Original Article

Osteoclast function, bone turnover and inflammatory cytokines during infective exacerbations of cystic fibrosis

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Abstract

Background: Raised levels of pro-inflammatory, pro-resorptive cytokines during pulmonary infection may contribute to osteoporosis in cystic fibrosis (CF). We assessed osteoclast number and activity during infective exacerbations and examined their relationship to serum inflammatory cytokines and bone turnover markers.

Methods: Serum samples from 24 adults with CF were obtained before, during and after treatment of infection. Osteoclastic cells were generated from peripheral blood mononuclear cells and their number and activity assessed. Serum osteocalcin, type 1 collagen cross-linked N-telopeptide (NTx), interleukin-6 (IL-6), tumour necrosis factor alpha (TNFα), receptor activator of NFkB ligand (RANKL) and osteoprotegerin (OPG) were measured.

Results: Osteoclast number and activity were increased at the start of exacerbation and decreased with antibiotic therapy. Significant correlations were demonstrated between osteoclast formation and serum TNFα, OPG, osteocalcin and NTx and between osteoclast activity and serum IL-6 and NTx.

Conclusions: The systemic response to infection is associated with increased bone resorptive activity in patients with CF.

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Keywords: Cystic fibrosis; Osteoporosis; Osteoclasts; Cytokines; Bone turnover

1. Introduction

Cystic fibrosis (CF) is associated with reduced bone mineral density (BMD) and increased risk of fracture [1–4]. The aetiology is multifactorial and includes vitamin D insufficiency, malnutrition, glucocorticoid use, reduced levels of physical activity and hypogonadism [5]. However, the most consistent correlate of low bone mass is the severity of disease, as defined by lung function and nutritional parameters [6]. The mechanism of this association remains only partially identified, but may relate to effects of the systemic inflammatory response to pulmonary infection on osteoclast function and formation [7–9]. Thus many of the cytokines released during infection also have stimulatory effects on osteoclast development and activity, resulting in increased bone resorption; these include interleukin-6 (IL-6), tumour necrosis factor alpha (TNFα) and receptor activator of NFkB ligand (RANKL) [10–12]. Inflammation and associated alterations in cytokine levels have been implicated in the pathogenesis of bone loss in postmenopausal osteoporosis [13], rheumatoid arthritis [14–16], Paget’s disease of bone, multiple myeloma [17], periodontitis [18] and inflammatory bowel disease [19,20].

In patients with CF, increased production of a range of pro-inflammatory, pro-resorptive cytokines has been reported during infective exacerbations and, in some studies, BMD or rates of bone loss have been shown to correlate with circulating levels of these cytokines [21–23]. Based on the hypothesis that bone resorption is increased during infection as a result of increased cytokine production, we studied osteoclast number and activity during infective exacerbations in patients with CF and investigated their relationship to changes in serum cytokine levels and biochemical markers of bone formation and resorption.
2. Methods

2.1. Patients

Patients were recruited from Papworth Adult Cystic Fibrosis Centre, UK using the following inclusion criteria: CF confirmed by gene analysis or abnormal sweat test, age \( \geq 18 \) years, forced expiratory volume in 1 s (FEV\(_1\)) \(< 75\%\) predicted, at least one course of intravenous (IV) antibiotic therapy for respiratory exacerbation in the preceding year and primarily infected with *Pseudomonas aeruginosa*. Patients were excluded if they had received oral glucocorticoids in the 3 months prior to recruitment or during the study period, had received bisphosphonates, were pregnant during the study period, had renal dysfunction (elevated serum urea and/or creatinine) or if they were post-transplantation. Six healthy controls were recruited (3 male, 3 female) from laboratory staff with a mean age of 23.3 [1.2] years. The study was approved by the Local Research Ethics Committee and informed written consent was obtained from all patients and controls.

In 9 patients peripheral blood samples were taken at baseline (clinically stable, defined as no exacerbation requiring treatment for \( \geq 1 \) month), day 1 (start of exacerbation prior to intravenous antibiotic therapy, exacerbation defined by Fuchs criteria [24]), day 14 (end of intravenous antibiotic therapy and patients clinically stable with clinical evidence of resolution) and day 42 (follow-up clinic appointment). However, due to an insufficient number of patients experiencing exacerbations after baseline samples had been taken, the protocol was changed so that blood samples in the subsequent 15 patients were taken at just three time points during exacerbation: day 1, day 14 and day 42 as defined above. All 24 patients had exacerbation during the study period and in all cases were clinically stable at day 42. Clinical evaluation of infective status before, during and after the study period and in all cases were clinically stable at day 42. Correlation analysis was performed using the Biosource (France) human osteocalcin (KAP1381) ELISA. Serum type-I collagen cross-linked N-telopeptide (NTx) was measured using the Osteomark (Unipath Ltd., UK) competitive inhibition human NTx (9021) ELISA. Intra- and inter-assay variability were calculated using standards and in-house controls and, expressed as coefficients of variation, were 4.3% and 5.8% for IL-6, 6.1% and 8.8% for sRANKL, 8.1% and 3.4% for OPG, and 9.3% and 10.1% for TNF\(\alpha\). The intra-assay variation for osteocalcin and NTx was 3.5% and 4.9% respectively.

2.2. Serum separation

Peripheral blood was collected into serum-gel monovettes (Sarstedt, UK), mixed well and allowed to clot. Serum was frozen at \(-80^\circ C\) within 2 h of separation.

2.3. Osteoclast generation

Peripheral blood mononuclear cells were plated at \(3.0 \times 10^5\) cells/ml on to Osteologic \(\text{TM}\) slides (BD Biosciences, UK) or 96-well plates and cultured in \(\alpha\)-Minimal Essential Medium (MEM) containing RANKL (50 ng/ml) (Insight Biotechnology) and macrophage-colony stimulating factor (25 ng/ml) (RnD Systems). Cultures were incubated at 37 \({^\circ C}\) in 5% CO\(_2\)-air for up to 10 days (Osteologic \(\text{TM}\) slides) or 14 days (plastic) before analysis of osteoclast formation and resorption [25]. The number of osteoclastic cells was assessed using a tartrate-resistant acid phosphatase (TRAP) stain, which is specific for osteoclastic cells. Positive staining cells were quantitatively assessed by microscopy. Osteoclast activity was assessed by measurement of the area resorbed on Osteologic \(\text{TM}\) slides [25]. The total area resorbed was quantified using image capture software or Adobe \(\text{®}\) Photoshop \(\text{®}\) (Adobe systems, Inc.) to calculate total area resorbed as a percentage of total area.

2.4. Enzyme linked immunosorbant assays (ELISA assays)

ELISA kits used were IL-6 (900-K16), TNF\(\alpha\) (900-K25), sRANKL (900-K142) from Peprotech Ltd. (Europe) and OPG (DY805) from R&D systems. Serum osteocalcin was measured using the BioSource (France) human osteocalcin (KAP1381) ELISA. Serum type-I collagen cross-linked N-telopeptide (NTx) was measured using the Osteomark (Unipath Ltd., UK) competitive inhibition human NTx (9021) ELISA. Intra- and inter-assay variability were calculated using standards and in-house controls and, expressed as coefficients of variation, were 4.3% and 5.8% for IL-6, 6.1% and 8.8% for sRANKL, 8.1% and 3.4% for OPG, and 9.3% and 10.1% for TNF\(\alpha\). The intra-assay variation for osteocalcin and NTx was 3.5% and 4.9% respectively.

2.5. Statistical analysis

An ANOVA was performed as a global assay to detect variance over the time course of an exacerbation. A non-paired Tukey’s multiple comparison test was used to compare the baseline measurements obtained in 9 patients with normal controls and to examine changes over the time course of the study in all 24 patients. Correlation analysis was performed using a two-tailed non-parametric Spearman’s rank test with paired data points. All graphs box and whisker plots show the median and interquartile range (IQR). For all tests \(p<0.05\) was considered significant.

3. Results

3.1. Patient demographics

24 patients (14 male, 10 female) were recruited with a mean age [SD] of 27.0 [6.0] years. All had pancreatic insufficiency. 22 were F508 del homozygous, one F508 del/Q220X and one G551D/unknown. Mean values [SD] at recruitment for FEV\(_1\) and BMI were 46.7% [15.4] and 21.1 [3.2] kg/m\(^2\), respectively. Demographic data in the 9 patients with true baseline samples were not significantly different from those in the remaining 15 patients. For the 9 patients that had baseline samples, the mean (SD) number of days between these baseline samples being taken and day 1 of their next exacerbation was 162 (157) days. For the 15 patients without baseline samples, the mean (SD) number of days between their day 1 exacerbation sample and their last exacerbation was 125 (122) days.
3.2. Osteoclast formation and bone resorption

No significant difference was seen in the number of TRAP positive cells formed in controls (median 39.1, IQR 45.1–54.6) and CF patients at baseline (median 29.6, IQR 37.0–53.8). Over the course of an infective exacerbation there was a significant variation in the number of TRAP positive cells formed in culture ($p=0.013$; Fig. 1A). A significant increase in the number of TRAP positive cells was seen at day 1 of exacerbation (median 48.3, IQR 52.8–59.1) and day 14 (median 52.9, IQR 48.9–58.8) compared to baseline ($p<0.05$). A significant decrease was seen by day 42 (median 44.3, IQR 39.4–48.6) compared with the day 1 and day 14 values, to a level comparable to baseline ($p<0.05$) and normal controls.

No significant difference was seen between resorption area in CF patients at baseline (median 7.9%, IQR 6.9–11.8) and normal controls (median 6.8%, IQR 4.7–9.4). Over the course of an infective exacerbation there was a significant variation in the resorption area (expressed as a % of total area) ($p=0.005$; Fig. 1B). A significant increase in resorption area was seen at day 1 (median 12.9%, IQR 11.6–17.0) and day 14 (median 12.0%, IQR 7.4–15.4) compared to baseline ($p<0.05$). Levels of resorption at day 42 were comparable to baseline and normal controls.

3.3. Serum markers of bone turnover

Serum osteocalcin levels in CF patients at baseline (median 7.3 ng/ml, IQR 3.9–11.9) were significantly lower than those seen in normal controls (median 12.5 ng/ml, IQR 9.3–16.9; $p<0.05$). No significant variation in osteocalcin levels was seen over the time course of an infective exacerbation (Fig. 2A).

Levels of NTx in CF patients at baseline (median 375.0 BCE/l, IQR 322.4–424.5) were not significantly different to those seen in normal controls (median 373.7 BCE/l, IQR 296.3–469.0). Significant variation in serum levels of NTx was of an seen over the time course of an infective exacerbation ($p=0.027$; Fig. 2B). A significant increase in NTx levels compared to baseline was seen at day 14 (median 613.2 BCE/l, IQR 476.8–691.4, $p<0.05$). By day 42 levels of NTx had decreased (median 512.5 BCE/l, IQR 393.1–697.7), although had not returned to baseline.

3.4. Serum cytokine levels

Serum levels of sRANKL in CF patients at baseline (median 39.9 pg/ml, IQR 16.3–48.2) did not differ significantly from levels in normal controls (median 38.7 pg/ml, IQR 19.3–49.1). The median level at day 1 was 36.9 pg/ml (IQR 816.4–2683). Significant variation in serum levels of sRANKL was seen over the time course of an infective exacerbation ($p=0.05$) with significantly increased levels at day 14 (median 132.9 pg/ml, IQR 37.6–729.1, $p<0.05$) compared to baseline. Serum levels decreased by day 42 (median 48.4 pg/ml, IQR 18.3–63.5) to a level comparable to baseline and normal controls.

Serum levels of OPG in CF patients at baseline (median 1664 pg/ml, IQR 1545–1740) were significantly lower than those in normal controls (median 2494 pg/ml, IQR 1805–3066, $p<0.05$). At day 1 the median level was 1560 pg/ml (IQR 8.7–317.8). Significant variation in serum levels of OPG was seen...
over the time course of infective exacerbation ($p = 0.03$) with a significant increase at day 14 (median 2207 pg/ml, IQR 1869–2895, $p < 0.05$) compared to baseline. The level had decreased by day 42 (median 1739 pg/ml, IQR 1486–1994, $p < 0.05$) to a level comparable to baseline.

Serum levels of IL-6 in CF patients at baseline (median 456.0 pg/ml, IQR 233.0–891.9) were significantly higher than levels in the controls (median 115.0 pg/ml, IQR 95.2–199.7). Significant variation in serum levels of IL-6 was seen over the time course of infective exacerbation ($p = 0.005$) with a significant increase at day 1 (median 756.0 pg/ml, IQR 175.2–1253.0) compared to baseline. A decrease in levels compared to baseline was seen by day 14 (median 125.0 pg/ml, IQR 68.1–195.0, $p < 0.05$) and day 42 (median 56.0 pg/ml, IQR 33–82.4, $p < 0.01$).

Serum levels of TNFα in CF patients at baseline (median 32.4 pg/ml, IQR 26.7–73.9) did not differ significantly from levels in normal controls (median 61.9 pg/ml, IQR 53.7–115.8). The median value at day 1 was 34.7 pg/ml (range 10.3–96.1). Significant variation in serum levels of TNFα was seen over the time course of infective exacerbation ($p = 0.003$) with significant increases at day 14 (median 113.8 pg/ml, IQR 48.3–137.6) and day 42 (median 113.0 pg/ml, IQR 82.5–136.3) compared to baseline.

3.5. Correlation between osteoclast formation, activity and serum levels of bone turnover markers and cytokines

The number of TRAP positive cells in culture showed positive correlations with resorption area ($r = 0.40$, $p = 0.02$; Fig. 3A), serum NTx ($r = 0.48$, $p = 0.014$) and serum TNFα ($r = 0.37$, $p = 0.03$) and a negative correlation with serum osteocalcin ($r = -0.38$, $p = 0.04$) and OPG ($r = -0.36$, $p = 0.05$). Positive correlations were also seen between serum levels of OPG and serum NTx ($r = 0.84$, $p = 0.005$; Fig. 3B) and IL-6 ($r = 0.41$, $p = 0.02$).

4. Discussion

The results of our study add new evidence to support the role of infection in the pathogenesis of CF-related low BMD. In particular, our results show evidence of increased production and activity of osteoclasts during infective exacerbations in association with increases in circulating levels of pro-resorptive cytokines and serum NTx, a marker of whole body bone resorption. These findings extend our previous work showing an increase in the production of potential osteoclast precursors in the peripheral blood during CF infective exacerbations [9]. The significant correlations observed in the present study between serum NTx and both TRAP positive cell production and resorption area add further evidence to the proposed association between infection and increased bone resorption. Our results are broadly consistent with and extend those reported in two previous studies in adults with CF. In one of these [8], serum samples were obtained at the start and end of a 2 week course of antibiotics for infective exacerbation. At the start of therapy, serum levels of NTx, IL-1β and IL-6 were elevated compared to healthy controls and fell during antibiotic therapy, although they remained higher than the control values at the end of the 2 week study period. The values for serum IL-6 and TNFα obtained in our study were higher than those reported previously, both in patients and controls, although all values were within the normal range provided by the manufacturer. Aris et al. [7] studied 17 adults with CF at day 1 of antibiotic therapy and subsequently on day 18 and day 40. Serum IL-1β, IL-6 and TNFα values declined during antibiotic therapy and NTx also decreased, having been elevated above the normal reference range at day 1. In both of these studies, baseline samples prior to the infective exacerbation were not obtained. In contrast, in the present study a number of patients ($n = 9$) had blood sampling prior to infection, providing a true baseline from which to demonstrate changes attributable to infective exacerbation and allowing for a true ‘non-exacerbating’ comparison to be made with normal controls. Unfortunately, because of the low rate of infective exacerbations in patients in whom a baseline sample had been obtained, it was necessary to change the protocol and thus true baseline samples were only available in 9 patients. However, in all patients samples were obtained at day 42, when there was clinical evidence of resolution of infection in all cases and most of the measured parameters had returned to or near baseline values.

Changes in serum levels of RANKL and OPG have not been previously reported in this patient group. RANKL is a major regulator of osteoclast production and activity, interacting with the RANK receptor to stimulate the formation of osteoclasts and
inhibit apoptosis of mature osteoclasts [26]. Osteoprotegerin is a soluble decoy receptor that binds to RANKL, thereby preventing it from binding to RANK and inhibiting its pro-resorptive effect. Levels of serum OPG in baseline samples from patients with CF were significantly lower when compared to normal controls whereas serum RANKL levels were similar in the two groups and day 1 levels were similar to those obtained at baseline for both cytokines. The low levels of OPG, in the presence of normal serum RANKL levels may have contributed to increased osteoclastogenesis and activity at the beginning of the infective exacerbation. An increase in serum levels of both cytokines during infective exacerbation was observed in our study, although this was only statistically significant for OPG; levels of both cytokines decreased after the completion of intravenous antibiotic therapy. An increase in OPG levels has been reported to accompany increased RANKL production in other situations associated with bone loss and may represent a compensatory phenomenon aimed at protecting the skeleton from excessive bone resorption [27].

In the present study baseline values of NTx, a marker of bone resorption, were similar in CF patients and controls, whilst baseline serum osteocalcin levels were significantly lower in CF patients. These findings are consistent with our previous histomorphometric study in patients with CF, which demonstrated decreased bone formation as the predominant change, whilst bone resorption was normal in most patients, although increased in some [28]. Aris et al. [7] reported higher levels of urinary NTx and free deoxypyridinoline, both markers of bone resorption, in clinically stable CF patients when compared to controls, but similar serum osteocalcin levels. The reasons for these differences are unclear but may be related to differences in clinical characteristics of the patient groups, for example disease activity, glucocorticoid use and/or measurement-related issues (i.e. serum vs. urinary NTx assays). Taken together, the current evidence supports a role for both reduced bone formation and increased bone resorption in the pathophysiology of bone loss associated with CF, the latter being most conspicuous during infective exacerbations.

Our study has several limitations. First, the number of patients studied was relatively small and the statistical power to demonstrate changes was thus limited and further compromised by our ability to obtain true baseline blood samples, prior to infection, in only 9 of the 24 patients. However, demographic characteristics and changes in cytokine and biochemical marker levels were similar in the patients with and without true baseline values. The power of the study to show significant changes may also have been reduced by differences between patients in the severity of infective exacerbation and the corresponding effect of this on the variance of some of the measured indices. The number of controls studied was also small. Secondly, levels of IL-6, osteocalcin and NTX, (but not RANKL, OPG or TNFα) exhibit circadian variations [29,30] and may also be affected by intake of food; fasting samples taken at the same time of day would therefore have been optimal. However, for practical reasons it was not possible to obtain fasting samples in most patients in this study, although in the majority sampling was performed in the morning hours. Finally, osteoclastic cells generated from peripheral blood mononuclear cells may not be fully representative of osteoclasts formed in vivo and peripheral blood mononuclear cells circulating at the time of infection may also have the ability to differentiate into dendritic cells or macrophages.

The demonstration of a direct association between infective exacerbations in CF and increased bone resorption has potential clinical implications and suggests that measures taken to reduce the risk of infection and to treat infective exacerbations promptly and effectively may improve bone health. Our findings also provide a rationale for the use of anti-resorptive drugs in the management of bone disease in patients with CF.

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