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Determination of chitinases family during osteoclastogenesis

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ABSTRACT

Mammalian chitinases consisting of CHIA, CHIT1, CHI3L1, CHI3L2 and CHID1 exert important biological roles in the monocyte lineage and chronic inflammatory diseases. Pathological bone resorption is a cause of significant morbidity in diseases affecting the skeleton such as rheumatoid arthritis, osteoporosis, periodontitis and cancer metastasis. The biologic role of chitinases in bone resorption is poorly understood. In this study, we evaluated the expression of the chitinases family during osteoclast differentiation. The expression of CHIA, CHI3L2 and CHID1 resulted unchanged during osteoclast differentiation, whereas CHIT1 and CHI3L1 increased significantly. We also observed that CHIT1 and CHI3L1 are involved in osteoclast function. Indeed, silencing CHIT1 and CHI3L1 with siRNA resulted in a significant decrease in bone resorption activity. In addition, transfection with CHIT1 or CHI3L1 siRNA and co-transfection with both decreased the levels of the pro-differentiative marker MMP9. Overall, these discoveries reveal a novel and crucial role for both CHIT1 and CHI3L1 in promoting bone resorption and identifying new potential candidate markers for therapeutic targeting.

Introduction

Generation, maintenance and integrity of skeletal mass require continuous renewal of bone by a combination of modeling and remodeling, which is mediated by osteoclasts and osteoblasts [1]. Osteolysis is a hallmark of numerous and etiologically different diseases of bone and joint. It is known that osteoclast plays a role in pathological bone resorption. Osteoclasts are multinucleated giant cells which arise from precursors of the mononuclear-phagocytic lineage. The osteoclasts' activity is controlled by local factors produced in the bone microenvironment. In addition, osteoclasts are autocrine/paracrine, intracrine regulatory cells able to produce factors such as IL-6, annexin II, TGF-beta and OIP-1/hSca, which influence their own formation and activity [2]. A critical initiating event in osteolysis is the activation of pro-inflammatory cytokine signaling within periprosthetic macrophages, which, in turn, leads to an imbalance in the levels of the key osteoclastogenesis regulators RANKL and OPG [3,4]. Matrix metalloproteinase-9 (MMP-9), a type IV collagenase highly expressed in osteoclasts, plays an important role in the degradation of the extracellular matrix [5]. Mammalian chitinases belong to the glycohydrolase family 18, which have evolved to hydrolyze chitin, a polymer of *N*-acetylglucosamine [6,7]. The family of chitinases includes members both with and without glycohydrolase enzymatic activity against chitin. Chitotriosidase (CHIT1) and acidic mammalian chitinase (CHIA or AMCASe) are the only two true chitinase-possessing chitinolytic (glycohydrolase) activities [8]. In contrast, chitinase-like-lectins (Chi-lectins) or chitinase-like proteins (C/CLPs), including chitinase 3-like-1 (CHI3L1, YKL40, HC-gp39), chitinase 3-like-2 (CHI3L2, CHIL2, YKL-39), chitinase domain containing 1 (CHID1), show enzymatic activity despite the retention and conservation of the substrate-binding cleft of the chitinases [9]. For the majority of the mammalian chitinases important biological roles in chronic inflammatory diseases have been identified [10–12]. So far, CHIT-1 is the best-characterized true chitinase from a clinical and biological perspective. Elevated levels have been reported in a variety of diseases including infections, chronic inflammation and degenerative disorders [13,14]. The sources of secreted CHIT1 are abnormal lipid-laden macrophages formed in tissues of patients with Gaucher's disease [15]. This molecule correlated strongly with disease symptoms and is used to monitor the efficacy of therapy [16]. Recently, it was hypothesized that cellular alteration in Gaucher's disease produced a pro-inflammatory milieu leading to bone destruction through enhancement of monocyte differentiation to osteoclasts and improvement of osteoclasts resorption activity [17]. Despite various theories were proposed to explain the disruption of bone homeostatic balance in Gaucher's disease, implying dysfunction of osteoclasts, osteoblasts and mesenchymal cells [18–20], to date the effect of CHIT1 remains nearly unexplored.

Abbreviations: CHIT1, chitotriosidase; CHI3L1, chitinase 3-like-1; MMP9, matrix metallopeptidase 9; siRNA, small interfering RNA; CPhoDs, calcium phosphate thin film discs; DDs, dentin discs.

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Only a study showed that, in periprosthetic soft tissue from patients with osteolysis the expression of alternative macrophage activation markers (CHIT1, CCL18) was increased in comparison to osteoarthritis controls [21]. Interestingly this finding indicated that the activation of alternative macrophage is involved in osteolysis and suggested a correlation between CHIT1 and osteolytic lesions [21]. Unlike CHIT1, several evidences reported increased levels of CHI3L1 protein and/or mRNA in patients with a wide spectrum of pathologies including rheumatoid arthritis, osteoarthritis (OA), giant cell arteritis and malignancies [22]. Moreover, it was observed that in patients with myeloma elevated serum concentrations of CHI3L1 aggravated bone destruction and were associated with an increase of bone resorption activity hastening the progression of bone disease [23].

As well as CHI3L1, CHI3L2 is significantly up-regulated in cartilage of patients with osteoarthritis [25]. Since proteomic analysis confirmed that CHI3L2 is secreted by human osteoarthritic cartilage in culture [26], it has been recognized as a biochemical marker for the activation of chondrocytes and the progression of osteoarthritis in humans [27].

CHID1 is a marker for alternative macrophage activation [28]. CHID1 is a type 1 transmembrane receptor, which is expressed by specialized tissue macrophages in placenta, skin, gut and pancreas; it is also expressed in cardiac and skeletal muscle and by sinusoidal endothelial cells in liver, spleen, bone marrow and lymph nodes [28]. Currently, there is a paucity of information about the biological functions of CHID1.

In this study, we have investigated the hypothesis that chitinases play a role in osteolysis by comparing the expression levels of CHIT-1 and CHIA and of the prototypical CLPs, CHI3L1, CHI3L2 and CHID1 during the differentiation of osteoclasts derivate from human monocyte/macrophages.

Materials and methods

Cells

Human monocytes were isolated from fresh buffy coat of healthy volunteers as previously described [29]. The buffy coats were kindly provided by the Transfusional Centre "Garibaldi" Hospital, Catania, S. Immuno-Haemathology and Transfusional Medicine. Monocytes then were purified from the lymphomonocytic population by positive isolation using magnetic beads coated with goat anti-mouse $CD14^+$ IgG (Dynabeads, Invitrogen) as previously described [29]. Analysis of monocytes was performed by multicolor FACS (Cytomics FC 500, Beckman Coulter) using the following antibodies (Beckman Coulter): anti-CD14 and anti-CD11c. Monocytes identified as $CD14^+$ $CD11c^+$ cells, showed purity greater than 80%.

In vitro macrophages and osteoclasts

Monocytes isolated from PBMCs were cultured at a density of 5×10^5 cells/cm^2in 24-well culture plates in conditioned IMDM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% of penicillin/streptomycin (Invitrogen, Milan, Italy). In order to obtained the osteoclasts, the conditioned medium was supplemented with 25 ng/ml soluble rhRANK ligand (Peproteck, BDA, Italy) and 20 ng/ml rhM-CSF (Peproteck, BDA, Italy), for 21 days with replacement of half of the medium every 3 days. The macrophages were obtained culturing the monocytes for 21 days in conditioned medium supplemented with rHuman M-CSF 5 ng/ml (Peproteck, BDA, Italy). Every 3 days fresh media containing the growth factors were added. Cells and supernatants were harvested every 3 days for enzymatic assay, qRT-PCR and Western blotting. The supernatants were stored at −20 °C. To confirm that macrophages achieved osteoclast differentiation, suitable markers were analyzed by qRT-PCR. Finally, in order to evaluate the ability of osteoclasts to digest bone, calcium phosphate thin film discs (CPhoDs) and dentine discs were added to the wells before cell seeding (osteoclasts at 21 days). Monocytes cultured with conditioned medium (T_0) for 24 h were used as a control.

Chitosan treatment

The cells were treated with 100 μg/ml of carboxymethyl chitosan (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The supernatant was collected and stored at −20 °C for successive analysis.

Morphology of differentiated osteoclasts by May Grunwald–Giemsa staining

In order to study the morphology of osteoclasts, a May Grunwald–Giemsa staining was performed on cell cultures. Images of the cells were recorded using a digital camera (OPTIKAM PRO5/PRO 5LT) combined with OPTIKA Vision Pro Plus software (TWAIN interface, SDK) mounted on an inverted light microscope (OPTIKA Microscopes, Italy). Five representative optical fields were acquired for each sample (data not showed).

Gene expression analysis by real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Italy). For reverse transcription-polymerase chain reaction (RT-PCR), 2 μg of total RNA was reverse-transcribed with RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Milan, Italy) in a 20 μl reaction solution. One-twentieth of the RT products were used for subsequent qRT-PCR analysis. The primers used are shown in Supplementary material Table S1 [10,12]. qRT-PCR was performed using Platinum SYBR Green qPCR SuperMix UDG with Rox (Invitrogen Life Technologies). The reaction was followed by a melting curve protocol according to the specifications of the ABI 7900 instrument (Applied Biosystems). Human GAPDH was used as a housekeeping gene for quantity normalization. Data are presented as mean % ± SD of at least three independent experiments. Differences were analyzed by Student's *t* test, with $p < 0.05$ being considered statistically significant.

Western blot

Cells were harvested by trypsinization and total proteins were extracted using Np40 cell lysis buffer (SIGMA-ALDRICH, Milan, Italy). The lysates were collected for Western blot analysis. Protein concentrations were determined according to the Bradford method [30].

Protein levels were visualized by immunoblotting with antibodies against human CHIT1 (sc-99033, Santa Cruz Biotechnology, USA), CHI3L1 (sc-30465, Santa Cruz Biotechnology, USA), MMP9 (sc-13520, Santa Cruz Biotechnology, USA) and β-actin (sc-69879, Santa Cruz Biotechnology, USA). Briefly, 30 μg of lysate supernatant was resolved by SDS/polyacrylamide gel electrophoresis on 4–20% Mini-PROTEAN® TGX™ (BIO-RAD, Milan, Italy) and transferred to a nitrocellulose membrane trans-Blot Turbo mini nitrocellulose (BIO-RAD, Milan, Italy) using a semidry transfer apparatus (BIO-RAD, Hercules, CA). The membranes were incubated with 5% milk in 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20 (TBST) buffer at 4 °C overnight. After washing with TBST, the membranes were incubated with a 1:2000 dilution of anti-CHIT1, anti-CHI3L1, anti-MMP9 or anti-β Actin antibodies for 1 h at room temperature with constant shaking. The filters were then washed and probed with horseradish peroxidase-conjugated anti-rabbit IgG-HRP (Santa Cruz Biotechnology, USA) for CHIT1, donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, USA) for CHI3L1 and MMP9 and goat anti-mouse IgM-HRP (Santa Cruz Biotechnology, USA) for β-actin at a dilution of 1:2000. Chemiluminescence detection was performed with the Amersham ECL detection kit according to the manufacturer's instructions.

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