Research paper

Generation of osteoclasts from type 1 Gaucher patients and correlation with clinical and genetic features of disease

Matthew C. Reed[*], Yehudit Bauernfreund[1], Niamh Cunningham[2], Brendan Beaton, Atul B. Mehta, Derralynn A. Hughes

Lysosomal Storage Disorders Unit, Department of Haematology, Royal Free Hospital and University College, London, UK

ARTICLE INFO

ABSTRACT

Gaucher disease (GD) is a rare autosomal recessive disorder caused by deficient activity of β-glucocerebrosidase resulting in the accumulation of glucosylceramide. Bone disease is a common feature with radiological evidence in up to 93% of patients. Severity of bone involvement ranges from osteoporosis to pathological fractures. The progressive course of type 1 GD is largely mitigated by treatment with enzyme replacement therapy (ERT) or substrate reduction. A number of studies have shown some patients suffer bone events while receiving ERT. Studies of biochemical markers of bone turnover have generated varied results and as a consequence are not generally used to assess bone disease in GD. *In vitro* osteoclast generation from peripheral blood samples of 74 Gaucher patients followed over a period of up to 10 years was correlated with bone events, reports of bone pain, anaemia, spleen status, bone mineral density, chitotriosidase activity, treatment with Gaucher specific therapies, bisphosphonates, mutation status and severity. Osteoclast generation, enumerated when cultured on glass, was significantly higher when differentiated from the peripheral blood of Gaucher patients which reported bone pain (116.4 ± 18.0 vs 69.0 ± 8.6, $p < 0.01$), had anaemia (153.7 ± 34.9 vs 78.5 ± 8.8, $p < 0.01$), had a splenectomy (137.6 ± 41.1 vs 60.8 ± 13.0, $p < 0.05$), *versus* those who did not. Osteoclast generation was also indicative of *in vivo* Gaucher specific therapy response as those naïve to therapy generated significantly more osteoclasts than those on therapy (111.2 ± 35.8 vs 45.1 ± 10.3, $p < 0.05$), as did patients receiving therapy but still suffering bone events (125.1 ± 31.37 vs 45.1 ± 10.33, $p < 0.05$). These findings demonstrate that the *in vitro* osteoclast assay may be a useful method for following bone disease progression in Gaucher patients.

1. Introduction

Gaucher disease (GD) is a rare autosomal recessive disorder caused by deficient activity of the enzyme β-glucocerebrosidase (Brady et al., 1966) resulting in the accumulation of the substrate, glucosylceramide, inside lysosomes of macrophages of the reticuloendothelial system (Rosenberg and Chargaff, 1958) and several other cell types (Pandey et al., 2017). The build-up of undegraded lipid may result in pathology by mechanisms such as dysfunction of mitochondria and formation of free radicals (Cleeter et al., 2013). There are also systemic effects on the immune system and cytokine release (Boven et al., 2004; Allen et al., 1997). Changes in non-reticuloendothelial cells including mesenchymal stem cells, osteoblasts and immune cells have been described and contribute to the heterogeneous pathology (Lecourt et al., 2013; Mucci et al., 2013; Burstein et al., 1987; Sotiropoulos et al., 2015). The resulting clinical phenotype is multi-system including bone disease, blood cytopenias, hepatosplenomegaly, lung disease and in some cases neurological damage (Cox and Schofield, 1997). Type 1 GD, which

accounts for 94% of cases (Charrow et al., 2000), is the least severe and is characterised by the absence of neurological involvement of the central nervous system (Cox, 2010), which characterises types 2 and 3.

Bone disease is a common feature of GD with radiological evidence in up to 93% of patients (Linari and Castaman, 2015). Features of clinical or radiological evidence of bone disease include osteopenia, osteonecrosis, osteosclerosis, osteolytic lesions, pathological fracture and bone remodelling (Stirnemann et al., 2010; Deegan et al., 2011). The severity of bone involvement ranges from absence to severe osteonecrosis and pathological fractures (Clarke and Hollak, 2015). Bone disease can be partially explained by bone marrow infiltration of Gaucher storage cells and displacement of normal marrow cells to the periphery (Maas et al., 2002). The variation in severity and characteristics of bone disease suggest involvement of mechanisms independent of Gaucher cell infiltration, possibly including vascular occlusion, effects on the generation and activity of osteoblasts and osteoclasts, affecting rates of bone formation and resorption and immune regulation (Stowens et al., 1985). A murine model of GD with bone involvement showed histological evidence of medullary infarction and associated avascular osteonecrosis in addition to osteopenia. Cellular examination demonstrated reduced proliferation of bone marrow stromal cells and reduced osteoblast differentiation (Mistry et al., 2010).

The progressive course and complications of type 1 GD (Maaswinkel-Mooij et al., 2000) are largely mitigated by treatment with enzyme replacement therapy (ERT) or substrate reduction (SRT) (Weinreb et al., 2002; Moyses, 2003). Long term studies have demonstrated clinical effects on bone in GD including increases in bone mineral density (BMD), improvement in bone pain and reduction of bone crises (Sims et al., 2008; Mistry et al., 2011). Bone disease has been assessed by evaluation of bone density by dual X-ray absorptiometry (DXA) and bone marrow burden and skeletal architecture using magnetic resonance imaging (MRI) (Stowens et al., 1985). BMD of those affected by type 1 GD is lower than expected and correlates well with other markers of disease severity (Pastores et al., 1996). Only serial assessment can provide information on the trajectory of bone mineral density acquisition or loss. Studies of biochemical markers of bone turnover have varied results with some studies finding reduced bone formation markers and elevated resorption markers, suggesting bone loss due to increased osteoclast activity (Drugan et al., 2002; Ciana et al., 2003) and others reduced bone formation markers but no change in resorption markers (van Dussen et al., 2011) suggesting reduced osteoblast activity. Studies following response to ERT and SRT either found no significant changes in bone formation markers over 4.5 years on ERT (Ciana et al., 2005) or conflicting data in which two studies found significant increases of the bone formation marker osteocalcin (Sims et al., 2008; Schiffmann et al., 2002).

Chitotriosidase, an enzyme secreted from Gaucher cells, estimates the overall Gaucher cell burden and in the absence of inactivating mutations is a reliable marker of disease severity (van Dussen et al., 2014). A greater decrease in chitotriosidase has been shown in patients whose bone marrow responds to ERT than in those whose bone marrow does not respond (Poll et al., 2002). Chitotriosidase activities are not significantly associated with bone events in Gaucher patients on ERT (Deegan et al., 2011) and may not reflect the dysregulation of osteoblast and osteoclast activity occurring in bone. Other potential biomarkers including CCL18, MIP1-β and glucosylsphingosine have been described but have not yet found their use in the serial assessment of bone disease (Boot et al., 2004; van Breemen et al., 2007; Murugesan et al., 2016).

Despite a positive haematological response, a number of studies have also shown some patients continue to suffer bone events while receiving ERT (Deegan et al., 2011; Baris et al., 2016; de Fost et al., 2008). Low bone density and anaemia have been found to be risk factors for fractures and avascular necrosis (AVN) respectively however the mechanisms underlying the bone manifestations, risk factors for severe bone disease and the most effective therapies for refractory

patients have not been fully elucidated (Khan et al., 2012). Previously we have shown that osteoclasts isolated from the peripheral blood (PB) of type 1 GD patients differentiate and resorb bone *in vitro* more rapidly than osteoclasts isolated from healthy controls (Reed et al., 2013). This enhanced rate of osteoclast formation was reversed with the addition of either imiglucerase or miglustat, as exemplars of enzyme replacement or substrate reduction therapy for GD (Reed et al., 2013). Preliminary data suggested that PB osteoclast assays correlated with bone disease severity as indicated by MRI (Reed et al., 2013). In a relatively small study of 11 males and 18 females, Bondar et al. (2017) found a correlation between BMD and *in vitro* osteoclast generation in GD1 patients receiving ERT at a single time point for each patient. In the current larger study of 74 GD1 patients followed over ten years, we have also examined the relationship of osteoclast generation to mutation, splenectomy, overall disease severity, bone pain and bone events. In addition, our cohort includes naïve patients enabling the assessment of the potential impact of therapy. Finally, we were able to follow changes in bone mineral density measurements in GD1 patients for up to a 10 year period enabling us to assess whether change in osteoclast generation reflect change in Z score.

Here we examine the relationship between osteoclast generation and bone pain, bone mineral density and anaemia, assess the effect of therapeutic agents *in vitro* and *in vivo* on the numbers of osteoclasts generated from the PB of type 1 GD patients and seek to assess the value of PB osteoclast generation as a marker of bone response in type 1 GD.

2. Materials and methods

2.1. Patients

Type 1 Gaucher patients attending the Royal Free Hospital had a 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 a density of 5×10^5 CD14$^+$ CD64$^+$ (BD Oncomark CD14 FITC, CD64 PE, BD Oxford, UK) monocytes per ml prior to culture in osteoclast medium: RPMI 1640 (Gibco, UK), 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). 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. Osteoclast function

PBMC's cultured in osteoclast medium on dentine discs for 21 days were washed $\times 2$ with PBS prior to being lysed using mammalian cell lysis buffer (Sigma). Lysis buffer was removed, dentine discs were washed $\times 2$ with H_2O and incubated at room temperature with 1% toluidine blue in 0.5% sodium tetraborate solution for 30 to 60 s. Toluidine solution was removed and discs were washed $\times 5$ with H_2O. The dentine discs were air dried overnight. Pit formation was