recombinant human progranulin (6 nM) induced phosphorylation of mitogen-activated protein kinase in both Hobit and osteocytic cells and induced cell proliferation and survival. Notably, risedronate, a nitrogen-containing bisphosphonate widely used in the treatment of osteoporosis, induced the expression and secretion of progranulin in the Hobit secretome. In addition, our proteomic study of the Hobit secretome revealed that risedronate induced the expression of ERp57, HSP60 and HSC70, three proteins already shown to be associated with the prevention of bone loss in osteoporosis. Collectively, our findings unveil novel targets of risedronate-evoked biological effects on osteoblast-like cells and further our understanding of the mechanisms of action of this currently used compound.

Introduction

Osteoporosis is a skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue due to an imbalance between bone resorption and bone formation, with a consequent increase in bone fragility and susceptibility to fracture. It has become a major medical problem in the last half century, largely as the result of an increased longevity and a changing lifestyle [1].

Bisphosphonates are synthetic analogs of inorganic pyrophosphate in which the oxygen atom bridging the two phosphates is replaced by a carbon atom; they are generally accepted as first-line therapy for osteoporosis because of their efficacy, safety, and ease of administration. They have a high affinity for calcium phosphate crystals, concentrate selectively in the skeleton, and decrease bone resorption. It has been reported that the chronic supply of nitrogen-containing bisphosphonates, such as alendronate, ibandronate, risedronate and zoledronate, significantly reduces the risk of vertebral fractures by 35–65% [2].

Bisphosphonates bound to bone hydroxyapatite are released under the acidic conditions present in the resorption lacunae leading to intracellular accumulation of the drugs. In the case of nitrogen-containing bisphosphonates, at the cellular level these compounds induce changes in the cytoskeletal structure of osteoclasts, reducing their resorption activity and promoting their apoptosis. This action is mainly caused by the inhibition of farnesyl pyrophosphate synthase (FPPS), an enzyme of the mevalonate biosynthetic pathway. FPPS is responsible for the formation of isoprenoid metabolites required for the prenylation of small GTPases that are important for cytoskeletal integrity and function of osteoclasts. In addition, the inhibition of FPPS by nitrogen-containing bisphosphonates leads to accumulation of isopentyl diphosphate (IPP), a metabolite immediately upstream of FPPS, which reacts with AMP to produce a new compound which also induces osteoclast apoptosis [3]. However, unlike their effects on osteoclasts, nitrogen-containing bisphosphonates promote osteoblast

Abbreviations: 2-DE, two-dimensional gel electrophoresis; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; FPPS, farnesyl pyrophosphate synthase; IPP, isopentyl diphosphate; LC, liquid chromatography; MALDI-ToF, matrix-assisted laser desorption/ionization-time of flight; MS/MS, tandem mass spectrometry; MLO-Y4, murine long bone osteocyte-Y4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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and osteocyte survival in vitro and in vivo, through the opening of connexin 43 hemichannels, followed by the activation of pro-survival MAPK cascade [4–6]. This pro-survival effect on cells of the osteoblastic lineage is exerted at concentrations ranging from 10^{-11} up to 10^{-6} M, which are several orders of magnitude lower than the ones required to inhibit osteoclast activity [7]. In addition, we have demonstrated that low doses of nitrogen-containing bisphosphonates promote the non-lytic release of ATP from human osteoblastic Hobit cell line in culture leading to autocrine activation of purinergic signaling through P2 nucleotide receptors and to upregulation of the molecular chaperone Hsp90 [8]. Regardless of the growing interest on the action of nitrogen-containing bisphosphonates on both osteoblasts and osteocytes, a more detailed picture of their effects at the molecular level is still missing.

We recently employed a label-free, shotgun proteomic approach to identify the protein species expressed by MLO-Y4 osteocytic cells [9]. Among the identified species we noticed that a secreted protein, i.e. progranulin (also known as PC cell derived growth factor, GRN or Acrogranin) [10], was increased upon low doses of the heterocyclic ring-containing risedronate treatment of MLO-Y4 cells [9]. Thus, we speculated that nitrogen-containing bisphosphonates may be involved in the modulation of bone cells secretome. Progranulin is a secreted glycoprotein that acts as an important regulator of wound repair, inflammation, cell growth, migration and transformation. It is overexpressed in a great variety of cancer cell lines and clinical specimens of breast, ovarian, and renal cancers as well as glioblastomas [11]. It has also been detected in the conditioned medium of mouse PC cells [12], human breast cancer [13] and multiple myeloma cells [14,15]. Progranulin interacts with the heparan sulfate proteoglycan perlecan and localizes in the basement membranes of tumor blood vessels suggesting a potential role in angiogenesis [16]. Moreover, progranulin appears to be an endogenous autocrine growth factor for urothelial cancer cells [17].

To provide a more comprehensive understanding of osteoblastic cell dynamics, we characterized the secretome of unstimulated Hobit osteoblastic-like cells and the role of risedronate on protein expression of osteoblasts-secreted proteins. To this end, we employed a proteomic approach combining one dimensional gel electrophoresis in gel digestion followed by micro and nanobore liquid chromatography (LC) tandem mass spectrometry (MS/MS) performed on protein samples obtained from conditioned medium of Hobit cells.

Materials and methods

Cell culture conditions and collection of conditioned medium

Culture media and antibiotics were purchased from Invitrogen (Carlsbad, CA). Hobit cells, a human osteoblast-like cell line immortalized with the SV40 T antigen was generously provided by Dr. Riggs, Mayo Foundation, Rochester, Minnesota. MLO-Y4 (murine long bone osteocyte-Y4) cells were kindly provided by Dr. L.F. Bonewald, Department of Oral Biology, School of Dentistry, University of Missouri, Kansas City, MO. Cells were cultured as already described [8,9], maintained at 37 °C in a 5% CO_2 atmosphere and grown to approximately 80% confluence in 150-mm^2 culture dishes (Sarsted, Verona, Italy). The cells were rinsed with serum-free medium at 37 °C, 3 times for 5 min and then incubated in the serum-free medium at 37 °C for 12 h or 24 h.

All the chemicals described below were from Sigma Aldrich Co. (Milan, Italy) unless otherwise specified. Risedronate was provided by Procter and Gamble Pharmaceuticals (Cincinnati, OH). Recombinant full-length human progranulin was purified essentially as described before with minor modifications [16]. Briefly, a pCEP-Pu vector bearing the sequence of the BM40 signal peptide and the full-length progranulin was electroporated into human embryonic kidney cells (293-EBNA) expressing the Epstein-Barr virus nuclear antigen (EBNA)-1. Mass cultures were selected in media containing G418 and puromycin. Serum-free conditioned media were concentrated in a dialysis bag with polyethylene glycol, dialyzed, and purified on Ni-NTA resin eluted with 250 mM imidazole. Purity was checked by silver staining or colloidal Coomassie blue staining [18].

Preparation of the secreted proteins

After incubation, the conditioned medium from the plates (12 h: 28×10^6 cells; 24 h: 50.5×10^6 cells) was carefully removed and centrifuged at 800 g at 4 °C for 10 min to remove suspended cells. PMSF (1 mM) was added to minimize enzymatic activity, and the medium was stored at −80 °C until analysis. As control the same amount of serum free medium, never been in contact with cells, and maintained 24 h at 37 °C in a humidified atmosphere containing 5% CO_2, was used. The cells were still 90–100% viable after the serum free growth as determined by trypan blue exclusion counting. After collecting the conditioned medium, the cell number was determined with a hemocytometer after detaching the cells with trypsin. The analyzed proteins were normalized to the same cell number or to a gel run in parallel and silver stained. The conditioned medium was dialyzed against 20 mM phosphate buffer, pH 7.4, in Spectra/Por CE dialysis tubing (Spectrum Laboratories, Breda, The Netherlands), 3.5–5 k MWCO, 31 mm flat width. HiTrap Q XL anion exchange column (GE Healthcare, Uppsala, Sweden) was then used to concentrate proteins from the conditioned medium. The HiTrap Q, connected to a peristaltic pump, was equilibrated with 20 mM phosphate buffer, pH 7.4 at 0.8 ml/min. The protein sample was loaded onto the cartridge at 0.8 ml/min, and then washed with the same buffer to elute salts from the cartridge while retaining the analyte. The proteins were eluted with 6 ml of 1 M NaCl in 20 mM phosphate buffer, pH 7.4 and then dialyzed and concentrated using Amicon® Ultra-4 centrifugal filter devices (cut off 3 kDa) (Millipore, Carrigtwohill, Ireland). The collected proteins were subjected to precipitation with cold acetone, resolubilized into buffer containing 7 M urea, 2 M thiourea and analyzed by SDS-PAGE. The bands were stained with Coomassie R350.

Western blotting

Protein extracts were separated on SDS/10% (w/v) polyacrylamide gel. Ten or twenty micrograms of proteins were transferred to nitrocellulose or to PVDF membranes (Schleicher and Schuell Bioscience) for the detection of progranulin. Western blots were carried out as already reported [8,9,19]. The antibodies used were the following: anti-granulins precursor polyclonal antibody and anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pERK1/2 (Cell Signaling Technology, Beverly, MA, USA), anti HSP70 (Affinity BioReagents, Golden, CO, USA, which detects different members of the HSP70 family including HSC70), anti HSP60 or anti ERp57 (Abcam, Cambridge, UK). pERK1/2 levels were normalized with respect to total ERK2 levels after incubation of the membrane with a stripping solution [8]. The blots were developed by the SuperSignal West Dura or the ECL procedure (Pierce Biotechnology, Rockford, IL, USA). Blots were quantified by Image Quant (Amersham Biosciences), after normalization to the same cell number.

Two-dimensional polyacrylamide gel electrophoresis

Thirty to 50 μg of total cell extracts were loaded onto 13 cm, pH 4–7 L IPG strips (GE Healthcare) in duplicate. IEF was conducted using an IPGPhor II system (GE Healthcare) according to the manufacturer's instructions. Focused strips were equilibrated with 6 M urea, 26 mM DTT, 4% w/v SDS, 30% v/v glycerol in 0.1 M Trisedronate–HCl (pH 6.8) for 15 min, followed by 6 M urea, 0.38 M iodoacetamide, 4% w/v SDS, 30% v/v glycerol, and a dash of bromophenol blue in 0.1 M Trisedronate–HCl, pH 6.8, for 10 min. The equilibrated strips were applied directly to 10% SDS-polyacrylamide gels and separated at 130 V. Gels were fixed and stained by Coomassie [20].