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Article

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Enhanced RANK ligand expression and responsiveness of bone marrow cells in Paget's disease of bone

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Paget's disease is characterized by highly localized areas of increased osteoclast (OCL) activity. This suggests that the microenvironment in pagetic lesions is highly osteoclastogenic, or that OCL precursors in these lesions are hyperresponsive to osteoclastogenic factors (or both). To examine these possibilities, we compared RANK ligand (RANKL) mRNA expression in a marrow stromal cell line developed from a pagetic lesion (PSV10) with that in a normal stromal cell line (Saka), and expression in marrow samples from affected bones of Paget's patients with that in normal marrow. RANKL mRNA was increased in PSV10 cells and pagetic marrow compared with Saka cells and normal marrow, and was also increased in marrow from affected bones compared with uninvolved bones from Paget's patients. Furthermore, pagetic marrow cells formed OCLs at much lower RANKL concentrations than did normal marrow. Anti-IL-6 decreased the RANKL responsiveness of pagetic marrow to normal levels, whereas addition of IL-6 to normal marrow enhanced RANKL responsiveness. Thus, RANKL expression and responsiveness is increased in pagetic lesions, in part mediated by IL-6. These data suggest that the combination of enhanced expression of RANKL in affected bones and increased RANKL sensitivity of pagetic OCL precursors may contribute to the elevated numbers of OCLs in Paget's disease.

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Introduction

Paget's disease is a highly localized process in which large numbers of abnormal osteoclasts (OCLs) induce increased bone resorption (1, 2). The primary cellular abnormality in Paget's disease resides in the OCLs. The OCLs are increased in number and size, have increased nuclei per multinucleated cell, contain paramyxoviral-like nuclear inclusions (3), and are hyperresponsive to 1,25-dihydroxyvitamin D₃, or 1,25-(OH)₂D₃ (4). They also express high levels of osteotropic factors that can regulate OCL activity, including IL-6 (5), IL-6 receptor, and NF-κB (6). In addition, A.P. Mee (7) has reported that the *BCL2* gene is overexpressed in OCLs from patients with Paget's disease, suggesting that the OCL life span may be prolonged in pagetic lesions. However, the basis for the increased OCL formation and the highly localized nature of Paget's disease is still unknown. Recently, RANK ligand (RANKL), a newly described member of the TNF family, has been identified as a critical osteoclastogenic factor (8-10). RANKL is expressed on marrow stromal cells and osteoblasts, and appears to mediate the effects of most osteoclastogenic factors.

In vivo studies have shown that mice lacking RANKL developed severe osteopetrosis (11). Factors such as 1,25-(OH)₂D₃, IL-1, IL-11, and prostaglandin E₂ appear to induce OCL formation indirectly by upregulating RANKL expression on marrow stromal cells (12). These data suggest that RANKL may be the common mediator for the effects of most osteotropic factors on OCL formation. Therefore, we have examined the relative levels of RANKL expression by marrow stromal cells from normal bones and affected bones from patients with Paget's disease, and the RANKL responsiveness of OCL precursors from normal bones and affected bones from Paget's patients.

Methods

These studies were approved by the Institutional Review Board at the University of Texas Health Science Center at San Antonio and the Auckland Ethics Committee.

OCL culture. Nonadherent human bone marrow cells were collected from eight normal volunteers. Samples were also taken from involved bones of six patients with Paget's disease, and both involved and uninvolved bone from two other Paget's patients. Cells were pre-

pared by density gradient centrifugation followed by adherence to plastic as described previously (13). All pagetic patients had elevated serum alkaline phosphatase levels and had not received bisphosphonate therapy for at least 6 months before marrow aspiration. Marrow cells (10^6 cells/mL) were cultured in α -MEM supplemented with 20% horse serum (HyClone Laboratories, Logan, Utah, USA) in the presence or absence of recombinant human RANKL (0–100 ng/mL), 25 ng/mL macrophage colony-stimulating factor (MCSF) (R&D Systems Inc., Minneapolis, Minnesota, USA), and 10^{-7} M dexamethasone (Sigma Chemical Co., St. Louis, Missouri, USA). Half of the media was replaced every 2 days. After 3 weeks of culture, the cells were fixed and then stained for cross-reactivity with the 23c6 mAb that identifies multinucleated cells that fulfill the functional characteristics of OCLs (14). In selected experiments, RANK Fc (0–100 ng/mL) (Immunex Corp., Seattle, Washington, USA) was added to the cultures to block the effects of RANKL (100 ng/mL) on OCL formation (15, 16).

Stromal cell cultures. A marrow stromal cell line, PSV10, derived from an involved bone from a patient with Paget's disease (17), and a normal marrow stromal cell line, Saka (18), were cultured in α -MEM supplemented with 10% FCS. Both of these cell lines support OCL formation. After reaching confluence, the cells were treated with $1,25\text{-(OH)}_2\text{D}_3$ (10^{-9} M) or vehicle. RNA was extracted using RNeasy (Tel-Test Inc., Friendswood, Texas, USA) following the manufacturer's instructions, and were then subjected to RT-PCR analysis for RANKL mRNA expression as described below.

Activity of c-Jun kinase. Nonadherent mononuclear bone marrow cells from two Paget's patients and three normal donors, prepared as described above, were cultured in α -MEM and 20% horse serum in the presence of varying concentrations of RANKL (25–100 ng/mL). After 30 minutes of incubation with RANKL, the cells were harvested in ice-cold PBS and centrifuged at 700 g for 5 minutes. The cells were resuspended in cell lysis buffer composed of 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, and 1 mM sodium vanadate, in the presence of 1 μ g/mL leupeptin, 1 mM PMSF, and 1% Triton X-100, followed by a brief sonication. Cell lysates were cleared by centrifugation for 15 minutes at 12,000 g. Activity of c-Jun NH₂-terminal kinase was measured using a solid-phase glutathione-S-transferase-c-Jun (GST-c-Jun) (1–89 amino acids) fusion protein as described (19). Briefly, c-Jun kinase was immunoprecipitated with its substrate conjugated to glutathione-Sepharose® beads at 4°C. After overnight incubation, the precipitates were washed twice with cell lysis buffer and then with kinase buffer composed of 25 mM Tris (pH 7.5), 5 mM β -glycerolphosphate, 1 mM sodium vanadate, 2 mM DTT, and 10 mM MgCl₂. After the last wash, pellets were resuspended in 50 μ L of kinase buffer. The reaction was carried out at 30°C for 30 minutes in the presence of

100 μ M ATP. The reaction was stopped by adding 25 μ L of 3 \times SDS loading buffer composed of 50 mM Tris (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. Proteins were separated on 12% SDS-PAGE gels and transferred to nitrocellulose filters. Phosphorylated c-Jun kinase was detected with a specific anti-phospho-c-Jun kinase Ab following the manufacturer's instructions (New England Biolabs Inc., Beverly, Massachusetts, USA). An indirect method was also used to measure c-Jun kinase, using anti-phospho-c-Jun kinase as described (20).

RANKL mRNA expression. We evaluated the relative levels of RANKL mRNA expression using semiquantitative RT-PCR. Briefly, RNA from the marrow stromal cell lines and total marrow samples from Paget's patients and normal individuals was reverse transcribed using Moloney leukemia virus reverse transcriptase for 15 minutes at 42°C, followed by heating for 5 minutes at 95°C. The reverse transcription reaction was carried out in 20 μ L, and half of the reaction mixture was used for detecting β -actin mRNA expression. The remaining PCR reaction mixture was used for measuring RANKL expression. The gene-specific primers for RANKL mRNA (a generous gift from Babatunde Oyajobi, University of Texas Health Science Center, San Antonio, Texas, USA) (GenBank accessions AF013171, partial, and AF019047, full-length) were 5'-ACT GGA TCC GGA TCA GGA TG-3' (sense) and 5'-AGC TGC GAA GGG GCA CAT GA-3' (antisense). PCR was performed by denaturing at 94°C for 1 minute followed by incubation at 60°C for 1 minute, for 30 cycles. These conditions were during the linear phase of the PCR reaction. The reactions were terminated by incubating at 60°C for 7 minutes. PCR products were separated in 1.2% agarose gel, and the relative amounts were quantitated by densitometry and corrected for β -actin mRNA levels. The primers for β -actin were 5'-GGC CGT ACC ACT GGC ATC GTG ATG-3' (sense) and 5'-CAT CTC TTG CTC GAA GTC CAG GGC-3' (antisense). The PCR results were reproducible in three independent experiments.

Western blot analysis. Five million stromal cells or marrow cells were incubated in lysis buffer for 10 minutes on ice, and the total cell lysates were cleared by centrifugation at 12,000 g for 15 minutes. After measurement of total protein by the Bradford reaction (using a kit from Bio-Rad Laboratories Inc., Hercules, California, USA), 30 μ g of each lysate was separated by SDS-PAGE and then transferred to nitrocellulose filters. Nonspecific binding sites were saturated by incubating the membranes in blocking buffer (5% nonfat milk in 10 mM Tris at pH 8.0, 150 mM NaCl, and 0.1% Tween-20) for 1 hour at room temperature. Membranes were incubated with rabbit polyclonal anti-RANK (1:1,500; Immunex Corp.) or a monoclonal anti-RANKL Ab (1 ng/mL) (R&D Systems Inc.). After washing, the secondary Ab conjugated to horseradish peroxidase was added to a blocking solution and incubated for 1 hour at room temperature. Specific bands

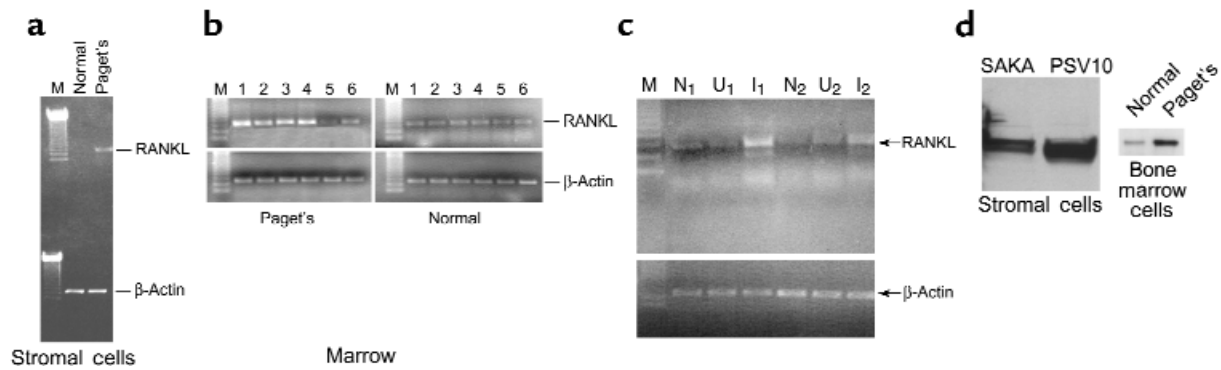


Figure 1

Expression of RANKL mRNA in bone marrow cells and stromal cells from patients with Paget's disease and normal individuals. Total RNA was extracted from normal (Saka) and Paget's (PSV10) stromal cells or from marrow samples from normal donors and involved bones from patients with Paget's disease. In addition, bone marrow aspirates were obtained from two normal individuals (N) and from uninvolved (U) and involved (I) bones from two patients with Paget's disease. RANKL mRNA expression was determined by RT-PCR as described in Methods. RANKL mRNA expression was elevated in six of eight patients with Paget's disease compared with eight normal donors (a, c), in the PSV10 cell line compared with Saka cells (b), and in the involved bones of both patients (I₁ and I₂) compared with uninvolved bones (U₁ and U₂) from these same patients (c). The levels of mRNA expression in uninvolved bones from patients with Paget's disease were similar to those from normal individuals tested at the same time (N₁ and N₂). Western blot analysis of marrow samples from a Paget's patient or a normal donor, and a marrow stromal cell line derived from a Paget's patient (PSV10 cells) or a normal donor (Saka cells) showed that RANKL expression was increased three- to five-fold in samples from Paget's disease patients compared with normal cells (d).

were visualized by chemiluminescence following the manufacturer's instructions (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA).

Statistical analysis. Results are reported as the mean \pm SEM for four determinations, and were compared using the Student's two-tailed *t*-test. Results were considered to be significantly different at $P < 0.05$.

Results

Expression of RANKL mRNA in stromal cell lines and marrow samples from Paget's patients and normal individuals. As shown in Figure 1b, RANKL mRNA expression was increased three- to fivefold in PSV10 cells compared with Saka cells. This high level of RANKL expression was also observed in bone marrow samples from pagetic lesions. RANKL expression was increased four- to fivefold in marrow from affected bones of patients with Paget's disease compared with normal marrow samples (Figure 1a). This high expression was found in six of eight Paget's patients used in this study (Fig-

ure 1, a and c). Because markedly increased OCL activity in Paget's disease is observed only in affected bones, we examined the expression level of RANKL in marrow samples from affected and unaffected bones from two Paget's patients. As shown in Figure 1c, RANKL mRNA expression was increased five- to sevenfold in bone marrow aspirated from the affected bones compared with the unaffected bones in both patients. The levels of RANKL mRNA expression in uninvolved bones were similar to those in the two normal samples, which were tested simultaneously. Furthermore, to confirm that RANKL protein was also increased in Paget's patients and stromal cells, we measured RANKL expression by Western blot. In this single experiment, Western blot analysis of marrow samples from a Paget's patient and a normal donor showed a threefold increase in RANKL expression (Figure 1d). Similarly, Western blot analysis of RANKL expression in PSV10 and Saka cells showed fivefold higher RANKL expression in PSV10 cells.

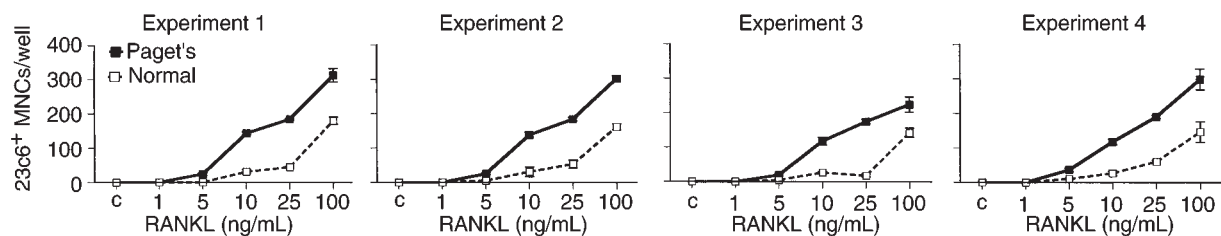
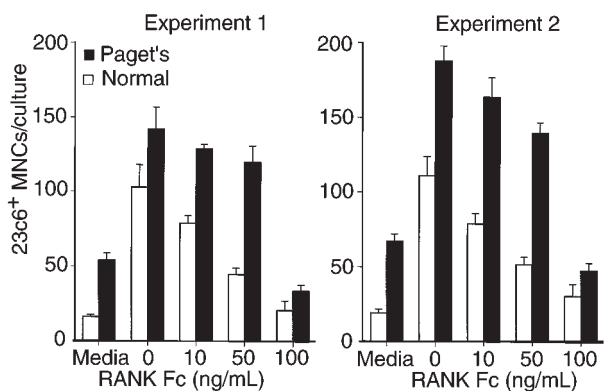


Figure 2

RANKL responsiveness of marrow cells from Paget's patients and normal individuals. Marrow cells were processed as described in Methods, and were treated with varying concentrations (0–100 ng/mL) of RANKL, MCSF (25 ng/mL), and 10^{-7} M dexamethasone. At the end of the culture period, multinucleated cells (MNCs), which cross-reacted with the 23c6 mAb, were scored. In the four experiments shown here, Paget's samples formed significantly more OCL-like cells at much lower concentrations of RANKL than did normal samples.

Figure 3

Effects of RANK Fc on RANKL-induced OCL formation in marrow cultures from Paget's patients and normal individuals. Bone marrow cells were cultured with RANKL (100 ng/mL) and varying concentrations of RANK Fc (0–100 ng/mL) as described in Methods. Relative inhibition of OCL formation was lower in marrow cultures from Paget's patients (solid bars) than in normal individuals (open bars) when 10–50 ng/mL of RANK Fc was added to the cultures ($P < 0.05$). Results are presented as mean \pm SEM for two independent experiments, done in quadruplicate.



Effects of RANKL on OCL formation. We next determined if pagetic OCL precursors responded to RANKL in a similar manner as normal cells. As shown in Figure 2, RANKL induced OCL formation in a dose-dependent manner in both pagetic and normal marrow cultures. However, OCL formation was significantly higher at lower concentrations of RANKL in pagetic marrow cultures than in normal marrow cultures. Furthermore, addition of RANK Fc (10–50 ng/mL) significantly decreased the relative levels of OCL formation in normal marrow cultures compared with marrow cultures from Paget's patients (Figure 3). For example, 50 ng/mL of RANK Fc decreased OCL formation in normal marrow cultures to levels that were $45 \pm 7\%$ of those formed in cultures lacking RANK Fc. In contrast, 50 ng/mL RANK Fc decreased OCL formation in Paget's marrow cultures only to levels that were $76 \pm 8\%$ of those formed in cultures lacking RANK Fc ($P < 0.05$). RANK Fc at a concentration of 100 ng/mL completely blocked RANKL-induced OCL formation in normal and Paget's marrow cultures. OCLs formed in cultures of marrow

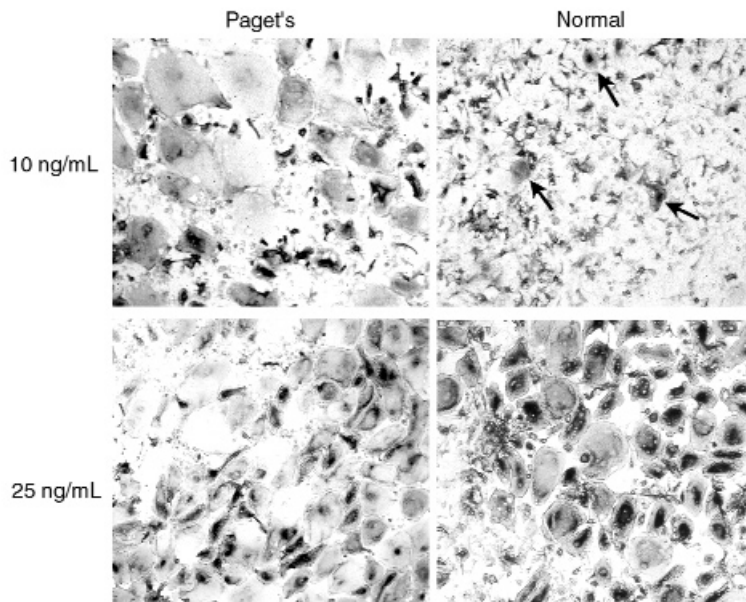
from Paget's patients were larger and contained more nuclei per multinucleated cell than those from normal marrow (Figure 4).

Activity of c-Jun kinase in Paget's and normal marrow samples. To confirm that there were differential effects of RANKL on OCL formation in Paget's marrow cultures, we measured c-Jun NH₂-terminal kinase activity in an equal number of marrow cells from Paget's patients and normal individuals. c-Jun NH₂-terminal kinase is an important component of the RANK/RANKL signaling pathway (19). As shown in Figure 5, RANKL (25 ng/mL) induced c-Jun kinase activity in pagetic bone marrow samples. In contrast, 100 ng/mL of RANKL was required to induce c-Jun kinase activity in normal marrow samples.

Effects of IL-6 on RANKL responsivity of OCL precursors. Because IL-6 is expressed at higher levels in marrow from affected bones of patients with Paget's disease than in normal marrow (5), we sought to determine whether IL-6 was involved in the enhanced responsivity of marrow cells from Paget's patients to RANKL. Therefore, we cultured bone marrow from Paget's patients with 10 ng/mL of RANKL, 25 ng/mL MCSF, and 10^{-7} M dexamethasone,

Figure 4

OCL-like cells formed in marrow cultures from patients with Paget's disease and normal individuals. Bone marrow mononuclear cells were prepared as described in Methods and then treated with 10 ng/mL or 25 ng/mL of RANKL plus 25 ng/mL MCSF and 10^{-7} M dexamethasone. OCL-like cells that were markedly increased in number and size formed in marrow cultures from Paget's patients at low concentrations of RANKL (10 ng/mL) compared with normal marrow (143 ± 4 for Paget's marrow vs. 31 ± 6 for normal marrow). At the higher concentration of RANKL (25 ng/mL), the numbers of OCL-like cells formed in pagetic marrow cultures were also higher than in normal cultures (184 ± 2 for Paget's marrow vs. 44 ± 2 for normal marrow). Similar results were seen in five independent experiments. Arrows point to OCL-like cells.



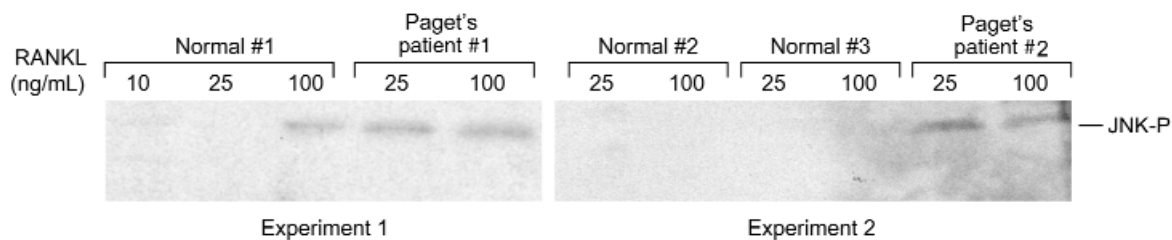


Figure 5

Activity of c-Jun kinase in Paget's and normal bone marrow cultures in response to RANKL. Bone marrow cells from two Paget's patients and three normal individuals were prepared as described in Methods and treated with RANKL (25–100 ng/mL). Phosphorylated c-Jun kinase (JNK-P) was measured as described in Methods, and the levels of c-Jun kinase activity in Paget's and normal individuals were compared. Lower concentrations of RANKL (25 ng/mL vs. 100 ng/mL) showed c-Jun kinase activity in marrow samples from Paget's patients compared with normal individuals. The results represent two independent experiments.

and varying concentrations of a neutralizing Ab to IL-6 (anti-IL-6) or nonspecific IgG. As shown in Figure 6a, addition of anti-IL-6 decreased the responsiveness of Paget's marrow cells to RANKL. This effect was dose dependent and not related to any toxicity of anti-IL-6. Addition of nonspecific IgG to these marrow cultures had no effect on RANKL responsiveness of normal or Paget's cells (data not shown). To confirm that IL-6 could affect the RANKL responsiveness of OCL precursors to RANKL, we added 200 pg/mL of IL-6 to normal marrow cultures treated with RANKL (10 ng/mL), MCSF (25 ng/mL), and dexamethasone (10^{-7} M). As shown in Figure 6b, IL-6 enhanced the responsiveness of normal marrow cells to RANKL. Concentrations of RANKL as low as 10 ng/mL could induce OCL formation in normal marrow cultures to levels similar to those induced by RANKL (10 ng/mL) in pagetic bone marrow cells. IL-6 by itself did not induce OCL formation under these culture conditions.

Expression of RANK in marrow samples from Paget's patients and normal individuals. To further investigate the mechanism responsible for the enhanced RANKL sen-

sitivity of OCL precursors from Paget's patients, we determined if RANK, the receptor for RANKL, was upregulated in bone marrow cells from Paget's patients. Western blot analysis of lysates of these cells confirmed that there was no difference in the levels of RANK expression between normal and pagetic marrow samples (data not shown).

Discussion

In this study, we found that RANKL mRNA is relatively overexpressed in a pagetic stromal cell line and in marrow samples from involved bones from Paget's patients compared with a normal stromal cell line and normal marrow cells. Furthermore, RANKL mRNA expression was also increased in bone marrow aspirated from involved bones, compared with the uninvolved bones from the same patients. Thus, RANKL appears to be highly expressed only in the involved bones of Paget's patients. The reason for the increased expression levels of RANKL is not known, but it does not appear to be related to high levels of IL-6 expression, because high

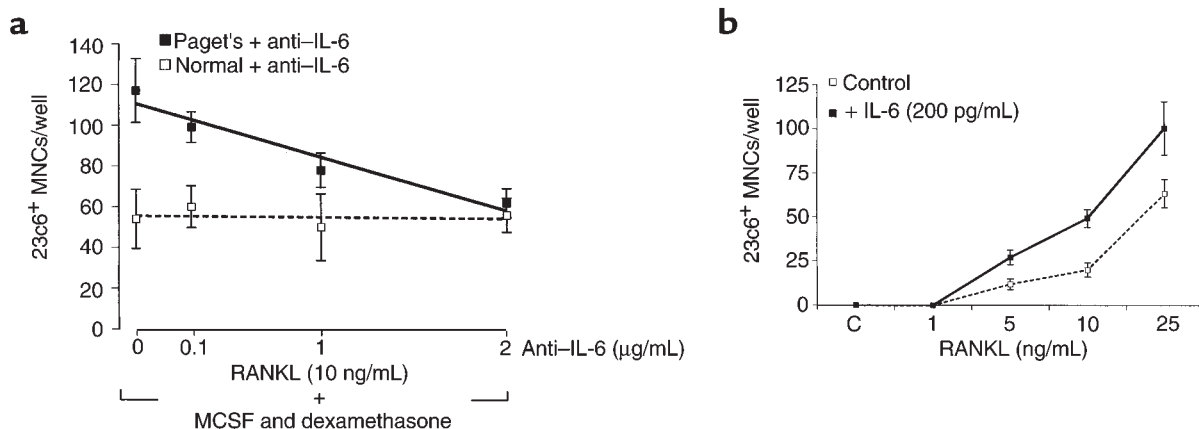


Figure 6

Effects of anti-IL-6 and IL-6 on the responsiveness of Paget's and normal marrow cultures to RANKL. (a) Marrow mononuclear cells from Paget's patients or normal donors were prepared as described in Methods and then cultured with 10 ng/mL of RANKL plus 25 ng/mL MCSF and 10^{-7} M dexamethasone in the presence of varying concentrations of a neutralizing Ab to IL-6. Increasing concentrations of anti-IL-6 decreased the RANKL responsiveness of marrow cells from Paget's patients to normal levels. (b) Addition of IL-6 (200 pg/mL) to normal marrow cultures enhanced RANKL responsiveness compared with cultures treated with media alone. Results are shown as mean \pm SEM for a typical experiment. Similar results were seen in three independent experiments.

concentrations of IL-6 do not increase RANKL expression in human stromal cells or osteoblasts (ref. 21 and unpublished data from Mena et al.). These results are in contrast to those of O'Brien et al. (22), who have shown that IL-6 in combination with soluble IL-6 receptor increased RANKL expression in a murine marrow stromal cell line, suggesting that human and murine stromal cells respond differently to IL-6.

In addition to increased expression of RANKL in pagetic lesions, OCL precursors from Paget's patients appear to be hyperresponsive to RANKL. Very low concentrations of RANKL induced OCL formation in Paget's marrow cultures, whereas much higher concentrations of RANKL were required for OCL formation in normal marrow cultures. Furthermore, anti-IL-6 decreased the RANKL responsiveness of OCL precursors from Paget's patients to normal levels, and IL-6 increased the RANKL responsiveness of normal marrow cells. We previously demonstrated that elevated levels of IL-6 are present in the bone marrow of patients with Paget's disease compared with marrow of normal individuals (5). Similarly, Hoyland and coworkers (6), using *in situ* hybridization, showed that pagetic OCLs express high levels of IL-6 mRNA, as well as IL-6 receptor mRNA. Similarly, recent studies by Wani et al. (23) showed that other factors, such as prostaglandin E₂, can also increase the sensitivity of OCL precursors from murine spleen cells to RANKL.

The increased RANKL sensitivity of OCL precursors from Paget's patients could also result from increased expression of the RANKL receptor, RANK, on OCL precursors from Paget's patients. Anderson et al. (15) have shown that overexpression of RANK can induce NF- κ B signaling even in the absence of RANKL. However, our results show that RANK expression levels are similar in marrow samples from normal individuals and Paget's patients.

These data suggest that the combined increased local expression of RANKL and hyperresponsivity of OCL precursors to RANKL, due in part to IL-6, may contribute to the increased numbers of OCLs in pagetic lesions and the localized nature of the disease. The mechanism responsible for the increased expression of RANKL in Paget's disease remains to be determined, but may reflect the effects of cytokines other than IL-6 upregulating RANKL expression in marrow stromal cells.

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