Clodronate treatment reduces serum levels of interleukin-6 soluble receptor in Paget’s disease of bone

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Abstract

Objective

Interleukin-6 (IL-6) and its soluble receptor (sIL-6R) stimulate osteoclast formation and activity. The primary cell abnormality in Paget’s disease of bone (PDB) involves osteoclasts. Pagetic osteoclasts overproduce IL-6 and IL-6 receptor in vitro. In vivo, IL-6 serum levels are very high in the acute phase of PDB. The aim of this study was to evaluate the modification in the serum levels of IL-6, sIL-6R and osteotropic hormones (parathormone, 25OHD₃ and 1,25(OH)₂D₃) as a in long-term response to clodronate treatment in patients with PDB.

Methods

16 patients (8 females) with polyostotic PDB were studied. IL-6, sIL-6R and osteotropic hormones serum levels were evaluated in active PDB and after clodronate treatment (300 mg injected intravenously for 5 consecutive days). The sequential changes in total alkaline phosphatase (tALP) serum levels were used to assess the maximal pharmacological response to treatment.

Results

In untreated pagetic patients, mean serum levels of IL-6 (3.20 ± 1.18 pg/ml) and sIL-6R (35.02 ± 8.33 ng/ml) were significantly increased. Serum osteotropic hormone levels fell within the normal range. Eight weeks after treatment, the maximal pharmacological response to clodronate was associated with a significant reduction of sIL-6R serum levels in all patients, without a significant variation in serum IL-6 and osteotropic hormone levels. Moreover, we observed a correlation between lower sIL-6R serum levels before clodronate therapy and complete remission of PBD, defined as a decrease of tALP serum levels within the normal range.

Conclusion

The decrease in serum sIL-6R levels could be one of the molecular mechanisms that play a role in the clinical response to clodronate treatment in PDB.

Key words

Paget’s disease of bone, clodronate, IL-6 soluble receptor.

**Introduction**

Paget’s disease of bone is a focal disorder of unknown aetiology, characterised by increased and grossly distorted bone remodelling, bone hyperactivity and abnormal bone structure (1, 2). There is general agreement that the primary cell abnormality in Paget’s disease involves the osteoclasts. Pagetic osteoclasts are markedly increased in both number and size, can have up to 100 nuclei per cell and contain paramixoviral-like nuclear and cytoplasmatic inclusions (3).

*In vitro* studies have demonstrated that pagetic osteoclast precursors show an increased sensitivity to 1,25(OH)$_2$D$_3$ (4). In particular, the concentration of this hormone required to induce osteoclast formation in long-term marrow cultures from patients with Paget’s disease is at least 1 log lower than that required for normal osteoclast formation (5). Demulder and co-workers have demonstrated that 1,25(OH)$_2$D$_3$ hypersensitivity is an intrinsic and exclusive property of the early osteoclast precursor, granulocyte-macrophage colony-forming unit (GM-CFU) derived cells (6). Nevertheless, in patients with Paget’s disease, serum 25OHD$_3$ and 1,25(OH)$_2$D$_3$ levels fall within the normal range (7).

Osteoclast-like multinucleated cells in marrow cultures from patients with Paget’s disease show markedly increased expression of interleukin-6 (IL-6) and IL-6 receptor messenger RNA compared to normal subjects (8). In addition, IL-6 serum levels are markedly increased in patients with Paget’s disease (9), in whom clinically uninvolved bones show increased bone remodelling not caused by secondary hyperparathyroidism (10, 11). Experimental evidence suggests that IL-6 may be the stimulatory factor enhancing osteoclast formation and activity both within the lesion and at sites distant from the pagetic lesion (3).

Furthermore, Udagawa and co-workers (12) have confirmed a role of the IL-6/IL-6R complex in osteoclastic differentiation in the basal condition, showing that the ability of IL-6 to induce osteoclast differentiation depends on signal transduction mediated by IL-6R expressed on osteoblastic cells, but not on osteoclast progenitors. On the other hand, Neale and co-workers (13) suggested that osteoclast differentiation from circulating mononuclear precursor in Paget’s disease of bone did not occur when M-CSF was substituted by IL-6 and sIL-6R, and they did not stimulate osteoclast differentiation in the presence of M-CSF. Moreover, as for IL-6, elevated concentrations of sIL-6R have been found in biological fluids in conditions of increased bone resorption, such as multiple myeloma and oestrogen deficiency (14).

Bisphosphonates are now well established as the first choice drug in the treatment of Paget’s disease of bone (15). The main action of bisphosphonates is to induce marked and prolonged inhibition of bone resorption by decreasing osteoclast activity. The molecular mechanisms of these metabolic effects have not yet been elucidated (16). The aim of this study was to evaluate the long-term effect of clodronate treatment on serum IL-6, sIL-6R and osteotrophic hormones (25OHD$_3$, 1,25(OH)$_2$D$_3$ and PTH) in patients with active Paget’s disease of bone.

**Materials and methods**

**Subjects**

Sixteen patients (8 women and 8 men; aged 39-79 years; mean age 57.62 ± 9.9 years; BMI 26.7 ± 1.29 Kg/m$^2$, mean age at diagnosis 48.18 ± 9.5 years) with polyostotic Paget’s disease of bone were studied. All patients enrolled were treated from the diagnosis with i.v. clodronate infusion according to the criteria proposed by Yates and co-workers (17) (mean number of treatment cycles per year 1.90 ± 0.66). Patients gave their written, informed consent before entering the study, which was conducted in accordance with the Declaration of Helsinki. The diagnosis of Paget’s disease was based on radiological and scintigraphic evidence in all patients. Exclusion criteria were abnormal liver transaminase serum levels, which might interfere with the interpretation
of total serum alkaline phosphatase, and treatment with corticosteroids, thiazide diuretics, estrogens or drugs acting on the immune system. All patients had normal absolute leucocyte and lymphocyte cell values. None of the pagetic patients had been treated with bisphosphonate or calcitonin for at least six months prior to this study.

Methods
Before starting clodronate treatment, a fasting venous blood sample was drawn and serum ionised calcium (sCa), magnesium (sMg), phosphate (sPO4⁻), total alkaline phosphatase (tALP), intact parathormone (iPTH), 25-hydroxycholecalciferol (25(OH)D3), 1,25-dihydroxycholecalciferol (1,25(OH)2D3), interleukin-6 (IL-6) and interleukin-6 soluble receptor (sIL-6R) levels were determined. Twenty-four hour urine samples were also collected and analysed for calcium (uCa), phosphate (uPO4⁻), creatinine (uCr) and hydroxyproline (Hp) values.

Patients were treated with clodronate (dichloromethylene bisphosphonic acid), a bisphosphonate without an amino group in its chemical structure. Bisphosphonate was administered iv (300 mg/die in 500 ml NaCl 0.9% over 3 hours for 5 days; total dose 1500 mg) to ensure optimal conditions for drug delivery to the skeleton.

At the end of treatment, the patients were evaluated at weekly intervals for 4 months and the sequential changes in tALP (normal range 98-275 U/L) were used to assess the long-term pharmacological response to treatment. Using the criteria proposed by Khan and co-workers, response was defined as a decrease by at least 25% in tALP compared to the pre-treatment value (Responders). Remission was defined as a decrease in serum tALP values to within the laboratory reference range (Complete responders) (18). At the time corresponding to the nadir value of tALP serum activity, sCa, sMg, sPO4⁻, iPTH, 25(OH)D3, 1,25(OH)2D3, IL-6 and sIL-6R levels were determined. Twenty-four hour urine samples were also collected and analysed for uCa, uPO4⁻, uCre and Hp values.

Ionised calcium was determined using an ion-selective electrode. Urinary calcium and serum magnesium were determined by atomic absorption spectrophotometry. Serum and urinary phosphate, urinary creatinine and total alkaline phosphatase were measured by an enzymatic colorimetric assay. Intact PTH serum levels were measured using an immunometric assay (Immulite Intact PTH – Diagnostic Products Corporation, Los Angeles, USA) (normal range 1.3 - 7.6 pmol/l). The intra- and interassay coefficients of variation (CV) were 4.7 - 7.0% and 5.0 - 5.5%, respectively. 25(OH)D3 serum levels were estimated using a modified competitive protein binding assay (Vitamin D3 screen – Buhlmann Laboratories AG; Allschwil, Switzerland) (normal range 20-200 nmol/l). The intra- and interassay CVs were 5.3 - 9.8% and 7.3 - 11.6%, respectively. 1,25(OH)2D3 serum levels were estimated using a radioimmunoassay test (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) (normal range 40 - 160 pmol/l). The intra- and interassay CVs were 5.0 - 8.0% and 9.0 - 10.0%, respectively. Urinary hydroxyproline was evaluated by the method of Prockop and Udenfriend, modified according to Cleary and Sanders (normal range 0.1 - 0.3 mmol/24h) (19, 20). Urinary hydroxyproline was adjusted to 24h urinary creatinine excretion. Serum levels of IL-6 and sIL-6R were determined by commercially available ELISA kits (Quantikine, R&D Systems, Minneapolis, USA) with a sensitivity for IL-6 and sIL-6R of 0.70 pg/ml and 7 ng/ml, respectively. The intra- and interassay CVs were < 5% for serum IL-6 and < 10 % for serum sIL-6R. The “reference values” for serum IL-6 and sIL-6R levels were established by evaluation in age- and sex- matched healthy control subjects and were found to be 1.8 ± 0.9 pg/ml for IL-6 (95% confidence interval for mean 1.2 - 2.5) and 30.5 ± 3.8 ng/ml for sIL-6R (95% confidence interval for mean 28.33 - 32.60).

All parameters were measured according to the manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed with an SPSS statistics package, version 9.0. The one-way ANOVA test was applied to assess statistical differences between groups. The difference from baseline within the clodronate regime treatment was examined by the Student’s t-test.

Table I. Biochemical parameters in serum and urine of patients with Paget’s disease of bone before and 8 weeks after clodronate treatment (300 mg/day e.v. for 5 days).

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tALP (UI/L)</td>
<td>1728.8 ± 461.6</td>
<td>467.8 ± 241.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1,25 (OH)2D3 (pmol/L)</td>
<td>86.97 ± 15.56</td>
<td>89.26 ± 21.41</td>
<td>n.s.</td>
</tr>
<tr>
<td>25(OH)D3 (nmol/L)</td>
<td>72.85 ± 12.98</td>
<td>73.07 ± 14.18</td>
<td>n.s.</td>
</tr>
<tr>
<td>Intact PTH (pmol/l)</td>
<td>3.64 ± 1.12</td>
<td>3.70 ± 1.09</td>
<td>n.s.</td>
</tr>
<tr>
<td>Interleukin-6 (pg/ml)</td>
<td>3.0 ± 1.18</td>
<td>3.70 ± 1.58</td>
<td>n.s.</td>
</tr>
<tr>
<td>sIL-6 &lt; 10 (ng/ml)</td>
<td>35.02 ± 8.33</td>
<td>28.47 ± 6.26</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ionised calcium (mmol/L)</td>
<td>1.21 ± 0.02</td>
<td>1.20 ± 0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>Magnesium (mmol/L)</td>
<td>0.85 ± 0.08</td>
<td>0.86 ± 0.07</td>
<td>n.s.</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>1.14 ± 0.19</td>
<td>1.12 ± 0.18</td>
<td>n.s.</td>
</tr>
<tr>
<td>Urinary parameters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium (mmol/24h)</td>
<td>3.80 ± 1.7</td>
<td>3.61 ± 1.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Phosphate (mmol/24h)</td>
<td>28.4 ± 6.9</td>
<td>28.9 ± 7.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hp/Cr (mmol/mmol)</td>
<td>54.4 ± 3.9</td>
<td>27.2 ± 2.6</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Each value is expressed as Mean ± DS. P value < 0.05 is considered statistically significant; n.s. = not statistically significant. sIL-6R = soluble interleukin-6 receptor; tALP = total serum alkaline phosphatase; Hp/Cr = hydroxyproline/creatinine ratio.
Results

The biochemical parameters in patients with Paget's disease before and after clodronate treatment are shown in Table I. In the active phase of Paget's disease, mean IL-6 and sIL-6R serum levels were significantly higher (P < 0.05) in pagetic patients compared to age- and sex- matched healthy subjects (3.20 ± 1.18 pg/ml; 95% confidence interval for mean 2.38 - 4.01 pg/ml and 35.02 ± 8.33 ng/ml; 95% confidence interval for mean 31.02 - 39.10 ng/ml for IL-6 and sIL-6R, respectively). In the study population, we also found high levels of serum tALP (1728.8 ± 461.6 UI/l) and urinary Hp/Cr ratio (54.4 ± 3.9 mmol/mmol). Levels of 1,25(OH)₂D₃, 25(OH)D₃, iPTH, sCa²⁺, sPO₄⁻, sMg, uCa and uPO₄⁻ were in the normal range.

Clodronate treatment suppressed bone remodelling, as evidenced by the significant decreases (P < 0.05) in tALP and Hp/Cr ratio levels in all pagetic patients (tALP serum levels after treatment 467.8 ± 241.1 UI/l) and urinary Hp/Cr ratio levels after treatment 27.2 ± 2.6 mmol/mmol). In agreement with the data in the literature, the maximum response to treatment was obtained 8 weeks after the i.v. clodronate infusion (16, 18). Ten patients were responders and 6 patients were complete responders to clodronate treatment, using the criteria of Kahn et al.

As reported in Figure 1, the clodronate treatment was associated with a significant reduction (P < 0.05) in sIL-6R serum levels in all patients without a significant variation (P > 0.05) in IL-6 serum levels. Mean sIL-6R levels before therapy were significantly lower in complete responder pagetic patients compared to responder patients (27.48 ± 9.05 ng/ml vs 39.54 ± 8.04 ng/ml; P < 0.05) (Fig. 2).

No differences in 1,25(OH)₂D₃, 25OH D₃, iPTH, sCa²⁺, sPO₄⁻, sMg, uCa or sMg, uCa or tPO₄⁻ were in the normal range.
uPO$_4^-$ levels were detected 8 weeks after clodronate treatment.

Discussion

Cytokine interleukin-6 is considered to be an important regulator of bone cell function (21). In Paget’s disease of bone IL-6 is produced in large amounts by pagetic osteoclasts and appears to be an autocrine-paracrine factor that increases osteoclast formation and activity in bone lesions. Moreover, IL-6 may be responsible for the increased bone remodelling seen in unaffected bones at sites distant from the pagetic lesions (3).

IL-6 exerts its action through a cell surface receptor system that consists of two transmembrane subunits: the IL-6 binding gp 80 (named IL-6R) and the signal-transducing component gp 130, which is not ligand binding. IL-6 binds to IL-6R, and this complex subsequently associates with two gp 130 molecules. The soluble form of IL-6R (sIL-6R) is a 55 kDa protein generated by proteolytic cleavage of the membrane-associated receptor (IL-6R) at the site adjacent to the transmembrane domain or by differential mRNA splicing (22-26). sIL-6R binds IL-6 with affinity similar to the membrane-bound receptor and enhances the biological effects of IL-6 in various cell types, including osteoclasts (27). The amplified effect of the IL-6 due to its soluble receptor plays a role in several pathological conditions characterised by increased bone resorption (14, 28).

In this study, patients with active Paget’s disease of bone had elevated IL-6 and sIL-6R serum levels. We did not observe a significant correlation between disease activity, as evaluated by tALP serum levels, and IL-6 or sIL-6R serum levels.

The pharmacological and clinical response to clodronate therapy was associated with a significant reduction in sIL-6R serum levels. In particular, we observed a correlation between lower sIL-6R serum levels before clodronate treatment and complete remission of disease, defined as a decrease in tALP serum levels to within the laboratory reference range.

Eight weeks after clodronate treatment, IL-6 serum levels did not vary significantly. Previous experimental studies reported that in vitro IL-6 release from monocyte-macrophage cells is inhibited when cell cultures are treated with clodronate for 20h (29). In vivo, no change in IL-6 serum levels was seen 48h after clodronate infusion in patients with malignancy (30). To our knowledge, no data on long term variations in IL-6 serum levels after clodronate treatment are available in the literature.

The observed reduction in sIL-6R serum levels was not accompanied by significant variations in serum Ca$^{2+}$, 25OHD$_3$, 1,25(OH)$_2$D$_3$, or iPPTH levels. In the short term after bisphosphonate treatment, alterations in the calcium homeostasis with a secondary increase in 1,25(OH)$_2$D$_3$, and iPPTH serum levels have been described (31). Eight weeks after clodronate treatment, these transient pharmacological effects disappeared in our patients.

These results may be related to the pharmacological intracellular effects of bisphosphonates. Bisphosphonates are pyrophosphate analogues in which the oxygen bridge has been replaced by a carbon with various side chains (P-C-P) (32). The pharmacological action of these compounds was originally ascribed to their physico-chemical effects on hydroxyapatite crystals, but it gradually become clear that cellular effects must also be involved. Bisphosphonates are internalised by osteoclasts, thereby interfering with specific biochemical processes (33). Clodronate can be metabolically incorporated into the non-hydrolysable analogue of ATP (AppCCl$_2$p). The intracellular accumulation of this metabolite inside the osteoclast inhibits ATP-dependent intracellular enzymes and osteoclast function (29).

Recently, Abildgaard and co-workers have described a significant reduction in sIL-6R serum levels in patients affected by multiple myeloma after long-term treatment with bisphosphonate (34).

In addition to affecting mature osteoclasts, bisphosphonates inhibit osteoclast differentiation and recruitment. In vitro the formation and differentiation of osteoclast-like cells in long-term cultures of human bone marrow has been induced by the IL-6/sIL-6R system and inhibited by bisphosphonates in a dose-dependent pattern (35, 36).

Our data suggest that the ability of bisphosphonate to inhibit several protein tyrosine phosphatases plays an important role in the modulation of the IL-6/sIL-6R system with regard to signal transduction on the cellular surface. In fact this complex binds two gp130 molecules (the signal transducing b-chain in the IL-6 receptor) to form a gp130 disulphide linked homodimer. Dimerization results in gp130 tyrosine phosphorylation; this process is required for signal transduction and clodronate could be able to down-regulate this cellular mechanism in osteoclasts.

In conclusion, this study describes one of the possible mechanisms of action of clodronate in Paget’s disease of bone. The pharmacological response to parenteral clodronate treatment could be in part related to the decrease in serum sIL-6R levels. Moreover, lower sIL-6R serum levels before clodronate treatment are associated with a complete remission of the disease. The sIL-6R serum level before therapy could perhaps also be a marker of a complete pharmacological response to clodronate treatment in the clinical management of PDB.

Acknowledgement

The authors are grateful to Ms Rosanna Scala for editorial advice.

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