The influence of serum cytokines and growth factors on osteoclast formation in Paget’s disease

S.D. NEALE¹, E. SCHULZE¹, R. SMITH¹,² and N.A. ATHANASOU¹,²

From the ¹Nuffield Department of Orthopaedic Surgery, University of Oxford, and ²Department of Pathology, Nuffield Orthopaedic Centre, Oxford, UK

Summary

Background: Osteoclasts are multinucleated cells (MNCs) that form from circulating mononuclear precursors in the presence of the receptor activator of nuclear factor κB-ligand (RANKL) and macrophage-colony stimulating factor (M-CSF).

Aim: To determine whether cytokines and growth factors influence RANKL/M-CSF induced osteoclastogenesis and bone resorption in Paget’s disease.

Design: Prospective case-control study.

Methods: Serum levels of M-CSF, interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNFα) were measured in 13 Paget’s disease patients and 8 normal controls. The effect of serum from Paget’s patients on osteoclast formation was also assessed.

Results: Serum levels of IL-1β, IL-6 and TNFα were low or undetectable in Paget’s disease patients and normal controls. Levels of M-CSF were significantly increased in Paget’s patients who were not currently under treatment. In Paget’s patients under treatment, serum M-CSF levels were not significantly different from normal controls. The addition of serum from untreated Paget’s patients dose-dependently increased RANKL-induced osteoclast formation and lacunar resorption in normal monocyte cultures; elevated IL-6 levels were found in the supernatant and the addition of a specific antibody to human IL-6 blocked the increase in osteoclast formation and resorption. Serum from untreated Paget’s patients also induced osteoclast formation in the absence of exogenous M-CSF; an antibody specific to human M-CSF abolished this effect.

Discussion: Both M-CSF and IL-6 play a major role in osteoclast formation and bone resorption in Paget’s disease and measurement of serum M-CSF may provide a useful indicator of disease activity.

Introduction

Paget’s disease is a disorder of bone remodelling in which there is an uncoupling of osteoclastic bone resorption and osteoblastic new bone formation.¹ This imbalance is reflected by an increase in the number and resorbing activity of osteoclasts at sites of Paget’s disease. Osteoclasts are multinucleated cells that are formed from circulating mononuclear precursors in the presence of the receptor activator of nuclear factor κB-ligand (RANKL) and macrophage-colony stimulating factor (M-CSF).²,³ Several growth factors and cytokines are known to influence RANKL/M-CSF induced osteoclast formation.⁴

A number of in vitro studies have shown that the increase in osteoclast numbers in Paget’s disease occurs as a result of increased local production of cytokines and growth factors.¹,⁵–⁷ A role for IL-6 in promoting the increased osteoclast formation in Paget’s disease is suggested by the finding of high levels of IL-6 in the culture medium of Pagetic marrow cultures, and the stimulatory effect of this medium on osteoclast formation in normal

Address correspondence to Dr N.A. Athanasou, Department of Pathology, Nuffield Orthopaedic Centre, Windmill Road, Headington, Oxford OX3 7LD. e-mail: nick.athanasou@ndos.ox.ac.uk

© Association of Physicians 2002
bone marrow cell cultures. However, it is not certain whether the serum concentration of IL-6 is increased in Paget’s disease patients, and whether osteoclastic multinucleated cells which form in Pagetic bone produce an increased amount of IL-6 or exhibit increased expression of mRNA for IL-6 and IL-6 receptor. Overproduction of IL-1 and TNF α may play a role in the increased bone turnover in Paget’s disease. IL-1 and TNF α are known to promote human osteoclast formation and osteoclastic bone resorbing activity. TNF α, in combination with M-CSF and IL-1, has also been shown to promote human osteoclast formation by a mechanism independent of RANKL.

In this study, we have sought to analyse the role of cytokines/growth factors in stimulating osteoclast formation in Paget’s disease. We have compared serum levels of IL-6, IL-1 β, TNF α and M-CSF in Paget’s disease patients and normal controls. We have also examined the effect of peripheral blood serum from patients with untreated Paget’s disease and normal controls on osteoclast formation and lacunar resorption in long-term human peripheral blood mononuclear cell cultures, and identified those growth factors and cytokines which stimulate osteoclast formation.

Methods

Measurement of peripheral blood serum cytokine and growth factor levels

Serum was collected from the peripheral blood of 13 Paget’s patients (8 male, 5 female) (one patient was examined before and after treatment) and eight controls (5 male, 3 female) who had normal serum calcium, phosphate and alkaline phosphatase (SAP) and were not under treatment (Table 1). The age range of the Paget’s disease patients was 41–84 years (median 71 years) and that of the controls, 45–86 years (median 73 years). Ethical approval was obtained and each patient/volunteer gave his/her informed consent. Aliquots were stored at −70 °C until ready for testing. Serum levels of IL-1 β, TNF α and M-CSF were measured using specific human enzyme-linked immunoassays (ELISAs) (R&D Systems). Levels of IL-6 were measured using a high sensitivity human IL-6 ELISA kit (Amersham Pharmacia Biotech). Corresponding recombinant human cytokines/growth factors were used as positive controls. All concentrations below the detection limits of the assays (IL-1 β, 1.5 pg/ml; TNF α, 4.0 pg/ml; IL-6, 0.48 pg/ml) were recorded as non-detectable. Each serum sample was assayed in duplicate.

Isolation of peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated by Ficoll-Paque sedimentation and adherence from peripheral blood. Each 4 ml of blood was diluted 1:1 with minimal essential medium (MEM) (Gibco), layered over 5 ml of Ficoll-Paque (Pharmacia Biotech), and centrifuged at 510 g for 20 min. The mononuclear cell-rich layer at the interface was removed, washed twice in MEM and the pellet then resuspended in MEM supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin sulphate, 2 mM L-glutamine.

Table 1  Clinical details of patients with Paget’s disease of bone

<table>
<thead>
<tr>
<th>Age (years)/Sex</th>
<th>Previous treatment history</th>
<th>Total SAP levels (IU/l)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>83/M</td>
<td>None</td>
<td>1079</td>
</tr>
<tr>
<td>71/M</td>
<td>None**</td>
<td>2214</td>
</tr>
<tr>
<td>84/F</td>
<td>None</td>
<td>780</td>
</tr>
<tr>
<td>68/M</td>
<td>None**</td>
<td>460</td>
</tr>
<tr>
<td>71/M</td>
<td>None**</td>
<td>2485</td>
</tr>
<tr>
<td>84/M</td>
<td>None**</td>
<td>1040</td>
</tr>
<tr>
<td>41/F</td>
<td>None</td>
<td>91</td>
</tr>
<tr>
<td>64/M</td>
<td>None**</td>
<td>222</td>
</tr>
<tr>
<td>69/M***</td>
<td>None</td>
<td>1038</td>
</tr>
<tr>
<td>69/M***</td>
<td>Pamidronate infusion (3×60 mg), 1 month previously</td>
<td>826</td>
</tr>
<tr>
<td>69/M***</td>
<td>Pamidronate infusion (3×60 mg), 3 months previously</td>
<td>461</td>
</tr>
<tr>
<td>75/F</td>
<td>None**</td>
<td>264</td>
</tr>
<tr>
<td>82/F</td>
<td>Alendronate (40 mg daily), 5 months previously</td>
<td>181</td>
</tr>
<tr>
<td>74/F</td>
<td>None</td>
<td>494</td>
</tr>
<tr>
<td>81/M</td>
<td>Alendronate (60 mg daily) for 6 months</td>
<td>105</td>
</tr>
</tbody>
</table>

*Normal local reference range 100–250 IU/l. **Oral Alendronate or intravenous Pamidronate 6 or more months previously, but not currently under treatment. ***Same patient.
(Gibco) and 10% fetal calf serum (FCS) (Gibco) (MEM/FCS). The number of cells in the cell suspension was counted in a haemocytometer after lysis of the red blood cells using a 5% (v/v) acetic acid solution.

**Osteoclast formation in vitro from PBMCs: the effect of peripheral blood serum from Paget’s patient and normal controls**

PBMCs (5×10⁵ cells/well) were added to either glass coverslips (6 mm) or pre-wetted dentine slices (4×4 mm) placed in 7-mm wells of a 96-well Multiwell plate. After 1–2 h incubation, the coverslips and dentine slices were removed from the wells, washed vigorously in MEM to remove the non-adherent cells, and placed in larger 16 mm wells containing either 1 ml MEM/FCS or 1 ml of this culture medium in which the FCS was replaced by the appropriate volume of human serum, resulting in a 1% or 10% final concentration in the culture medium. These cultures were incubated at 37 °C in 5% CO₂ for up to 21 days in the presence of 10⁻⁸ M dexamethasone (Sigma-Aldrich) with or without 30 ng/ml soluble RANKL (Amgen) and/or 25 ng/ml recombinant human M-CSF (R&D Systems). The culture medium was changed every 3–4 days, at which time fresh factors were added.

The coverslips were removed from the cultures after 14 days incubation and stained immunohistochemically using an indirect immunoperoxidase method with the monoclonal antibody 23C6 (Serotec) to determine expression of the vitronectin receptor (VNR), an osteoclast-associated antigen. After 21 days incubation, the dentine slices were removed from the wells and left overnight in 1 M ammonium hydroxide to remove the adherent cells. After rinsing in distilled water, the dentine slices were stained with 0.5% (v/v) toluidine blue prior to examination by light microscopy. The surface of each dentine slice was examined for evidence of lacunar resorption, and the percentage surface area resorbed on each dentine slice measured using an image analysis system.

**Analysis of the effect of M-CSF and IL-6 on osteoclast formation in Paget’s disease**

To determine the effect of M-CSF and IL-6 on serum-enhanced osteoclast formation from mononuclear phagocyte precursors, 10 μg/ml anti-human M-CSF antibody or 10 μg/ml anti-human IL-6 antibody (R&D Systems) was added to cultures containing 30 ng/ml RANKL, 10⁻⁸ M dexamethasone and 10% Paget's or normal serum in the presence and absence of M-CSF. Anti-human IL-6 and anti-human M-CSF antibodies were added at the beginning of the incubation period and every media change thereafter. To measure the level of IL-6 released, the supernatant was collected during the medium change from cultures incubated in the presence and absence of anti-human IL-6 antibody. The supernatants were collected at days 3, 7, 10 and 14, centrifuged to remove cell debris, and stored at −20 °C. Levels of IL-6 were measured using a specific human IL-6 ELISA (R&D Systems).

**Statistics**

Each experiment was carried out in triplicate. Significance was determined using the Mann–Whitney Rank Sum test with p<0.05 considered as statistically significant.

**Results**

**Measurement of peripheral blood serum cytokine and growth factor levels**

We did not find detectable levels of IL-1β or TNFα in the serum of either Paget’s disease patients (n=13) or normal controls (n=8). IL-6 levels ranged from non-detectable in both groups to 4.5 pg/ml in the serum of Paget’s disease patients and 3.7 pg/ml in the serum of normal controls. Mean serum IL-6 levels in Paget’s patients who had high or low SAP levels, and those Paget’s patients not currently under treatment, were not significantly different from those of normal controls (p=0.12, p=0.07 and p=0.05, respectively) (Table 2).

The concentration of M-CSF was increased two-fold in the serum of Paget’s disease patients who were not currently under treatment compared to normal controls (p=0.012) (Table 2). The mean (±SEM) serum M-CSF level was 944.4±117.4 pg/ml in untreated Paget’s disease patients and 450.9±51.1 pg/ml in normal controls. This increase in serum M-CSF was noted in Paget’s patients whether their SAP levels were > 500 IU/l or < 500 IU/l. (Table 2). The serum M-CSF level of Paget’s patients who were under treatment was significantly lower than that of untreated Paget’s patients (p=0.017), and not significantly different from that of normal controls (p=0.358).
The serum M-CSF levels of one patient who underwent three 60 mg intravenous infusions of the aminobisphosphonate Pamidronate over the course of 3 weeks, were 1375 pg/ml prior to treatment, and 370 pg/ml and 437.5 pg/ml 1 and 3 months after treatment, respectively. The corresponding SAP levels were 1038, 826 and 461 IU/l.

Osteoclast formation in vitro from PBMCs: the effect of peripheral blood serum from Paget’s patient and normal controls

The addition of serum from Paget’s disease patients (n = 3) could induce the formation of a few osteoclasts capable of lacunar resorption in the absence of exogenous M-CSF. As expected, the addition of a specific antibody to human M-CSF abolished osteoclast formation and lacunar resorption in PBMC cultures to which either 10% Paget’s or 10% normal serum but not M-CSF had been added. An average of four VNR-positive MNCs per low-power field was noted on each coverslip. Osteoclast differentiation was not induced by Paget’s or normal serum when RANKL was omitted from the cultures.

Role of IL-6

In cultures of normal monocytes to which 10% Paget’s disease patient serum (n = 3) and M-CSF were added, we detected strikingly high IL-6 levels in the supernatant. After three days incubation, the mean level of IL-6 in PBMC cultures to which 10% Paget’s serum had been added was 11908.0 pg/ml compared with 7561.2 and 2569.6 pg/ml in PBMC cultures containing 10% normal serum and 10% FCS, respectively (n = 2 experiments). After seven days incubation, IL-6 levels were decreased, measuring 544.2, 256.2 and 106.9 pg/ml in PBMC cultures containing 10% Paget’s serum, 10% normal serum and 10% FCS, respectively. IL-6 was not detected in day 10 or day 14 supernatants, nor was it detected in the supernatants of all PBMC cultures to which a specific antibody to human IL-6 was added. The addition of an anti-IL-6 antibody markedly inhibited the Paget’s serum-induced increase in osteoclast formation and lacunar resorption (n = 3 experiments; Figure 3). In PBMC cultures to which

### Table 2  Serum M-CSF and IL-6 concentrations in Paget’s disease patients and normal controls

<table>
<thead>
<tr>
<th></th>
<th>M-CSF levels (pg/ml)</th>
<th>IL-6 levels (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paget’s patients not currently under treatment*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAP &gt; 500 IU/l (n = 6)</td>
<td>843.8 ± 136.2**</td>
<td>2.06 ± 0.55</td>
</tr>
<tr>
<td>SAP &lt; 500 IU/l (n = 5)</td>
<td>1045.0 ± 194.7**</td>
<td>1.10 ± 0.14</td>
</tr>
<tr>
<td>Both (n = 11)</td>
<td>944.4 ± 117.4**</td>
<td>1.58 ± 0.31</td>
</tr>
<tr>
<td>Paget’s patients currently under treatment (n = 4)</td>
<td>369.2 ± 39.7</td>
<td>0.73 ± 0.24</td>
</tr>
<tr>
<td>Normal controls (n = 8)</td>
<td>450.9 ± 51.1</td>
<td>1.10 ± 0.37</td>
</tr>
</tbody>
</table>

Results are mean levels of release ± SEM. *Paget’s disease patients not under treatment within the last 6 months before blood was taken. **p < 0.05 compared with normal controls.
10% Paget’s serum and M-CSF had been added, the mean (± SEM) percentage surface area of lacunar resorption in the presence of anti-IL-6 antibody was 14.8% ± 5.4%, compared with 38.0% ± 4.8% in PBMC cultures which did not contain anti-IL-6 antibody (p = 0.018). In the absence of M-CSF, the addition of anti-IL-6 had no effect on osteoclast formation and lacunar resorption.

**Discussion**

Both osteoclast numbers and activity are increased in bones affected by Paget’s disease. In this study, the serum of untreated Paget’s patients markedly stimulated osteoclast formation and resorption. Osteoclast formation was significantly increased in PBMC cultures incubated with Paget’s but...
not normal serum. We also found that the serum concentration of M-CSF is significantly higher in patients with untreated Paget’s disease relative to normal controls and treated Paget’s patients, and that the serum from Paget’s patients could induce osteoclast differentiation in the absence of exogenous M-CSF; this was due to the presence of endogenous M-CSF, as an antibody specific to M-CSF completely inhibited this effect.

M-CSF, a growth factor for cells of the monocyte-macrophage lineage, plays a critical role in the process of osteoclast formation and bone resorption. Studies of the disease osteopetrosis in op/op mice, which is characterized by a severe reduction in osteoclast numbers, have shown that M-CSF is essential for osteoclast formation. In vitro studies have shown that M-CSF is required for both the proliferation and differentiation of murine and human osteoclast precursors. In combination with the recently discovered osteoclast differentiation factor, RANKL, M-CSF induces human osteoclast formation in vitro in the absence of stromal cells. Osteoclast formation from circulating precursors under these conditions is stimulated by corticosteroids.

In this study, circulating levels of M-CSF in patients with untreated Paget’s disease were significantly higher than in normal controls and Paget’s patients who were under treatment. M-CSF is produced by many cell types, including osteoblasts, marrow fibroblasts and endothelial cells. In Paget’s disease, the bone marrow contains abundant well vascularized cellular fibrous tissue, and the bone surface is lined by plump osteoblasts. It is possible that these stromal cells in the abnormal bone microenvironment in Pagetic lesions represent the source of the increased serum M-CSF formed in untreated Paget’s patients. Over-expression of M-CSF by these cells, which have been shown to exhibit enhanced RANKL expression, may result in the proliferation and differentiation of more osteoclast precursors, which are hypersensitive to RANKL, at the disease-affected site and the formation of increased numbers of osteoclasts.

Our in vitro studies provide support for this theory: peripheral blood serum from Paget’s disease patients markedly increased osteoclast formation and lacunar resorption compared with control cultures incubated in the presence of fetal calf serum.

The serum concentration of IL-6 in our Paget’s patients was low and not significantly different from that of normal controls who had no evidence of bone disease. IL-1β and TNFα levels were also not detectable in the peripheral blood serum of patients with Paget’s disease of bone. However, in the cultures with an increase in osteoclast formation and bone resorption induced by untreated Paget’s patient serum, we found elevated IL-6 levels in the supernatant. The addition of a specific antibody to IL-6 inhibited the increase in osteoclast formation induced by untreated Paget’s patients serum. Our results suggest a role for both M-CSF and IL-6 in the regulation of osteoclast formation in Paget’s disease. IL-6 stimulates the development of osteoclast progenitor cells and, in combination with soluble IL-6R, increases osteoclast formation in mouse and human bone marrow cultures.

A role for IL-6 in promoting the increased osteoclast formation in Paget’s disease was suggested by the finding of elevated levels of IL-6 in the culture supernatant of long-term Pagetic marrow cultures. The addition of this supernatant to normal marrow cultures enhanced osteoclast formation, and an antibody specific to IL-6 blocked this effect. Furthermore, an increase in the concentration of IL-6 in marrow plasma and peripheral blood serum was noted in patients with Paget’s disease compared to normal marrow plasma or peripheral blood. Although, unlike other investigators, we did not find elevated serum IL-6 levels in Paget’s disease patients, we did find elevated IL-6 levels in the supernatant in PBMC cultures to which untreated Paget’s patients serum was added. The majority of this release of IL-6 occurred during the first four days of culture. Further, the addition of a specific anti-IL-6 antibody to normal monocyte cultures blocked the increase in osteoclast formation induced by Paget’s serum. These results suggest a role for IL-6 in the local stimulation of osteoclast formation and bone resorption in Paget’s disease.

Bisphosphonates are very effective at treating Paget’s disease. In our study, treatment with the aminobisphosphonate Pamidronate significantly decreased the circulating concentration of M-CSF in patients with Paget’s disease. One patient underwent three 60 mg infusions of intravenous APD over the course of 3 weeks. The serum M-CSF levels of this patient before and after treatment correlated with the level of disease activity. Inhibition of bone resorption by bisphosphonates has mainly been attributed to an inhibitory effect on the function of mature osteoclasts and induction of apoptosis. Bisphosphonates have also been shown to inhibit osteoclastic activity indirectly by preventing recruitment and differentiation of osteoclast precursor cells. The reduction in bone resorption induced by bisphosphonate treatment is followed by a decrease in osteoblast activity, most likely due to the close coupling that exist between bone formation and resorption. The lower M-CSF levels noted in bisphosphonate-treated patients could thus be due to a decrease in M-CSF production by osteoblasts; this may represent another mechanism whereby
osteoclast numbers in Paget’s disease are reduced following bisphosphonate treatment.

In summary, serum M-CSF levels are significantly increased in Paget’s patients who are not currently under treatment and have active disease; serum levels of IL-1β, IL-6 and TNFα were low or undetectable in Paget’s disease patients and normal controls. In Paget’s patients under treatment, serum M-CSF levels were not significantly different from normal controls. Moreover, the addition of serum from untreated Paget’s patients dose-dependently increased RANKL-induced osteoclast formation and lacunar resorption in normal monocyte cultures; the addition of a specific antibody to human IL-6 blocked this increase in osteoclast formation and resorption. Furthermore, serum from untreated Paget’s patients promoted RANKL-induced osteoclast formation in the absence of exogenous M-CSF, and an antibody specific to human M-CSF abolished this effect. These results suggest that both M-CSF and IL-6 play a role in inducing osteoclast formation and bone resorption in Paget’s disease, and that measurement of serum M-CSF may provide a useful indicator of disease activity.

Acknowledgements

The authors thank all the Paget’s disease patients and normal volunteers who kindly donated blood samples for this study, and Dr Matthew Brown and Professor John Wass for their co-operation. The authors also thank Dr D. Lacey, Amgen Inc., for providing the RANKL. This work was funded by the National Association for the Relief of Paget’s Disease. Dr. Schulze is a Research Fellow funded by German Academic Exchange Service, University Programme III.

References


