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Enhanced differentiation of osteoclasts from mononuclear precursors in patients with Gaucher disease

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ABSTRACT

Gaucher disease (GD) is an autosomal recessive disorder caused by deficiency of β-glucocerebrosidase. Storage of glucosylceramide in reticuloendothelial cells results in multiorgan pathology including bone disease. Established skeletal disease may remain problematic despite Gaucher-specific treatment. Both osteopenia and osteonecrosis have been described but the underlying pathophysiology, in particular the role of monocyte-derived osteoclasts is not well defined. The objective of this study was to explore the effect of glucocerebrosidase deficiency, inhibition and replacement on osteoclast development and function.
In cultures derived from GD patients, or where GBA was chemically inhibited multinucleate giant cells expressing markers of osteoclast differentiation occurred earlier and in greater numbers compared to normal controls and the functional capacity of osteoclasts for bone resorption was enhanced. Increases in osteoclast number and activity correlated with radiological markers of active bone disease. Abnormalities were reversed by addition of specific therapies and were attenuated by co-culture with cells derived from healthy controls (HCs). Numbers of osteoblast lineage cells in the peripheral blood were mismatched to osteoclast precursors indicating uncoupling of osteoblast–osteoclast regulation which may further impact on bone remodelling. Elucidation of the underlying mechanisms of these changes will suggest rational therapies for the most disabling aspect of this condition.

Introduction

Gaucher disease (GD), results from inherited deficiency of the lysosomal enzyme beta glucocerebrosidase and consequent accumulation of glucosylceramide throughout the reticuloendothelial system [1]. Clinical manifestations are due both to direct effects of the accumulation of glucosylceramide laden macrophages [2] and also the indirect systemic effects of immunological disturbance and altered cytokine profiles [3,4].

Effects on the skeleton are radiologically evident in up to 90% GD patients and result in significant pain and disability [5]. Common features include abnormalities of remodelling [6], diffuse and localised osteopenia [7], osteosclerosis [6,8] and avascular necrosis [5,9]. Whilst bone infarction is likely to result from infiltration by Gaucher cells, the pathophysiology underlying the other skeletal manifestations remains unclear. In particular, although recent reports have suggested an osteoblast abnormality [10,11] GD-related effects on the monocyte-derived osteoclast, have not been well defined. The majority of GD1 children and adults, exhibit osteopenia [12,13] and a recent study of 1552 patients in the International Gaucher Registry has demonstrated an increased risk of fractures in GD patients with lumbar spine Z scores < 1.0 [14]. Areas of osteopenia may coexist with sclerosis thus suggesting possible dysregulation of osteoclast mediated bone resorption and osteoblast bone formation.

In a murine model of GD osteopenia was demonstrated to arise from a defect in bone formation due to inhibition of PKC in osteoblasts by GL-1 and in this model TRAP-labelling of surfaces, indicative of the rate of bone resorption, remained unaltered [11]. These results are subtly at variance with those produced in an *in vitro* model of GD where specific chemical inhibition of bone marrow mesenchymal stem cell glucocerebrosidase using conduritol b epoxide (CBE) leads to impairment of MSC proliferation but not their capacity to differentiate into osteoblasts; moreover conditioned media from CBE-treated MSCs lead to an increase in osteoclast derived resorption areas [10]. Similarly factors produced by CBE-treated peripheral blood mononuclear cells (PBMC) induced differentiation of osteoclast precursors into active osteoclasts [15]. An early histomorphometric study following *in vivo* tetracycline labelling revealed massive invasion by Gaucher cells. Osteoblastic surfaces were covered by 'plump' osteoblasts and the total trabecular resorption surfaces and number of osteoclasts were increased [16] also consistent with finding increased cathespin K, an osteoclast cysteine protease in GD [17].

Examination of markers of bone turnover has not been conclusive. Ciana et al. found reduced serum levels of carboxyterminal propeptide of type I procollagen (PICP) and elevation of carboxyterminal telopeptide

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of type I collagen (ICTP) [18] concurring with Drugan et al. who measured serum osteocalcin and CTX levels in [19] untreated type 1 GD concluding bone resorption exceeded bone formation. However van Dussen et al. recently recorded decreased osteoclacin in 50% of their GD patients suggesting a relative defect in bone formation although other markers of bone formation were unchanged [20].

The availability of enzyme replacement therapy (ERT) with recombinant glucocerebrosidase has revolutionised the clinical course of patients with GD [21,22]. ERT has reduced new bone-related events such as bone pain, crises, and infarction but has more limited effect on established skeletal pathology [23]. The timing of improvements of bone density with ERT are commensurate with the slow kinetics of bone turnover [18,23,24]. Oral substrate reduction therapy (SRT), for example with miglustat (Zavesca®), reversibly inhibits glucosyl ceramide synthetase thereby reducing accumulation of glucosylceramide and allowing its degradation by residual enzyme [25]. Recent studies have suggested that miglustat may reduce the occurrence of bone pain and increase bone mineral density in patients with type I GD with first changes noticeable as early as 6 months [26] and a newer agent eliglustat tartrate has also reported improvement in lumbar BMD and Z scores in patients treated for two years in a phase two study [27]. Three studies have reported measurement of bone degradation and turnover markers during enzyme replacement therapy with inconsistent results [18,28,29] and we have recently observed little change in markers of bone metabolism including seven patients switching treatment modality from ERT to SRT [30].

Abnormalities of osteoblast and osteoclast function have been demonstrated in a number of disorders including Paget's disease [31], rheumatoid arthritis [32] and multiple myeloma [33]. *In vitro* osteoclast culture from PBMC has been shown to be increased in several diseases where *in situ* alterations in osteoclast numbers and activity are pathological features [34,35]. Numerous factors including pro-osteoclastogenic cytokines and their inhibitors [36,37], T-cells [38], fatty acids [39] and platelets [40] are involved in the regulation of *in vitro* and by implication *in vivo* osteoclastogenesis. Whilst perturbations of some of these factors have been reported in GD [41] effects on osteoclastogenesis at the cellular level and their relationship to clinical bone disease has not been studied in cells derived from patients. Therefore to further understand the pathogenesis of GD-related bone disease and the bone-directed effects of GD-specific therapy we have examined the kinetics of generation, resorptive activity and effects of therapy on osteoclasts derived from the peripheral blood of GD patients.

Methods

Patients

All patients with type 1 GD were attending the Royal Free Hospital with confirmed genetic diagnosis of GD. The investigation has received institutional ethical approval and patients gave informed consent. The demographics of the patients included in the study are found in Table 1.

Generation of osteoclasts from human peripheral blood mononuclear cells (PBMC)

Human PBMC were isolated by ficoll density gradient centrifugation, and suspensions adjusted to an absolute CD14 + CD64 + (BD Oncomark CD14 FITC, CD64 PE, BD Oxford, UK) monocyte count of 5×10^5/ml prior to culture in RPMI 1640 (Gibco, UK) supplemented with 100 iu/ml penicillin, 100 μg/ml streptomycin sulphate, 2 mmol/l L-glutamine (Gibco, UK), 10% foetal bovine serum (Gibco, UK), 25 ng/ml MCSF (GenScript Corporation, NJ, USA), and 30 ng/ml RANKL (Peprotech EC Ltd., UK) (osteoclast medium). Mononuclear cells were cultured either on 6 mm glass cover slips (Richardsons, Leicester, UK) or 6 mm dentine slices (Immunodiagnostic Systems, UK) at 37 °C in 5% CO_2 washed vigorously in PBS at 2 h to remove non-adherent cells including lymphocytes, and media replenished at three day intervals.

Table 1
Demographics.

Characteristic	Study cohort	
Gender	Male 27 (69.2%)	
	Female 12 (30.7%)	
Age	42 years (range 19–84 years)	
Splenectomised	8 (20.5%)	
Presentation Zimran SSI	9 (range 1–22)	
Receiving ERT[a]	33 (84.6%)	
Duration ERT	10 year (range 1–16)	
Receiving miglustat	2 (5%)	
Mutations	N370S/L444P	10 (25.6%)
	N370S/N370S	7 (23.0%)
	N370S/84GG	3 (7.6%)
	N370S/D409H	2 (5.1%)
	N370S/c1388 + 1G>A	2 (5.1%)
	N370S/Other	12 (30.7%)
	Other	3 (7.6%)

[a] Treatment status at PBMC sampling. All other patients subsequently received ERT.

Identification of osteoclasts

Osteoclasts were identified cytochemically by expression of tartrate-resistant acid phosphatase (TRAP) (Sigma, UK) [42] or by colocalisation of vitronectin receptor (CD51/61, Dako Cytomation Stockport, UK with Alexa Fluor 488 f(ab′)2 anti-mouse IgG, Invitrogen, UK) with F-actin rings (Texas Red-X Phalloidin, Invitrogen UK). The number of TRAP positive, or VNR/F-actin ring positive multinucleate (>/= 3 nuclei) cells per 6 mm coverslip was determined by light microscopy or fluorescence microscopy. Enumeration of TRAP+ cells is expressed as total osteoclasts per coverslip, and of VNR/F-actin ring positive cells as positive cells per ten high power fields (hpf).

Demonstration of abnormal membrane trafficking

Osteoclasts were labelled with BODIPY-Lac-Cer by pulsing with either BODIPY-LacCer in 1% serum for 30–60 min, removing cell surface fluorescence by washing 3 times 1 min with 10% serum and chasing for 60–90 min depending. Cells were incubated with 200 nM Lysotracker Red for the final 15 min.

Fluorescence microscopy

Fluorescent cells were observed using an Olympus IX70 confocal fluorescence microscope. Alexa Fluor 488 excited at 490 nm and observed at 520 nm, and Texas Red-X Phalloidin excited at 590 nm and observed at 610 nm. Lysotracker Red excited at 546 nm and observed at 590 nm; and BODIPY-LacCer excited at 450–490 nm and viewed at 520 nm. Images were collected using an Olympus LSR Ultra view camera.

Assessment of osteoclast activity by bone resorption

Dentine slices were washed and sonicated in 0.25% NH_4OH prior to staining in 1% toluidine blue/0.5% sodium tetraborate. The size of resorption pits was assessed by scanning electron microscopy (Philips 501 Scanning electron microscope).

OPG by Elisa

OPG was assessed by Elisa (Immunodiagnostic Systems Ltd. (IDS), Tyne & Wear, UK) according to the manufacturer's instructions.