CLINICAL STUDY

High-dose glucocorticoids increase serum levels of soluble IL-6 receptor α and its ratio to soluble gp130: an additional mechanism for early increased bone resorption

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Abstract

Objective: Glucocorticoids (GCs) at pharmacological doses stimulate bone resorption. Mechanisms of this action are unclear. The osteoclastogenic cytokine interleukin (IL)-6 acts through an oligomeric receptor consisting of two subunits, gp80 (or IL-6 receptor α , IL-6R α) and gp130; both exist in membrane and soluble forms. Soluble IL-6R α (sIL-6R α) enhances, while gp130 inhibits IL-6 signalling. *In vitro*, GCs enhance many effects of IL-6 by up-regulation of IL-6R α . The aim of the present study was to assess acute changes of IL-6 system in the peripheral blood of patients given high-dose GCs.

Subjects and methods: Serum levels of IL-6, sIL-6R α , gp130 and bone turnover markers were assessed before and each day during treatment in 24 multiple sclerosis (MS) patients undergoing high-dose (prednisolone, 15 mg/kg per day), short-term (3 to 5 days) intravenous GC therapy for relapse at the Regional Multiple Sclerosis Centre.

Results: An immediate and marked fall of osteocalcin and an early increase of C-terminal telopeptide of type I collagen were already noticed at day 2 ($P < 0.001$ and $P < 0.02$, respectively); both became more apparent in the subsequent days. IL-6 was always below or near the detection limit of our ELISA. gp130 showed a slight increase. sIL-6R α significantly increased, peaking at day 4 ($P < 0.01$). However, inter-individual variability of response was noticed. Four patients showed a slight decrease, while no change was observed in one patient and an increase was noticed in the remaining nineteen (maximum change ranging from +10% to +67% with respect to baseline). In these patients, a significant increase of sIL-6R α /gp130 ratio was apparent. No correlation was found between bone turnover markers and any measured component of the IL-6 system.

Conclusions: sIL-6R α and sIL-6R α /gp130 ratio are precociously increased in the peripheral blood of the vast majority of patients given high-dose, intravenous GCs. The increase of systemically available sIL-6R α conceivably results in the enhancement of IL-6-dependent osteoclastogenesis. The role of such a mechanism in the bone loss observed in inflammatory and immune-mediated diseases (where abundancy of IL-6 in the bone microenvironment is expected) requires further investigation.

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Introduction

Glucocorticoid (GC)-induced osteoporosis is the leading form of secondary osteoporosis. Chronic GC treatment is associated with atraumatic fractures in 30 to 50% of patients. Increased risk of fracture is already apparent after 3 months of therapy. GCs affect bone through immediate and sustained decrease of bone formation together with early and transient increase of bone resorption (1–2). Mechanisms of increased bone resorption have been scarcely investigated. Weinstein *et al.* have recently shown that GCs may act directly on murine osteoclasts to prolong their lifespan (3).

However, data on direct actions of GCs on osteoclast differentiation, activity and apoptosis are conflicting. GC receptors have been inconsistently found in human osteoclasts depending on cellular model and detection technique (4–5). Nongenomic effects are conceivable, but have not been documented so far (6). Most authors agree that GCs may act on osteoclasts indirectly, cells of the stromal-osteoblastic lineage being the main target of GC action in the bone microenvironment (1). Up- and down-regulation of RANKL and osteoprotegerin (OPG) expression in these cells, hence an increased RANKL/OPG ratio, has been recently proposed as a mechanism of increased bone resorption (7).

Another possible pathway involves up-regulation of IL-6 signalling. IL-6 has long been known as a potent osteoclastogenic cytokine, involved in the pathological bone resorption of a number of bone diseases, including post-menopausal osteoporosis, Paget's disease of bone and skeletal metastases (8). The main source of IL-6 in the bone microenvironment is likely to reside in the stromal and osteoblastic cells. IL-6 acts through an oligomeric membrane receptor consisting of two subunits, known as gp80 (or IL-6 receptor α , IL-6R α) and gp130. gp130 is the signal-transducing molecule; it is shared by different oligomeric receptors for a family of cytokines including IL-6, IL-11, oncostatin M, leukemia inhibitory factor and others. IL-6R α is the specific ligand-binding subunit (9). Udagawa *et al.* have suggested that the ability of IL-6 to induce osteoclast differentiation depends on signalling mediated by IL-6R α expressed on osteoblastic cells but not on osteoclast progenitors (10). The major effect of IL-6 could be an up-regulation of RANKL (11–12). Nonetheless, recent work suggests that IL-6 may also exert RANKL-independent pro-osteoclastogenic effects (13).

Soluble forms of both IL-6R α (sIL-6R α) and gp130 (sgp130) have been described in several biological fluids, including blood. sIL-6R α and sgp130 are generated by either proteolytic cleavage of membrane molecules or translation of alternatively spliced mRNAs. Interestingly enough, IL-6 may bind to sIL-6R α forming an IL-6/sIL-6R α complex which is able to bind membrane gp130, leading to signal transduction. Activation mediated by ligand/soluble receptor subunits is called trans-signalling (14). In contrast, sgp130 may bind soluble and membrane-bound IL-6/IL-6R α complexes and prevent interaction with membrane gp130, hence signal transduction. Therefore, sgp130 appears to be an endogenous antagonist of IL-6 (15).

GCs up-regulate expression of both IL-6R α (10) and gp130 (16) in cells of the stromal-osteoblastic lineage. Accordingly, they are able to enhance the osteoclastogenic activity of IL-6 at least under peculiar experimental conditions *in vitro* (10, 17). To the best of our knowledge, no data have been reported on the effects of GC administration on serum sIL-6R α and sgp130 in relation to bone resorption. The aim of the present study was to assess serum sIL-6R α , sgp130 and bone turnover markers in a group of multiple sclerosis (MS) patients undergoing high-dose, short-term intravenous GC therapy for relapse.

Materials and methods

Study population

The study was performed in the Department of Internal Medicine and the Centre for Research and Therapy on Multiple Sclerosis at San Luigi Hospital, Orbassano (Turin, Italy). All participants gave written informed consent before enrollment. The study protocol and

informed consent documents were prepared according to the Declaration of Helsinki and subsequent relevant integrations, and approved by the local ethical review board. Patients with relapsing MS were diagnosed according to criteria by Poser *et al.* (18). Exclusion criteria were: history of diseases affecting bone, prolonged immobilization (> 3 weeks) and/or treatment with any drug documented to influence bone metabolism in humans in the previous 6 months.

Twenty-four patients (16 female, 8 male; age: median 36.5, range 25–61 years) were recruited. Demographics, clinical data and baseline laboratory results of the study population are reported in Table 1. 14 out of 24 patients were on interferon (IFN)- β therapy at recruitment, the mean duration of previous treatment being 23 months; none of these patients stopped IFN- β therapy at the beginning of the study. No calcium and vitamin D supplements were given. Methylprednisolone (15 mg/kg per day) diluted in 250 ml of saline was infused for 2 h in the morning, for 3 to 5 days, according to individual clinical response as assessed by experienced neurologists of the Centre and as a function of adopted protocols. No concomitant medication was administered.

Biochemical analysis

Each morning, after overnight fast and before GC infusion, a sampling cannula was inserted in a vein in antecubital fossa and flushed with 1 ml sterile heparinized saline. Blood collection was performed after 30 min resting. Second-void urine samples also were collected.

Each day measurements of serum calcium, phosphorus, PTH, osteocalcin (OC), carboxyterminal telopeptide of type I collagen (CTX), IL-6, sIL-6R α and sgp130 were performed. On day 1, serum 25-hydroxyvitamin D (25-OHD) was also measured.

Table 1 Demographics, clinical data and baseline laboratory results of the study population. Data are expressed as mean $\pm$ S.E.M. (range).

	Values
Age (years)	38 $\pm$ 2 (25–61)
Number of females/males	16/8
BMI (kg/m ²)	23.0 $\pm$ 0.6 (19.5–33.2)
Smokers	3/24
Disease duration (years)	9 $\pm$ 1 (1–23)
EDSS	2.9 $\pm$ 0.4 (1–8)
Patients on treatment with interferon- β	14/24
Serum calcium	2.19 $\pm$ 0.05 (1.70–2.47) mmol/l [8.8 $\pm$ 0.2 (6.8–9.9) mg/dl]
Serum phosphorus	1.03 $\pm$ 0.03 (0.65–1.32) mmol/l [3.2 $\pm$ 0.1 (2.0–4.1) mg/dl]
PTH	61 $\pm$ 5 (30–129) pg/ml [61 $\pm$ 5 (30–129) ng/l]
25-OHD	24 $\pm$ 4 (7–83) ng/ml [60 $\pm$ 10 (17–207) nmol/l]

Serum calcium, phosphorus and albumin were measured by Aeroset System (Abbott Laboratories, Abbott Park, IL, USA), routinely used in the hospital laboratory. Serum calcium levels were always corrected for serum albumin as follows: corrected calcium (mmol/l) = total calcium (mmol/l) + 0.02(40-albumin (g/l)). Serum PTH was measured by chemiluminescence immunoassay for intact PTH (Nichols Institute Diagnostics, San Clemente, CA, USA). 25-OHD was measured by RIA (Immunodiagnostic Systems, Boldon, UK). ELISAs were used for OC, CTX (Osteometer Biotech, Herlev, Denmark), IL-6, sIL-6R α and sgp130 (R&D Systems Quantikine, Abingdon, UK). Ranges, minimum detectable concentrations, intra- and inter-assay coefficients of variation were as follows: OC: 2–68.1 ng/ml; 0.4 ng/ml; 2.6% and 9.1%; CTX: 0.156–2.562 ng/ml; 0.01 ng/ml; 2.5% and 9%; IL-6: 3.12–300 pg/ml; 0.7 pg/ml; 2.7% and 5.4%; sIL-6R α : 31.2–2000 pg/ml; 11 pg/ml; 2.6% and 5.9%; sgp130: 0.25–16 ng/ml; 0.08 ng/ml; 2.3% and 5%.

Statistical methods

Statistical analysis of data was performed with Statistica 6.0 software package (Statsoft Inc, Tulsa, OK, USA). Results are presented as mean $\pm$ S.E.M. Assessment of changes during GC treatment was performed by Wilcoxon matched pair test; for multiple comparisons, Bonferroni correction was used. Correlations among variables were assessed by Spearman R analysis.

Results

In our series, serum 25-OHD levels resulted below the lower limit of normal range (<20 ng/ml (<50 nmol/l)) in 12/24 patients. Six patients showed hypocalcaemia (<2.10 mmol/l (<8.2 mg/dl)); in one of them hypophosphoraemia (<0.81 mmol/l (<2.5 mg/dl)) was also present. Increased PTH levels were found in seven patients: in two of them calcemia was below the lower limit of the normal range, while it was normal in the remaining five. None of the patients had hypercalcaemia or hyperphosphoremia (Table 1). At baseline, CTX levels were higher in males than in females (0.827 ± 0.136 (mean $\pm$ S.E.M.) vs 0.421 ± 0.058 ng/ml, $P = 0.01$), and PTH showed an inverse correlation with sIL-6R α levels (Spearman $R = -0.47$, $P = 0.03$).

In the course of GC administration, no apparent changes of serum phosphorus and calcium were observed. PTH showed a slight decrease (Fig. 1), while a progressive increase was noticed for urinary calcium to creatinine ratio (Fig. 2). As far as bone turnover markers are concerned, an immediate and marked fall of OC (Fig. 3a) and an early increase of CTX (Fig. 3b) were already noticed at day 2 ($P < 0.001$ and

Figure 1 Effects of GC administration on PTH. PTH was measured on morning fasting blood samples before (day 1) and each day during treatment (days 2–5). Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ by Wilcoxon matched pair test.

$P < 0.02$, respectively); both became more apparent in the subsequent days. Serum IL-6 was always below or near the detection limit of our ELISA (0.7 pg/ml). Changes of sIL-6R α and sgp130 were evaluated in terms of absolute values, percent increase or decrease with respect to baseline values and area under the curve (AUC). Changes of sIL-6R α /sgp130 ratio were also assessed. sgp130 showed a slight increase (Figs 4a and 5a), with great inter-individual variability: negative changes were observed in five patients, and positive changes in 19, with maximum percent variations ranging from -6 to -16% and from $+6$ to $+34\%$ versus baseline, respectively. No difference in demographics, clinical and biochemical variables was found between the two subgroups.

As shown in Figs 4b and 5b, as a group phenomenon sIL-6R α significantly increased, peaking at day 4 ($P < 0.01$). Again, inspection of individual patterns

Figure 2 Effects of GC administration on urinary calcium to creatinine ratio. Calcium and creatinine were measured on second-void urine samples before (day 1) and each day during treatment (days 2–5). Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ by Wilcoxon matched pair test.

Figure 3 Effects of GC administration on serum bone markers. OC (a) and CTX (b) were measured on morning fasting blood samples before (day 1) and each day during treatment (days 2–5). Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Wilcoxon matched pair test.

showed an ample inter-individual variability: when maximum percent changes were considered, four patients showed a slight decrease (from -10 to -33% with respect to baseline), while no change was observed in one patient and an increase was noticed in the remaining 19 (from $+10$ to $+67\%$ with respect to baseline); the latter were defined as 'responders'. When demographics, clinical and biochemical data were considered, no significant difference was found between responders and non-responders, apart from baseline sIL-6R and PTH levels, which were respectively lower and higher in the former (sIL-6R: 35 ± 2 vs 48 ± 5 ng/ml, $P = 0.009$; PTH: 66 ± 6 vs 46 ± 7 pg/ml, $P = 0.05$). When the whole population was considered, sIL-6R/sgp130 ratio was unchanged, but a significant increase was observed in the subgroup of responders (Fig. 6).

Finally, we calculated total and incremental AUCs of OC, CTX, sIL-6R and sgp130. Due to variable duration of GC treatment, AUCs were adjusted as a function of days to allow for analysis. No correlation was found between bone turnover markers and all other analytes, in terms of either absolute or percent peak changes versus baseline values, or AUCs.

Discussion

The mechanisms of GC action in the bone microenvironment have been extensively studied in the last two decades. Inhibition of bone formation has long been recognized as the key pathogenetic event (1).

The immediate and marked fall of serum OC is clearly consistent with this view and confirms previous results published by us and others. Studies investigating the effects of GCs on bone resorption have yielded conflicting results (19–28). Discrepancies could be accounted for by different experimental conditions: the list of variables is long and includes human versus animal models, endogenous versus exogenous GC excess, pharmacokinetics and pharmacodynamics of the various GCs employed, doses and administration routes, underlying disease for which GCs were administered and, most importantly, study design (cross-sectional versus longitudinal). There is increasing evidence that early effects (days/weeks) may be opposite to late effects (months/years). It is agreed nowadays that in patients exposed to pharmacological doses of GCs increased bone resorption occurs early and is transient (1). Yet, few studies have addressed the changes of osteoclast function at the very beginning of treatment (20). Our previous and present findings, together with those of other studies, prompt the interest, even for strategies of prevention, for elucidating the subserving mechanisms.

Prior emphasis to explain these events had been placed on the effects of GCs on other hormonal axes and mineral metabolism, including PTH. However, the role of hyperparathyroidism (due to hypocalcaemic actions of GCs complemented by a direct action on parathyroid glands) has been recently questioned (29). We have found a decrease of PTH in the course

Figure 4 Effects of GC administration on serum sgp130 and sIL-6Rα (absolute values). sgp130 (a) and sIL-6Rα (b) were measured on morning fasting blood samples before (day 1) and each day during treatment (days 2–5). Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ by Wilcoxon matched pair test.

Figure 5 Effects of GC administration on serum sgp130 and sIL-6R α (percent values). sgp130 (a) and sIL-6R α (b) were measured on morning fasting blood samples before (day 1) and each day during treatment (days 2–5). Data are expressed as mean $\pm$ S.E.M.

of GC administration. Similarly, non-significant decrease of PTH in the course of GC therapy was reported by Pearce *et al.* (25). In fact, studies investigating serum PTH levels in GC-treated patients have yielded inconsistent results (29), possibly due to the fact that single random measurements do not reflect the overall pattern of PTH secretion. Bonadonna *et al.* have recently shown that GC treatment induces a redistribution of spontaneous PTH secretory dynamics by reducing the amount released in tonic fashion and increasing the amount released as pulses (30).

While the pathogenetic role of the classical systemic actions of GCs remains in the background, direct effects on bone cells have gained importance. IL-6 is a well-recognized osteoclastogenic cytokine. It promotes the differentiation of osteoclasts from progenitor cells and the action of mature osteoclasts (8). The cellular and molecular mechanisms of IL-6 action on cells of the osteoclastic lineage are not fully understood. IL-6 could have direct effects, since available data support the presence of gp130 on osteoclast membrane, at least in some models (31). IL-6 also exerts indirect effects on osteoclastogenesis by altering the ratio of

RANKL/OPG produced by stromal and osteoblastic cells (11–12). In different *in vitro* models adding IL-6 and sIL-6R α in the medium yielded an increased release of more RANKL than OPG (11). For osteoclastogenesis, an important role of sIL-6R α has been convincingly demonstrated *in vitro* (10–11, 17). To the best of our knowledge, the present report is the first that has addressed the changes of serum levels of sIL-6R α and sgp130 in patients given high-dose GCs. Our data clearly show that the vast majority of such patients (about 80%) display increased sIL-6R α and sIL-6R α /sgp130 ratio in the first days of treatment. It is obvious that many sources may contribute to the increase of circulating sIL-6R α . Numerous studies have documented that GCs are able to increase IL-6R α expression in a variety of tissues (10, 32–33). With regard to the bone microenvironment, Vermes *et al.* have demonstrated that availability of the specific receptor subunit sIL-6R α is required for IL-6 signalling in human osteoblasts, the membrane form being inactive (34). Accordingly, it is logical to think that the pro-osteoclastogenic action of IL-6, at least in the part mediated by osteoblast activity, is a function of IL-6/sIL-6R α complexes, that bind gp130 to activate specific intracellular pathways. Since the potentiating role of sIL-6R α for IL-6 signalling is referred to as a general phenomenon, and GCs increase IL-6R α expression in many cells including osteoblasts, the early increase of osteoclast population and function in patients given GCs may find a further explanation in the increased availability of IL-6R α . Interestingly enough, in patients with higher levels of sIL-6R α GCs administration yielded a decrease of sIL-6R α and sIL-6R α /sgp130 ratio. We have no explanation for divergent patterns, since our responders and non-responders did not show differences in the history of disease, clinical presentation and response to treatment.

O'Brien *et al.* have recently emphasized the importance of the early phase of increased resorption, which is essential for the development of GC-induced bone loss in a murine model (35). Would such a key-role be confirmed in patients, sIL-6R α could represent a promising target for new anti-resorptive drugs. Interestingly enough, a humanized monoclonal antibody

Figure 6 Effects of GC administration on serum sIL-6R α /sgp130 ratio in the responders ($n = 19$). sgp130 and sIL-6R α were measured on morning fasting blood samples before (day 1) and each day during treatment (days 2–5). Data are expressed as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ by Wilcoxon matched pair test.

against the human IL-6R α (called myeloma receptor antibody, MRA, or tocilizumab) is currently under investigation in a number of diseases including adult rheumatoid arthritis, systemic juvenile idiopathic arthritis and multiple myeloma. MRA has been shown to compete for both the membrane-bound and the soluble forms of the human IL-6R α , and to inhibit IL-6 function (36–37). To our knowledge, potential effects of MRA on GC-induced bone resorption has not yet been assessed.

Limitations of our study should be noted. First, we did not find any correlation between the increase of the bone resorption marker and the increase of serum sIL-6R α levels and sIL-6R α /sgp130 ratio. The lack of correlation could be accounted for by the small sample size, the inter-individual variation and, most importantly, the multiplicity of mechanisms that mediate GC-induced bone resorption and early changes of relevant markers. In particular, direct actions of GCs on osteoclasts would be independent of any significant change in local and circulating concentrations of hormones, cytokines and their receptors. Second, serum IL-6 was always below or near the detection limit of our ELISA. We cannot exclude that a more sensitive assay could detect changes in the very low range of concentrations. Finally, serum levels of IL-6, sIL-6R α and sgp130 may not reflect their concentrations in the bone microenvironment. IL-6 is constitutively expressed by human osteoblasts *in vitro*, is measurable in bone marrow plasma and is detectable by immunohistochemistry in bone specimens (38). Since GCs admittedly exert opposite effects (i.e., inhibitory and stimulatory, respectively) on cellular expression of IL-6 and sIL-6R α , direct evidence of such effects in the bone microenvironment should be searched for.

In conclusion, we have reported for the first time that sIL-6R α and sIL-6R α /sgp130 ratio are precociously increased in the peripheral blood of the vast majority of patients given high-dose, intravenous GCs. The increase of systemically available sIL-6R α conceivably results in the enhancement of IL-6-dependent osteoclastogenesis. Consistently with previous *in vitro* studies, our data suggest a role for IL-6 signalling in the GC-induced increase of bone resorption. The role of such a mechanism in the bone loss observed in inflammatory and immune-mediated diseases (where abundancy of IL-6 in the bone microenvironment is expected) requires further investigation.

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