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RANKL and cathepsin K in diffuse sclerosing osteomyelitis of the mandible

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BACKGROUND: Diffuse sclerosing osteomyelitis (DSO) of the mandible is characterized by mixed bone resorption and formation.

METHODS: Immunohistopathology of DSO in the clinically acute and subacute phases was compared with healthy bone.

RESULTS: Receptor activator of nuclear factor κB ligand (RANKL) was found in DSO lesions. When it was used *in vitro* to stimulate monocytes, cathepsin K expression was observed in mononuclear prefusion precursors and in multinuclear giant cells. Similarly, exacerbations of DSO were characterized by RANKL and induction of cathepsin K in mononuclear precursor cells, which subsequently seem to differentiate into osteoclasts or foreign body giant cells. The proportion of bone to soft tissue increased with the duration of disease.

CONCLUSIONS: RANKL-driven osteoclastogenesis and acidic cysteine endoproteinase cathepsin K seem to play important roles in DSO as osteoclast-mediated bone resorption may represent the primary disease process later followed by new bone formation.

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Introduction

Diffuse sclerosing osteomyelitis (DSO) of the mandible is characterized by an inflammatory and tissue destructive host response and pain (1–4). It is initiated as an oral infectious process (5–7), but changes later to a sterile chronic osteomyelitis (8–10). Monocyte recruitment in particular is probably one of the important steps in the DSO disease process. Although there is no formal proof of their local production in DSO lesions, it can be speculated that the pro-inflammatory cytokines interleukin-1β and tumour necrosis factor-α play important roles also in this disease condition in that they induce vascular endothelial cell adhesions molecules like intercellular adhesion molecule-1 and hyaluronan receptors CD44 (11). This probably allows efficient capture of the circulating monocytes into the inflammatory endothelium. This adhesion step is in inflammation in general followed by transmigration stimulated by locally produced chemokines. Monocyte chemoattractant protein-1 (CCL2), macrophage inflammatory protein-1α (CCL-3), Regulated upon activation, normal T-cell expressed and presumably secreted (RANTES) (CCL-5) and stromal cell-derived factor-1α (CXCL12) are often mentioned in this respect (12–13). Monocytes, apart of their role in inflammation, also form the precursor cells of the multinuclear giant cells. Such foreign body giant cells and osteoclasts are formed as a result of costimulation with macrophage-colony stimulating factor and receptor activator of nuclear factor κB ligand (RANKL; see below). The inflammatory nature of DSO has been ascertained by the alleviation of DSO-associated pain with glucocorticosteroids (9). Clodronate has also been successfully used as pain medication (14). Related but more generalized changes include chronic recurrent osteomyelitis (CROM) and a syndrome characterized by synovitis, acne, pustulosis, hyperostosis and osteitis (SAPHO).

From the cell biological point of view, DSO is an interesting disease model as bone resorption and formation are normally coupled to each other. Earlier work on the topology of the lesion disclosed organization of the changes into discrete zones in the cylindrical specimens analysed (2). We hypothesized that these changes might also indirectly represent different phases of the osteomyelitic reaction and to some extent reflect

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the coupling mentioned above. The exact molecular mechanisms involved in coupling are not known in detail yet, but the core binding factor alpha 1 subunit (or Runx2), which dimerises with core binding factor beta subunit to form core-binding factor, seems to play a role. Core-binding factor alpha 1 subunit was first recognized for its essential role in bone formation during development. It was therefore somewhat surprising that it was later in adult skeleton coupled to osteoclast differentiation and bone resorption, possibly mediated through the RANKL/RANK/osteoprotegerin system. DSO is characterized by bone destruction, but also by new bone formation in a somewhat haphazard manner, indicating deranged coupling (15–18). In DSO sclerotic changes are mainly found in the subperiosteal parts of the bone specimens followed, in a centripetal direction, by (i) coarse trabeculae and necrotic foci, (ii) partly calcified thin trabeculae reminiscent of fibrous dysplasia and (iii) by granulation tissue with giant cells (10, 19). In spite of these well-described lesional changes and topology, little is known how DSO changes over time, because repeated biopsies are rarely clinically indicated or ethically acceptable. In this study an approach was carried out to indirectly analyse changes over time by a comparison of a rare set of biopsies obtained during surgical treatment performed during acute and subacute phases of DSO over a long period of time by one of the authors (Marjut Montonen).

Inflammation promotes formation of osteoclasts responsible for bone resorption. Osteoclasts produce proteolytic enzymes, mainly cathepsin K, which is an acidic cysteine endoproteinase able to cleave across multiple sites of the non-helical and helical parts of the collagen monomers. Various pro-inflammatory cytokines, like tumour necrosis factor-α and interleukin-1 have been considered to play key roles in osteoclastogenesis. Although they are important, today's consensus is that in particular receptor activator of nuclear factor kappa B ligand (RANKL)-RANK signalling system plays a central role (20–25). Other factors and pro-inflammatory cytokines contribute directly or via induction of RANKL. Osteoclastic cathepsin K is a much more efficient collagenolytic enzyme than the conventional mammalian collagenases, which cleave only across the specific initial cleave site at ^{775}Gly-^{776}Ile(Leu). Pathological bone tissue destruction can be also associated with low local pH and induction of cathepsin K-immunoreactive mono- and multinuclear cells, which lead to dissolution of the bone mineral followed by cathepsin K-driven collagenolysis of the decalcified bone matrix (26–28). This study aimed to evaluate, if cathepsin K is induced and perhaps involved in the pathological bone changes in DSO.

Materials and methods

Subjects and samples

The diagnosis of the DSO ($n = 13$, seven women, six men) was based on clinical and radiological findings, corroborated by histopathology ($n = 8$) and scintigraphy ($n = 12$). At the time of the bone biopsy, eight DSO patients were in the acute painful phase, in which the biopsies were taken within 1 month from the onset of the exacerbation (Table 1). Two of the patients were operated twice so altogether 10 biopsies were available from the acute phase DSO. Five patients were in the subacute phase. In them biopsies were taken after at least 1 and up to 12 months had passed from the beginning of the latest exacerbation. Control bone samples were obtained ($n = 13$, six women, seven men) in connection to prophylactic removal of asymptomatic impacted lower third molar. Therefore, the mean age of the DSO patients was clearly higher (43.7 years, range 17–69) than that of controls (25.1 years, range 16–48). None of the patients had been on antibiotics or glucocorticosteroids during the month preceding the biopsy, but symptomatic use of non-steroidal anti-inflammatory drugs was allowed and used by patients in exacerbation. Bone samples were taken by osteotomy. After approximately 1.5 cm long horizontal and vertical burr cuts to the buccal bone plates, cortical and cancellous bone pieces were removed by the chisel. Ethics Committee approved the study. All patients gave their informed consent.

Preparation of the tissue specimens for histological and immunohistochemical examinations

The specimens were fixed in 10% neutral-buffered formalin for 3–5 days, demineralized in aqueous 0.33 M ethylenediaminetetraacetic acid for 4–12 weeks at +20°C, rinsed, dehydrated in an ascending ethanol series and xylene, embedded in paraffin, cut at 5–7 μm and stained with haematoxylin and eosin. Sections were examined with a Leitz Orthoplan microscope under transmitted and polarized light.

RANKL stimulations of human peripheral blood mononuclear cells

Monocytes were isolated from buffy coat of three healthy blood donors of the Finnish Red Cross by the use of Ficoll-Paque PLUS (Pharmacia Biosciences, Uppsala, Sweden) density gradient at 2000 *g* for

Table 1 Relationship with duration of DSO, the number of specimens corresponding to the time elapsed since the latest exacerbation and the total number of specimens

Duration of the disease (years)	<1/4	1	2	3	4	5	6	7	8	9	15
Time elapsed since the latest exacerbation <1 month (acute phase, number of specimens = 10)	2			2	1	2		1		1	1
>1 month <12 months (subacute phase, number of specimens = 5)			2		1	1			1		
Total no of specimens ($n = 15$)	2	0	2	2	2	3	0	1	1	1	1

20 min at +22°C. Washed cells were cultured in six-well plates with four coverslips per well at 1×10^7 cells/well in 2 ml of Macrophage-Serum Free Medium (GibcoBRL, Paisley, Scotland) supplemented with 1% penicillin and streptomycin. After 1 h the lymphocytes were washed away and the adherent monocytes were further cultured for 3 and 14 days in the presence of 25 ng/ml macrophage-colony stimulating factor M-CSF (R&D) and 40 ng/ml RANKL (Alexis Biochemicals, Lausen, Switzerland) as described in detail elsewhere (19).

Cathepsin K immunofluorescence staining
Cells cultured on coverslips were fixed in 3% paraformaldehyde in 10 mM phosphate buffered 150 mM saline, pH 7.4, for 20 min, permeabilized using 0.2% Triton-X in phosphate buffered saline, washed and incubated in normal goat serum. Sections were incubated in 2 μg/ml polyclonal goat anti-human cathepsin K IgG in 10 mM phosphate buffered 150 mM saline, pH 7.4, containing 1.25% bovine serum albumin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 60 min followed by Fluorescein-5-isothiocyanate (FITC)-conjugated donkey anti-goat IgG for 45 min. The cytoskeletal actin was stained using Alexa Fluor 633 phalloidin reagent (Molecular Probes, Leiden, The Netherlands) and nuclei were visualized using TO-PRO-3 reagent (Molecular Probes, Leiden, The Netherlands) before cells were mounted in Dako Fluorescent Mounting Medium (DAKO, Glostrup, Denmark). After washes with phosphate buffered saline the slides were air-dried and mounted. Normal goat IgG was used at the same concentration as and instead of the primary antibody as a negative control. The specificity of this antibody has also been confirmed using antigen absorption test (20). All steps were performed at room temperature. Tartrate resistant acid phosphatase was stained according to manufacturer's instructions (TRAP staining kit, Sigma, St. Louis, MO, USA). Cells were observed using Olympus motorized revolving AX 70 system microscope coupled with 12 bits digital image camera (Sensicam, Kelheim, Germany).

In vitro analysis of bone resorption
After 14 days of culture 4×10^5 cells were then transferred to well 7.5 mm in diameter containing a dentin slice 5 mm in diameter (Immunodiagnostic Systems). Cells were incubated on dentin in the presence of media with M-CSF and RANKL (replaced twice a week) for 7 days. The cells were then brushed away and the dentin slices were stained with toluidine blue to visualize the resorption pits using a Leica TCS SP2 confocal microscope.

Immunohistochemistry
Six micrometre paraffin sections were cut onto chrom-alum-gelatin-coated slides, kept overnight on +60°C hot plate, followed by deparaffinization, rehydration and pepsin treatment for 45 min to reveal hidden immunoreactive epitopes. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in absolute methanol for 30 min. The sections were incubated at room temperature in: (i) normal goat or horse serum (diluted 1:50 in phosphate buffered saline containing 0.1% bovine serum albumin) for 20 min, (ii) 2 μg/ml polyclonal rabbit anti-human cathepsin K IgG or 20 μg/ml monoclonal mouse anti-human $IgG_{1/\kappa}$ RANKL (R&D Systems, Minneapolis, MN, USA) in phosphate buffered saline containing 1.25% bovine serum albumin for 60 min, (iii) biotinylated goat anti-rabbit or horse anti-mouse IgG (Vectorlabs, Burlingame, CA, USA) diluted 1:100 in phosphate buffered saline containing 0.1% bovine serum albumin for 30 min, (iv) avidin-biotin-peroxidase complex (ABC, diluted 1:100 in phosphate buffered saline) for 30 min and (v) 0.023% 3,3-diaminobenzidine tetrahydrochloride and 0.006% H_2O_2 for 5 min. Some sections were counterstained with haematoxylin before mounting. Normal rabbit IgG or mouse $IgG_{1/\kappa}$ was used at the same concentration as and instead of the primary antibody as a negative staining control. In between the steps the slides were washed 3×5 min in phosphate buffered saline, pH 7.4.

Results

Histological findings in acute phase DSO
Acute phase DSO was characterized by chronic inflammation (Fig. 1a), multinuclear giant cells (Fig. 1b), sequestra (Fig. 1c) and woven bone trabeculae lined by many osteoclasts (Fig. 1d).

Histological findings in subacute phase DSO
Subacute phases contained less inflammatory cells (Fig. 1f). Reversal lines, indicating repeated periods of bone resorption and deposition, were prominent in the most long-standing cases (Fig. 1e).

Control bone samples
Controls samples represented for the most part normally organized lamellar bone (data not shown). All biopsies were taken with clinical indications and not in a standardized manner. Therefore, sampling did not allow reliable histomorphometry.

RANKL induced osteoclast formation
The RANKL stimulation lead to maturation of the M-CSF maintained human peripheral blood monocytes first to TRAP+ mononuclear prefusion cells (Fig. 2a), followed by their fusion into multinuclear (Fig. 2b) cathepsin K+ cells (Fig. 2c) able to resorb bone (Fig. 2d).

RANKL in DSO
The $RANKL^+$ cells were found in control bone samples as well as in DSO of the mandible. RANKL staining disclosed that in control samples $RANKL^+$ cells were seen in the bone marrow space in mononuclear cells (Fig. 3a), but in DSO also in multinuclear osteoclasts close to bone (Fig. 3b). In general, the intensity of RANKL staining was particularly strong in the multinuclear osteoclasts.

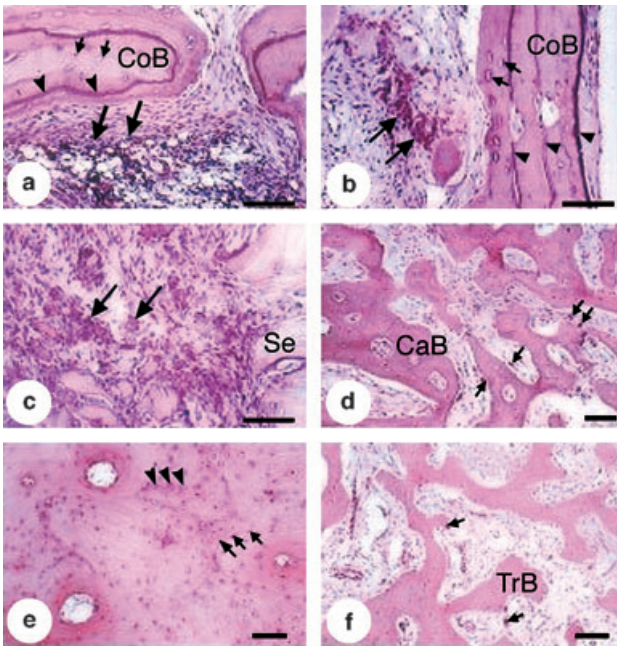

Figure 1 Histological features of DSO. Acute phase (a–e) showing a prominent mononuclear inflammatory cell infiltrate (big arrows) subjacent to lamellar cortical bone (CoB) (a), a collection of multinuclear giant cells (big arrows), probably representing post-resorption osteoclasts (b), and a sequestrum (Se) having undergone partial resorption to fragments surrounded by chronic inflammatory cells (arrows) (c). Deposition lines (arrowheads) in the cortical bone (CoB) are accentuated and the osteocyte (small arrows) patterns regular (a and b). The cancellous bone (CaB) is formed of woven bone trabeculae without marked osteoblastic activity but many osteoclasts (arrows) are present on the endosteal bone surface (d). In a long-standing case having undergone acute exacerbation, osteocytes (arrows) tend to accumulate along irregular reversal lines traversing the sclerotic bone. Some lacunae are empty or obliterated (arrowheads) (e). In a case representing subacute phase DSO, the bone is composed of irregular trabeculae (TrB) throughout; osteoclasts (arrows) are visible but chronic inflammatory cells are sparse (f). Demineralized material. Haematoxylin and eosin stain. Bar = 100 μm (a–f).

Cathepsin K immunostaining

Using cathepsin K immunostaining it was possible to confirm and extend the findings described in haematoxylin-eosin stained slides. Cells of interest became clearly visible in the immunostained slides. Cathepsin K reactivity was observed in osteoclasts located in the resorption lacunae (Fig. 4a,b). Nuclei were seen as oval defects in the intensely stained cytoplasm. Cathepsin K was also found in multinuclear giant cells (Fig. 4c), often occurring in association with necrotic bone as well as in some mononuclear cells (Fig. 4d). The negative staining control confirmed the specificity of the staining.

Discussion

In this study we observed the different durations of DSO lesions were associated with different histopathologies so that acute exacerbations differed from subacute with temporary remissions. The cut-off line for the acute and subacute was drawn based on the clinical status and time since the beginning of the latest exacerbation, not on the topology of the biopsy specimen as was done in an earlier thesis work on this subject. In the nicely written report of carefully studied DSO patients Stellan Jacobsson in his now already classic stone mark thesis work (2) noticed topographical organization of disease-related changes to distinctive zones in cylindrical bone biopsies. We speculated that these zones might also indirectly represent different phases of the osteomyelitic reaction. Thus, bone with inflammatory cells might represent acute phases, whereas the sclerotic bone with densely and irregularly packed osteons could represent the other end of a continuum, the resting or healing phase of the osteomyelitic process. Indeed, the acute lesions with short duration of DSO and short time after latest exacerbation were found to be predominated by mononuclear inflammatory cells representing

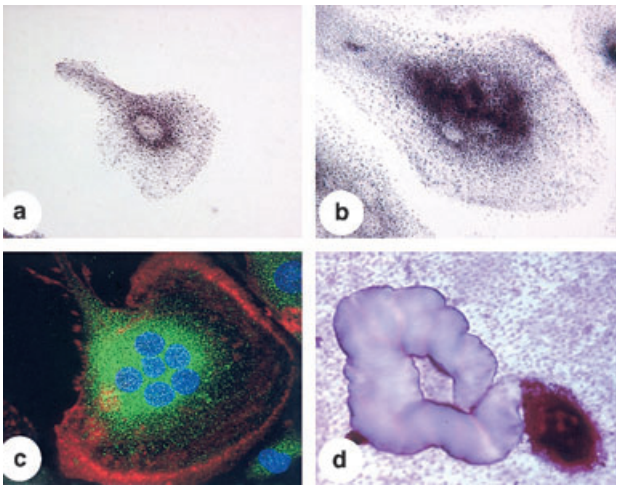

Figure 2 Cytological staining of macrophage-colony stimulating factor and receptor activator of nuclear factor κB ligand costimulated cultures of human monocytes. Under the influence of receptor activator of nuclear factor κB ligand human monocytes differentiate into mononuclear prefusion cells, which are tartrate resistant acid phosphatase positive (a) and fuse to multinuclear cells (b). These multinuclear cells also contain cathepsin K (green colour) as is shown for one such cells, in which the nuclei are blue (DAPI staining) and the actin ring is red (Alexa Fluor 633 phalloidin staining) (c). These cells display osteoclast phenotype in that they are able to form resorption pits (d).

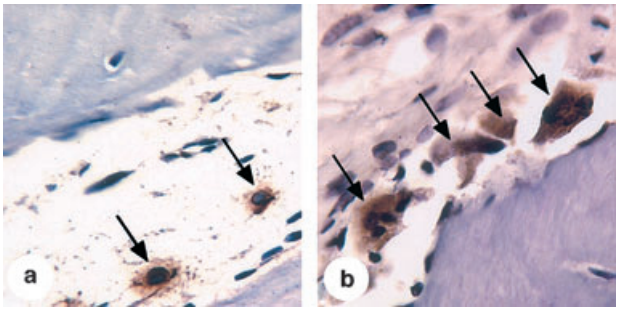

Figure 3 Immunostaining for receptor activator of nuclear factor κB ligand in DSO. Some of the bone marrow cells (arrows) were receptor activator of nuclear factor κB ligand positive in control bone samples (a), whereas DSO samples were also characterized by intensively staining receptor activator of nuclear factor κB ligand positive osteoclasts (arrows) (b).

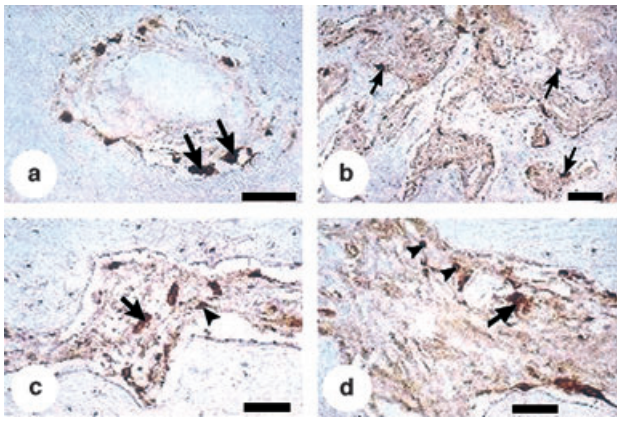

Figure 4 Immunostaining for cathepsin K in DSO. Osteoclasts (arrows) in resorption lacunae on the endosteal bone surface are strongly reactive (a) and even more frequent (b) than evident histologically (see Fig. 1f illustrating the same specimen). Multinuclear (arrow) and occasional mononuclear cells (arrowhead) with cytoplasmic extensions (C) or a roundish morphology (arrowheads), frequently in the vicinity of endosteal bone surface or osteoclasts (arrow) are also stained (d). These cells may thus represent committed, mononuclear prefusion progenitor cells. Demineralized material. Non-counterstained sections. Bar = 100 μm (a and b) and 50 μm (c and d).

lymphocytoid and macrophage-like cells associated with osteoclasts and bone resorption, but relatively weakly developed osteoblast response. In contrast, the subacute lesions were characterized by much milder inflammatory cell infiltrates and by the presence of woven rather than lamellar bone.

In this study we also found RANKL in DSO lesions. However, the differences between the DSO lesions and control biopsies in the proportion of cells containing RANKL or in the staining intensity of RANKL were not as prominent as one could have expected although such a difference seemed to exist. If it is accepted that the different topographical zones of DSO lesions and different phases of disease represent the same process, respectively, over space and time (i.e. the early, inflammatory and destructive vs. late and reparative phases), then it can also be speculated that probably the early, inflammatory phase leads first to recruitment of progenitors, differentiation and activation of osteoclasts. This would lead to bone resorption. Inflammation and coupling of bone resorption to bone formation probably together lead to subsequent reversal reaction, osteoblast activation and new bone formation. These changes can probably be quite fast as RANKL, together with M-CSF, stimulated *in vitro* formation of cathepsin K and TRAP positive osteoclasts capable of bone resorption in only a few days. The putative proinflammatory cytokines, which may play a role in monocyte recruitment, tumour necrosis factor-alpha and interleukin-1 beta, can probably indirectly or directly enhance both osteoclastogenesis and osteoclast activity making a further contribution to this end. It therefore seems that the clear differences in the histopathology during different phases of DSO are more dependent on deranged coupling, perhaps in part related to the dynamic nature of the lesions, than to excessive production of RANKL alone. These findings are in agreement with work by Kartsogiannis et al. (29), who demonstrated that in intramembranous mouse bones RANKL was expressed in some of the osteoclasts and, in particular, in osteoclasts, which were apparently actively resorbing bone. These findings extend this earlier report to human osteoclasts involved in pathological bone lysis and suggest that DSO osteoclasts are actively engaged in bone resorption.

The present work demonstrates an association between the acidic endoproteinase cathepsin K and pathological intramembranous bone destruction and remodelling in DSO. Cathepsin K, discovered 1994, can both solubilize collagen and cleave directly cross the collagen super helix at multiple sites. Its site of action is not restricted to the initial and specific cleavage site utilized by all the conventional collagenases belonging to the matrix metalloprotainase (MMP) family, which comprise collagenase-1 (MMP-1, formerly known as fibroblast-type collagenase), collagenase-2 (MMP-8, formerly known as neutrophil collagenase, but now known to occur also in a mesenchymal and differently glycosylated form) and the more recently discovered collagenase-3 (MMP-13) and MT1-MMP (MMP-14, the first of the recently discovered membrane-type MMPs). Cathepsin K has a low optimal pH, which does not permit the function of the neutral endoproteinases of the MMP-family. *Vice versa*, MMPs are most active at neutral or slightly alkaline pH and are not able to act at low, acidic pH. The action of the two classes of enzymes is mutually exclusive (30). Recent finding on pathological bone destruction suggest that cathepsin K may not only act intracellularly in the acidic lysosomal compartment, but is also produced and released into the extracellular space by mononuclear cells, which may represent prefusion osteoclast progenitors (26). Cathepsin K containing multinuclear cells have been described in multinuclear cells in osteomyelitis of enchondral long bone (31) and is now shown in DSO, where the process is probably driven by both RANKL-dependent and -independent osteoclast and foreign body giant cell formation (32). Cathepsin K inhibitors have already been developed for the treatment of metabolic bone diseases, such as osteoporosis. They may be useful also in DSO.

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