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Impact of sphingolipids on osteoblast and osteoclast activity in Gaucher disease

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ABSTRACT

Gaucher disease (GD) is an inherited disorder in which mutations in the GBA1 gene lead to deficient β-glucocerebrosidase activity and accumulation of its substrate glucosylceramide. Bone disease is present in around 84% of GD patients, ranging from bone loss including osteopenia and osteonecrosis to abnormal bone remodelling in the form of Erlenmeyer flask formation. The range of severity and variety of types of bone disease found in GD patients indicate the involvement of several mechanisms. Here we investigate the effects of exogenous sphingolipids on osteoclasts, osteoblasts, plasma cells and mesenchymal stem cells (MSC) and the interactions between these cell types. Osteoclasts were differentiated from the peripheral blood of Gaucher patients and control subjects. Osteoblasts were differentiated from mesenchymal stem cells isolated from bone marrow aspirates of Gaucher patients and control subjects. The human osteoblast cell line SaOS-2 was also investigated. Osteoclasts, osteoblasts and a human myeloma plasma cell line NCI-H929 were cultured with relevant exogenous sphingolipids to assess effects on cellular viability and function. Calcium deposition by osteoblasts differentiated from Gaucher patient MSC's was on average only 11.4% of that deposited by control subject osteoblasts. Culture with glucosylsphingosine reduced control subject MSC viability by 10.4%, SaOS-2 viability by 17.4% and plasma cell number by 40%. Culture with glucosylceramide decreased calcium deposition by control MSC-derived osteoblasts while increasing control subject osteoclast generation by 55.6%, Gaucher patient osteoclast generation by 37.6% and plasma cell numbers by up to 29.7%. Excessive osteoclast number and activity and reduced osteoblast activity may have the overall effect of an uncoupling between osteoclasts and osteoblasts in the GD bone microenvironment.

1. Introduction

Gaucher disease (GD) is an inherited disorder in which mutations in the GBA1 gene lead to deficient β-glucocerebrosidase (GC) activity and the consequent accumulation of its substrate glucosylceramide [1]. GD is typically divided into three types distinguished by the presence of neurological features in types 2 and 3 and an absence of such features in type 1 [2], the most common form of GD comprising ~94% of the GD patient population [3]. Common features of type 1 GD include hepatosplenomegaly, cytopenias and bone disease [4]. Present in around 84% of GD patients [5] bone disease can manifest in a number of forms ranging from bone loss including osteopenia, osteonecrosis osteosclerosis, osteolytic lesions, pathological fracture and abnormal bone remodelling in the form of Erlenmeyer flask formation of the femoral head [6].

GD bone disease can be partially explained by bone marrow infiltration of Gaucher cells, hypothesised to cause displacement of marrow cells to the periphery [7] which may lead to marrow infarcts including osteonecrosis of joints and to elicit an inflammatory response which may affect bone metabolism [8]. However, the range of severity and variety of types of bone disease found in GD patients indicate the involvement of several mechanisms which cannot be explained by Gaucher cell infiltration alone. Previous research by our group [9] and also Mucci et al. [10] have shown increased *in vitro* osteoclast generation and activity when differentiated from PBMC's isolated from GD patients. In addition, murine and zebrafish models of GD demonstrated impaired osteoblast differentiation and bone mineralisation [11, 12] and altered osteocyte function and viability in a CBE murine cell line model [13].

Studies have shown an increased risk of multiple myeloma, a bone marrow malignancy, for type 1 GD patients [14, 15]. In de Fost et al., multiple myeloma risk was estimated to be 51.1 fold elevated and

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prevalence of monoclonal gammopathy of uncertain significance also increased. This may be relevant as the onset of MGUS can coincide with skeletal fragility, and bone destruction might occur at a very early stage [16]. > 80% of MM patients develop bony lesions leading to pain and fractures with lytic lesions most common in the spine, skull and long bones with widespread osteopenia also a common feature [17]. Patients with MM exhibit increased numbers of osteoclast progenitor cells in the peripheral blood [18], with osteoclast numbers increased in the bone marrow of patients with monoclonal gammopathy, increasing further in patients with malignant disease [19]. Evidence suggests myeloma cells stimulate osteoclast generation *via* direct interaction [20]. In turn, osteoclasts have been shown to support myeloma cell survival and enhance proliferation [21, 22]. A flow cytometry based study of bone marrow from MM patients noted an increased number of colony forming mesenchymal stem cells (MSC's) which correlated with disease burden at time of diagnosis, suggesting MSC's and osteoblasts may play an important role in the proliferation and survival of myeloma cells [23].

Sphingolipids are a major category of lipids and are present in all mammalian cells [24]. Analysis of the lipid composition of GD patient plasma and urine found elevated levels of 20 plasma and 10 urinary lipids including species of phosphatidylcholine, sphingomyelin and ceramides [25]. With several publications also showing substantially higher levels of glucosylsphingosine [26, 27] in GD plasma and serum these findings demonstrate the deficiency of GC affects the synthesis and degradation pathways of many sphingolipids.

Recent research in unrelated diseases have shown that sphingolipids are highly bioactive and have been theorised to play a role in a number of conditions including cancer [28]. Ceramide may be a mediator of pro-apoptotic pathways and an anti-inflammatory agent [29–31]. Glucosylsphingosine has been suggested to mediate cellular dysfunction [32] whereas glucosylceramide has also been linked with immunosurveillance [33] and cell proliferation [34].

Sphingolipids have been found to modulate osteoclast formation and function. Ceramide has been shown to reduce osteoclast activity by inhibiting actin ring formation [35] whilst lactosylceramide increases RANK expression in osteoclasts [36]. Furthermore sphingosine-1-phosphate (S1P) has been found to be a chemotactic factor, regulating precursor osteoclast migration between the bone marrow and the blood [37]. Research is also revealing potential roles of sphingolipids in osteoblast recruitment and activity. S1P produced by osteoclasts is a chemoattractant for MSC's expressing its receptors, S1PR1 and S1PR2, resulting in their migration to the bone marrow and thus also acting as a coupling factor between osteoclasts and osteoblasts [38].

Here we investigate the effects of exogenous sphingolipids on osteoclasts, osteoblasts, plasma cells and mesenchymal stem cells and the interactions between these cell types.

2. Methods

2.1. Patients

Type 1 Gaucher patients attending the Royal Free Hospital had confirmed genetic diagnosis of GD. The investigation received institutional ethical approval and patients gave informed consent.

2.2. Osteoclast generation from human peripheral blood mononuclear cells (PBMC)

Human PBMC were isolated by density gradient and suspensions adjusted to 5×10^5 $CD14^+$ $CD64^+$ (BD Oncomark CD14 FITC, CD64 PE, BD Oxford, UK) monocytes per ml prior to culture in R10-OC. PBMC's were cultured on 6 mm glass coverslips (Richardsons, Leicester, UK) or 6 mm dentine slices (Immunodiagnostic Systems, UK) at 37 °C in 5% CO_2, washed in PBS after 2 h and cultured in osteoclast medium for 21 days, medium replaced every 3–4 days.

2.3. Osteoclast identification

Osteoclasts were identified cytochemically by expression of tartrate-resistant acid phosphatase (TRAP) (Sigma, UK). TRAP positive multinucleate (≥ 3 nuclei) cells were enumerated by light microscopy and expressed as the averaged total osteoclasts per 6 mm coverslip. Samples were blinded prior to enumeration.

2.4. Alizarin red staining (AR)

For quantification of mineralisation a protocol previously described was followed [39]. Briefly, cells were fixed in 4% paraformaldehyde and stained with 40 mM alizarin red S, pH 4.1, incubated at RT for 20 min. AR was washed 5× in water and solubilised with 10% acetic acid, incubated at RT for 30 min. Cells were detached, transferred to a microfuge tube and vortexed for 30 s, overlaid with mineral oil and transferred to an 85 °C water bath for 10 min, cooled in ice and centrifuged at 2000*g* for 15 min. 500 µl supernatant was transferred to a microcentrifuge tube to which 200 µl 10% ammonium hydroxide was added and vortexed. 150 µl of sample/standard was transferred to each of 3 wells of a 96 well plate. A range of AR concentrations were added to the 96 well plate to produce a standard curve.

2.5. Culture medium

A10 – MEMα +10% FBS, 10 mM HEPES, 2 mM L-Glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin. R10 – RPMI 1640 supplemented as for A10. D10 –DMEM supplemented as for A10. MSC proliferation medium– MesenCult® Proliferation Kit (Human) (STEMCELL Technologies, Grenoble, FR) with MesenCult™ Stem Cell Stimulatory Supplements, further supplemented with 10 mM HEPES buffer, 2 mM L-Glutamine and 100 units/ml penicillin, 0.1 mg/ml streptomycin. A10-OB – A10 + 5 mM β-glycerophosphate disodium salt hydrate, 200 µM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 10 nM dexamethasone. D10-OB – D10 + 4 mM β-glycerophosphate disodium salt hydrate, 100 µM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate. R10-OC – R10 + 25 ng/ml rhMCSF (Cambridge Bioscience, Cambridge, UK) and 30 ng/ml rhRANKL (Peprotech EC, London, UK).

2.6. Sphingolipids

Sphingosine (Enzo life sciences, Exeter, UK), sphingosine-1-phosphate, glucosylsphingosine (Insight Biotechnology, Wembley, UK), ceramide (Oxford Bioscience, Oxford, UK), lactosylceramide, glucosylceramide (Universal Biologicals, Cambridge, UK). 10 mM stock solutions were made by addition of 100% methanol to lyophilised solid. Sphingolipids were added to a final concentration of 1 µM to culture medium for all experiments.

2.7. Isolation of MSC's from bone marrow

Bone marrow aspirates were collected with A10 and preservative free heparin. The cell suspension was layered onto ficoll-paque plus and centrifuged at 650 *g*, 4 °C for 25 min. The white cell layer was extracted, adjusted to 50 ml with A10 and centrifuged at 400 *g* at 4 °C for 5 min. Cells were re-suspended in MSC proliferation medium, transferred to a 75cm^2 tissue culture flask and incubated at 37 °C, 5% CO_2. After 24 h all medium, containing non-adherent cells, was removed. The flask was washed 2× with PBS. Adherent cells were cultured in MSC proliferation medium and incubated at 37 °C, 5% CO_2. At ~80% confluence MSC's were detached using 0.25% trypsin-EDTA and re-seeded into three 75cm^2 tissue culture flasks for further expansion.