CLINICAL STUDY

Raised serum levels of interleukin-8 and interleukin-18 in relation to bone metabolism in endogenous Cushing's syndrome

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Abstract

Objective: It is well known that patients with endogenous Cushing's syndrome (CS) have decreased bone mass and enhanced risk for osteoporotic fractures, secondary to decreased bone formation and increased bone resorption. Immunological mediators, such as cytokines, have recently been shown to influence bone metabolism, and in the present study we examined serum levels of several cytokines, with known or potential effects on bone homeostasis, in 33 consecutive recruited untreated CS patients and 33 age-, sex- and body mass index-matched healthy controls.

Methods: Cytokine levels were measured by enzyme immunoassay and bone mass by dual-energy X-ray absorptiometry.

Results: Our main findings were (i) interleukin (IL)-8 and IL-18 levels were significantly increased in CS patients compared with controls. (ii) Levels of both IL-8 and IL-18 were positively correlated to serum cortisol. (iii) For serum levels of the 'classical' resorptive cytokines, i.e. IL-6 and tumor necrosis factor α , no significant differences were found between CS patients and controls. (iv) Raised IL-18 levels were correlated with decreased osteocalcin levels in CS patients.

Conclusions: Our results demonstrated that CS patients have markedly elevated levels of the proinflammatory cytokines IL-8 and IL-18 in spite of high levels of the immunosuppressive hormone cortisol. These cytokines may be involved in the pathogenesis of disturbed bone homeostasis in CS.

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Introduction

Secondary osteoporosis is a major problem in several endocrine and chronic inflammatory disorders, but the pathogenic mechanisms have been only partly elucidated (1–3). Among others, we have recently shown that bone mass as well as bone area are reduced in endogenous Cushing's syndrome (CS), most probably related to decreased bone formation and increased bone resorption (1, 4, 5). While some of these disturbances in bone metabolism may be a direct consequence of cortisol excess (6), other factors may be involved (e.g. secondary hyperparathyroidism) (7, 8), and the mechanisms leading to altered bone homeostasis in CS have not been fully clarified (5).

Systemic and locally produced hormones and growth factors regulate bone metabolism and turnover and recently various cytokines have also been found to be involved in this process (9, 10). Several

cytokines have bone-resorptive properties *in vitro*, e.g. interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNF α) (11–15). Enhanced activity of these cytokines has also been related to postmenopausal (hypogonadotropic) osteoporosis, although the results have been somewhat conflicting (14–18), possibly reflecting the heterogeneity of postmenopausal osteoporosis.

In the present study, we expanded our series of patients with CS (5) and related bone parameters to a panel of 'classical' and 'recently' discovered cytokines and chemokines with known or potential effects on bone homeostasis. In addition to further elucidating the pathogenesis of disturbed bone metabolism in CS, such an approach, examining a patient population with well-described hormonal changes and markedly disturbed bone homeostasis, might also shed light on the relationship between cytokines and bone metabolism in more general terms.

Table 1 Patient characteristics, bone mineral density and biochemical bone markers in patients with CS and age-, sex and BMI-matched controls. Except for age, BMI and serum cortisol (means $\pm$ s.e.m.) data are given as median and 25th–75th percentiles.

	CS (<i>n</i> = 33)	Controls (<i>n</i> = 33)	<i>P</i> value
Age	43 $\pm$ 2	44 $\pm$ 2	NS
BMI	28.5 $\pm$ 0.7	27.5 $\pm$ 0.5	NS
Sex (men/women) (%)	27/73	24/76	NS
Diagnosis (pituitary/adrenal)	26/7		
Serum cortisol (nmol/l)	631 $\pm$ 42	398 $\pm$ 33	<0.001
Bone mineral			
Total bone area (CS, <i>n</i> = 23)	2205 (1892,2325)	2312 (2236,2682)	0.009
Total BMC (CS, <i>n</i> = 23)	2335 (2035,2585)	2828 (2589,3265)	<0.001
Total BMD (CS, <i>n</i> = 23)	1.10 (1.03,1.13)	1.19 (1.14,1.28)	<0.001
BMD LS (CS, <i>n</i> = 32)	1.02 (0.99,1.13)	1.24 (1.10,1.34)	<0.001
BMD FN (CS, <i>n</i> = 32)	0.86 (0.74,0.91)	0.97 (0.90,1.12)	<0.001
BMD UD (CS, <i>n</i> = 26)	0.31 (0.28,0.35)	0.34 (0.32,0.38)	0.008
BMD 33% (CS, <i>n</i> = 26)	0.70 (0.65,0.79)	0.75 (0.71,0.82)	0.025
Bone markers			
Osteocalcin	3.1 (1.8,4.7)	3.7 (2.7,4.9)	NS
CTX-1	4009 (2920,4956)	2848 (2308,3464)	0.001

LS (lumbal columna); FN (femoral neck); UD (ultra distal radius/ulna); TR (truncus).
NS, not significant.

Patients and methods

Patients and controls

Thirty-three consecutive recruited patients with endogenous CS (Table 1) (mean age 43 ± 2 years, body mass index (BMI) 28.5 ± 0.7 kg/m²) were systematically evaluated before treatment and compared with 33 age-, sex- and BMI-matched healthy controls (mean age 44 ± 2 years, BMI 27.5 ± 0.5 kg/m²). The patients had a typical history and objective findings of CS. The diagnosis was confirmed by established abnormal diurnal rhythm of serum cortisol, resistance to a conventional 2-day dexamethasone suppression test and elevated 24-h urine free cortisol levels. Hypogonadism in men was defined as androgen index <25% (normal range 25–200%) and in women as secondary amenorrhea for at least 6 months. The study was approved by the local ethical committee and conducted according to the Declaration of Helsinki II. Informed consent was obtained from all patients.

Osteodensitometric measurements

Bone mineral density (BMD), bone mineral content (BMC) and area were measured in the lumbar spine (L2–L4, anterior–posterior), the left femoral neck and non-dominant forearm, using dual-energy X-ray absorptiometry (DEXA; Lunar DPX-L, software version 1.31; Lunar Corporation, WI, USA), as previously described (5). BMC is expressed in g and BMD is expressed in g/cm². For each bone region measured, the area in cm² was determined. The software uses a 'threshold' technique to determine if a data point is air, tissue or bone. The cuts define a region, separating out data points into each of the defined locations. The

software adds up the bone data points for a region and calculates the area based on the sample size of the bone. In addition, total body determination of BMC and BMD was evaluated in 23 of the patients and all 33 age- and BMI-matched controls (5).

Biochemical measurements

Blood samples were drawn after an overnight fast into pyrogen-free vacuum blood collection tubes without any additives (Becton Dickinson, San Diego, CA, USA). Tubes were immediately immersed in melting ice, allowed to clot for 2 h and centrifuged for 15 min (1000 *g* at 4 °C) before storing serum at –80 °C in multiple aliquots until analyzed. Samples were thawed only once.

Analyses of bone-related markers and hormones in serum

The bone formation marker osteocalcin was measured by immunoradiometric assay with a commercial kit from Incstar Corporation, Stillwater, MI, USA. The assay measures intact osteocalcin 1–49. Degradation products of the C-terminal telopeptides of type I collagen (CTX-1) were measured in serum with a commercial enzyme immunoassay (EIA; Crosslaps) (19) from Osteometer Bio-Tech A/S, Herlev, Denmark. Serum and free cortisol in 24-h urine samples was measured by radioimmunoassay, using commercial kits from Orion Diagnostics, Espoo, Finland.

Cytokine measurement in serum TNF α (detection limit 3 pg/ml) and IL-10 (detection limit 0.5 pg/ml) were quantified by EIA (BioSource Europe, Nivelles, Belgium) as described (20). IL-1 β (detection limit 0.1 pg/ml), IL-1 receptor antagonist (IL-1Ra;

detection limit 22.0 pg/ml), IL-6 (detection limit 0.094 pg/ml), IL-8 (detection limit 2 pg/ml), IL-18 (detection limit 15 pg/ml), and monocyte chemotactic protein (MCP-1) (detection limit 8 pg/ml) were measured by EIAs as recommended by the manufacturer (R&D Systems, Minneapolis, MN, USA). Serum levels of soluble TNF receptors (sTNFRs), p55 and p75, were analyzed by EIAs (detection limit between 150–300 pg/ml) as described by Liabakk *et al.* (21). Intra- and interassay coefficients of variation were <10% for all assays.

Statistical analysis

Mann–Whitney rank sum test for unpaired data was used for comparisons between variables. Relationships between variables were tested using Spearman's rank correlation test. Throughout, we report two-tailed *P* values, and values <0.05 were considered significant. However, particular attention should be directed towards smaller *P* values (i.e. <0.01) because a considerable number of *P* values have been calculated. Percentual differences between groups in the text are based on medians.

Results

Osteodensitometry

As previously reported (5), total bone area, total BMC and total BMD were significantly reduced in patients with CS compared with controls (5%, 17% and 8% respectively, $P < 0.001$). Further, CS patients had significantly decreased BMD in the lumbar spine (18%, $P < 0.001$), femoral neck (11%, $P < 0.001$), ultra distal radius (9%, $P = 0.01$) and radius 33% (7%, $P < 0.03$) (Table 1). Similar results were observed for BMC in these regions (data not shown). No men were found to be hypogonadal, whereas 18 women had secondary amenorrhea. These women had significantly higher frequency of osteopenia/osteoporosis (osteopenia defined as a T-score -0.5 to 2.5 and osteoporosis as a T-score < -2.5 measured by DEXA) in femoral neck ($P = 0.0364$; data not shown).

Serum levels of bone-related markers

Serum levels of osteocalcin were not significantly decreased (21%, $P = 0.11$), while CTX-1 was significantly increased (47%, $P < 0.005$) in CS patients compared with controls (Table 1).

Serum levels of cytokines

When examining serum cytokine levels in CS patients and healthy controls, several significant findings were revealed (Fig. 1). First, CS patients had markedly increased levels of the proinflammatory cytokine

IL-18 (67%, $P < 0.05$) and in particular of the CXC chemokine IL-8 (215%, $P < 0.001$), compared with healthy controls. Secondly, while serum levels of TNF α did not differ between CS patients and controls, the patients had decreased levels of both types of sTNFRs although the differences in soluble p55 TNFR did not reach statistical significance (p75: 17%, $P < 0.05$; p55: 16%, $P = 0.06$). Finally, investigating the components of the IL-1 system, we found that while CS patients had slightly, but significantly decreased IL-1 β levels (44%, $P < 0.05$), IL-1Ra was significantly elevated (27%, $P < 0.05$). No differences were found between the CS patients and controls with respect to IL-6, IL-10 or MCP-1 levels. Similar patterns of cytokine levels were found in both adrenal ($n = 7$)- and pituitary ($n = 26$)-dependent CS (data not shown).

Relationships between cytokine levels, bone-related markers and BMD

In CS patients, but not in controls, the increased IL-8 and IL-18 levels were significantly positively correlated with serum cortisol (Fig. 2). Moreover, in CS patients, but not in healthy controls, IL-18 levels were significantly negatively correlated with osteocalcin ($r = -0.48$, $P < 0.01$). No significant correlations were found between cytokine levels and CTX-1 in patients or controls. When correlating these cytokines with BMD, no significant correlations were found in CS patients while IL-8 was negatively correlated to total BMD ($r = -0.42$, $P < 0.05$), lumbar spine ($r = -0.44$, $P < 0.05$) and ultra distal radius ($r = -0.54$, $P < 0.001$) in controls.

Discussion

To some degree most patients with CS have hypogonadotropic hypogonadism, either directly because of the pituitary adenoma in Cushing's disease, or indirectly related to inhibition of gonadotropins by glucocorticoid (6). As expected, the women with hypogonadism had significantly decreased bone mass in the femoral neck compared with the eugonadal female patients. Compelling and consistent evidence indicates that sex steroids directly regulate the IL-6 gene. Estrogens inhibit IL-6 and loss of this sex steroid may cause an upregulation of IL-6 production which, in turn, may result in enhanced bone resorption (14). However, in the present study, CS patients had IL-6 levels within normal limits. Furthermore, although IL-1 β and TNF α have been suggested to induce bone disturbances in various disorders (2, 22), we found no elevation in serum levels of these 'classical' resorptive cytokines in CS patients. However, although IL-1 β was not raised in CS patients, those with high IL-1 β levels had decreased BMD. Moreover, and most importantly, serum levels may not necessarily reflect the

Figure 1 Serum levels of (A) IL-1 β , (B) IL-1Ra, (C) MCP-1, (D) TNF α , (E) p55 TNFR, (F) p75 TNFR, (G) IL-6, (H) IL-8 and (I) IL-18 in 33 patients with CS (●) and 33 age-, sex- and BMI-matched healthy controls (○).

cytokine levels in the bone microenvironment and our results do not exclude a role for IL-1 β or other 'classical' resorptive cytokines in the pathogenesis of disturbed bone metabolism in CS.

Major findings in the present study were the elevated levels of the proinflammatory cytokines IL-8 and IL-18 in CS patients, significantly correlated with cortisol levels. Moreover, this elevation of IL-18 was associated with decreased osteocalcin levels. In patients with elevated levels of the immunosuppressive hormone cortisol these findings may seem surprising. In fact, cortisol administration *in vivo* has been associated

with enhanced levels of the anti-inflammatory cytokine IL-10 and decreased levels of proinflammatory cytokines (23). However, it was recently shown in animal models that acute stress, through adrenocorticotrophic hormone, stimulates the expression of IL-18 in glucocorticoid-producing cells of the adrenal cortex. Furthermore, this production was not inhibited by corticosterone, suggesting an immunostimulatory role for IL-18 during acute stress (24). Nonetheless, the effect of cortisol on IL-18 levels has not been investigated, and the outcome of short-time *in vivo* cortisol administration may not necessarily reflect the situation

Figure 2 Correlations between serum levels of cortisol (s-cortisol) and (A) IL-8 and (B) IL-18 in 33 patients with CS and 33 age-, sex- and BMI-matched healthy controls.

in CS patients with persistently elevated cortisol levels over several months or years. Although the biological significance of these correlations between high levels of cortisol and IL-8 and IL-18 is at present unclear, it may possibly suggest that some immunopathogenic mechanisms may be operating in CS (24, 25).

Several cytokines are known to influence bone homeostasis by increasing bone resorption (e.g. IL-6 and $\text{TNF}\alpha$) (11, 12, 26), but few studies have addressed the role of IL-8 and IL-18 in this process (26, 27). However, both IL-8 and IL-18 can be produced by osteoblasts (28, 29) and IL-8 by osteoclasts in response to proinflammatory stimuli (2). Thus a local rise in IL-8 levels could potentially contribute to regional bone loss through suppressive effects on osteoblasts and recruitment of osteoclasts to new

sites of resorption. However, the responses to inflammatory stimuli are suppressed by glucocorticoids in these cells, as well as other cells that are potential sources to systemic IL-8 levels. Thus mechanisms other than those demonstrated *in vitro* may contribute to the elevated IL-8 levels found in CS in this study. Moreover, IL-8 may also indirectly contribute to disturbed bone homeostasis in various inflammatory disorders by modulating parathyroid hormone secretion (30). IL-18, originally identified as interferon γ -inducing factor, is related to the IL-1 family in terms of its structure, processing, receptor, signal transduction pathway and proinflammatory properties (25, 27) but, in contrast to the resorptive abilities of IL-1 β , the effects of IL-18 seem to be bone protective; first by inhibiting osteoclast formation through effects on

granulocyte macrophage colony-stimulating factor (29), but also by upregulating osteoblastic production of osteoprotegerin (31). Nevertheless, few studies have been performed and the exact role of IL-8 and IL-18 in bone homeostasis will have to be investigated further.

In conclusion, although CS patients have increased levels of the anti-inflammatory and immunosuppressive hormone cortisol, the present study demonstrates that these patients also have elevated serum levels of the proinflammatory cytokine IL-18 and the CXC chemokine, IL-8. As for IL-18, this rise in cytokine level was significantly correlated with decreased osteocalcin levels in these patients. Although serum levels might not necessarily reflect the cytokine levels in the bone microenvironment, our findings may suggest that some immunopathogenic mechanisms may be operating in CS, particularly in the induction of disturbed bone homeostasis.

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