Regulated expression of MCP-1 by osteoblastic cells in vitro and in vivo

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Summary. Inflammation is characterized by the recruitment of leukocytes from the vasculature. Recent studies have implicated chemokines as an important class of mediators that function principally to stimulate leukocyte recruitment, and in some cases, leukocyte activity. There are four defined chemokine subfamilies based on their primary structure, CXC, CC, C and CX3C. Members of the CC chemokine subfamily, such as monocyte chemoattractant protein 1 (MCP-1), are chemotactic for monocytes and other leukocyte subsets. The studies described below focus on the expression of MCP-1 in vitro and in vivo in an osseous environment. These studies indicate that MCP-1 is typically not expressed in normal bone or by normal osteoblasts in vitro. Upon stimulation by inflammatory mediators, MCP-1 is up-regulated. This expression is temporally and spatially associated with the recruitment of monocytes in both osseous inflammation and during developmentally regulated bone remodelling. Furthermore, exogenous MCP-1 applied to inflamed bone enhances the recruitment of monocytes. Because monocytes produce factors that influence osseous metabolism, including but not limited to prostaglandins, platelet-derived growth factor, interleukin-1 or tumor necrosis factor, chemokines that initiate their recruitment are likely to be highly important.

Key words: Bone, Chemokine, Cytokine, Osteoblast, Osteoclast, Review

Introduction

Chemokines are low molecular weight secretory proteins that function principally as stimulators of leukocyte recruitment. These secondary pro-inflammatory mediators are induced by primary proinflammatory mediators (e.g., IL-1 and TNF). There are four defined chemokine subfamilies based on their primary structure, CXC, CC, C and CX3C. The best characterized chemokines are CXC and CC families. Members of the CC chemokine subfamily, such as monocyte chemoattractant protein 1 (MCP-1), RANTES, MIP-1-γ and MIP-1-α, are chemotactic for monocytes, subsets of lymphocytes and natural killer cells, (Graves and Jiang, 1995), whereas CXC chemokines generally, but not exclusively, induce neutrophil chemotaxis and recruitment to tissue.

MCP-1, a monomeric polypeptide, is typically secreted in two predominant forms with molecular weights of 9 and 13 kD. Lectin blots indicate that the disaccharide, galactose-β-3D-N-acetyl galactosamine is present on the 13 kD MCP-1 isoform but not the 9 kD isoform (Fig. 1). MCP-1 induces recruitment of monocytes, a subset of T lymphocytes, eosinophils, and basophils (Graves and Jiang, 1995). Significant expression of MCP-1 in normal cells usually requires stimulation with pro-inflammatory agents (Williams et al., 1992). In contrast, MCP-1 appears to be constitutively produced by different human tumors and may account for much of the monocyte chemotactic activity produced by tumor cells (Bottazzi et al., 1983; Graves et al., 1989; Van Damme et al., 1992; Zhang et al., 1997). MCP-1 was first isolated by Valente and co-workers (Valente et al., 1988) and sequenced by Yoshimura and colleagues and Furutani and co-workers (Yoshimura et al., 1989; Furutani et al., 1989). Functional assays demonstrated the similarity between the chemotactic factors produced by human bone tumors (MG-63) and primate smooth muscle cells (Graves et al., 1989). The murine form of MCP-1, JE, was initially identified as an immediate early gene induced in response to platelet-derived growth factor (Cochran et al., 1983) and its properties have been described in a series of articles by Rollins and co-workers (reviewed in Rollins, 1997).

It is striking that MCP-1 is expressed in a number of inflammatory conditions associated with monocyte recruitment, including delayed hypersensitivity reactions, atherosclerosis pulmonary fibrosis, bacterial infection, arthritis, and renal disease (Graves and Jiang, 1995). MCP-1 stimulates monocyte chemotaxis, as well
as many of the cellular events associated with chemotaxis (i.e., Ca^{2+} flux and integrin expression). In monocytes, MCP-1 weakly induces cytokine expression (Jiang et al., 1992). In addition, this chemokine elicits a respiratory burst at very high concentrations, which leads to the generation of oxygen radicals (Rollins et al., 1991). MCP-1 is also a potent inducer of histamine release from basophils and has been implicated as an important mediator in allergic inflammation (Bischoff et al., 1992). Injection of MCP-1 in vivo results mainly in monocyte recruitment.

In early studies, MCP-1 was shown to bind freshly isolated monocytes through high affinity cell surface binding sites (Yoshimura and Leonard, 1990; Valenta et al. 1991). Subsequently, an MCP-1 receptor (CCR2) was cloned (Chao et al., 1994) and identified as a member of the large family of G-protein-coupled protein receptors that contain 7 transmembrane-spanning domains. Two forms of the receptor were identified (CCR2-A and CCR2-B) that differ only in the C-terminal cytoplasmic domain of the protein and apparently arise by alternative splicing events. Both forms of the receptor mediate ligand-dependent Ca^{2+}-mobilization, cellular migration and inhibition of adenyl cyclase consistent with identical ligand specificity. Since the alternative C-terminal domains have been shown to display different specificity for Gt-protein subtypes, some distinct function for each of the CCR2 subtypes is suggested (Kuang et al., 1996). The CC chemokine receptors identified to date all demonstrate multiple ligand specificities. In addition to MCP-1, CCR2 binds the related chemokines, MCP-2, MCP-3 and MCP-4 (reviewed in Baggiolini et al., 1997). Similarly, MCP-1 has been shown to bind the receptor CCR4, which also binds RANTES and MIP-1alpha. This cross-reactivity between ligands and receptors suggests a measure of redundancy in the chemokine system for the trafficking and activation of leukocytic cells. However in CCR2-knockout mice, the recruitment of monocyte/macrophages in response to some inflammatory stimuli is

![Fig. 1. MCP-1 isoforms differ by the presence of carbohydrate residues. The MCP-1 13 kD isoform (odd numbered lanes) and the MCP-1 9 kD isoform (even numbered lanes) were subjected to electrophoresis by SDS-PAGE and transferred to nitrocellulose. A. Immunoblot: MCP-1 antibody (lanes 1, 2) or normal rabbit serum (lanes 3, 4). B. Wheat germ agglutinin (lanes 1, 2) or wheat germ agglutinin plus hapten (lanes 3, 4). C. Lectin blot (lanes 1, 2) or peanut agglutinin plus hapten (lanes 3, 4). The results with peanut agglutinin indicate that the 13 kD MCP-1 isoform but not the 9 kD isoform has the disaccharide galactose-91-3D-N-acetyl galactosamine, while result with wheat germ agglutinin indicates the presence of sialic acid residues on the larger isoform. Figure 1 is reproduced with permission from Biochem. Biophys. Res. Commun. (179, 1400-1404, 1991).](image)

![Fig. 2. MCP-1 antisem to block chemotactic activity and recognize MCP-1 produced by a human osteoblastic cell line. A. Serum free medium conditioned by MG-63 osteoblastic cells was diluted as indicated, incubated with MCP-1 antisera (closed circle) or with buffer (open circle), and then tested for induction of monocyte chemotactic activity. B. Serum free media conditioned by MG-63 cells was diluted (1:2), incubated with MCP-1 antisera (closed circle) or with buffer (open circle) and the tested for induction of monocyte chemotactic activity. C. MG-63 cells were metabolically labelled with 35S methionine, lysed and the resulting lysate was incubated with normal rabbit serum (lane 1), MCP-1 antisera (lane 2) or MCP-1 antisera plus excess purified MCP-1 (lane 3). The results demonstrate that MCP-1 expression occurs in these cells and can account for the majority of the monocyte chemotactic activity that they produce. Figure 2 is reproduced with permission from the American Association for the Advancement of Science (Science 245, 1490-1492, 1989).](image)
markedly impaired as is the ability of macrophages to clear infection by the intracellular bacteria Listeria monocytogenes (Kurihara et al., 1997; Kuziel et al., 1997). Furthermore, disruption of the MCP-1 gene also leads to an impairment in the monocyte recruitment process and shares features with the CCR2 knockout model (Lu et al., 1998). Thus despite multiple ligand specificity, some functions of the chemokines and chemokine receptors may remain unique and essential.

MCP-1 and bone

Our laboratories have recently focused on the expression of MCP-1 by bone cells (Fig. 2). The studies have elucidated mechanisms for the regulated expression of MCP-1, which may be generally applicable to many cell types. Most importantly, the expression of MCP-1 in osteoblastic cells may serve an important function in controlling osseous metabolism.

![Image of MCP-1 promoter and luciferase construct]

**Fig. 3.** Characterization of the IFN-γ response elements in the MCP-1 promoter. A. Sequence of the currently known cis-acting elements involved in the regulation of MCP-1 gene transcription in response to IFN-γ stimulation. MCP-1 promoter/luciferase constructs were as described in (Valente et al., 1998). IRIS: IFN-γ-response inhibitory sequence; GAS: Gamma interferon activation site; Sp1: position of functional Sp1 binding site. Numbers refer to nucleotide positions relative to the ATG start codon. B. Identification of the IFN-γ response region in MG-63 cells. A deletion series of the human MCP-1 5′-flanking sequence constructed in a luciferase reporter vector was transfected with the control renilla expression vector CMV-pRL (Promega) into MG-63 cells and stimulated with either medium or 1000nM rhIFN-γ for 4 hours. Luciferase activity was normalized to renilla activity. Results are expressed as fold increase of luciferase activity over unstimulated (medium) control and represent the mean and SEM of 3 separate experiments carried out in duplicate. C. Time course of IFN-γ stimulation of MCP-1 luciferase activity in transfected MG-63 cells. MG-63 were transfected with the indicated constructs and stimulated with 1000u/ml rhIFN-γ for 2, 4 and 8 hours and normalized luciferase activity determined as in B. The results are expressed as fold increase over unstimulated controls, and represent the mean of 3 experiments carried out in duplicate + SEM. Figure 3 is reproduced with permission from The Journal of Immunology (J. Immunol. 161, 3719-3728, 1998).

MCP-1 expression by osteoblastic cells in vitro

It is believed that monocytes play an important role in regulating osseous metabolism. Significant monocyte recruitment occurs at sites of bone injury and remodeling. Monocytes affect bone through the production of factors that can stimulate both bone resorption and formation. This is supported by evidence that monocyte products can directly stimulate osteoblastic cells (Williams et al., 1992). Our laboratory and that of Van Damme and colleagues were the first to show that MCP-1 could be expressed by osteoblastic cells in vitro (Graves et al., 1989 and Van Damme et al., 1989). The studies described below have addressed the regulation of MCP-1 expression in osteoblastic cell lines and normal human osteoblast cells.

Williams et al. (1992) tested normal human osteoblasts for the release of monocyte chemoattractants. Results showed that IL-1 stimulation induced these cells to produce monocyte chemotactic activity. Without IL-1 stimulation, osteoblast-conditioned medium did not stimulate monocyte chemotaxis. The addition of IL-1 to normal human osteoblasts induced the production of chemotactic activity within 6 hours, which increased at later time points. Since the increase in chemotactic activity was time-dependent, this indicated that it was not simply caused by the presence of IL-1 in conditioned medium. In fact, these studies confirmed earlier reports that IL-1 alone has little or no direct chemotactic activity for monocytes. Evidence that MCP-1 antiserum inhibits virtually all IL-1 stimulated monocyte chemotactic activity produced by osteoblasts suggest that MCP-1 is one of the principal chemokines produced by the cells.

In vitro studies have shown that for most normal cell

<table>
<thead>
<tr>
<th>ADDITION</th>
<th>MCP-1 (ng/ml)</th>
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<tr>
<td></td>
<td>24h</td>
</tr>
<tr>
<td>Control</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PTH (10 nM)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>1.25(OH)2D3 (20nM)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Hydrocortisone (140 nM)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>PDGF-BB (15ng/ml)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>TGF-β (2ng/ml)</td>
<td>7±1.1</td>
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<tr>
<td>IL-6 (20ng/ml)</td>
<td>19±0</td>
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<tr>
<td>TNF-α (20ng/ml)</td>
<td>48±2.6</td>
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<tr>
<td>IL-18 (50 u/ml)</td>
<td>63±1.7</td>
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Confluent serum-free cultures of normal human osteoblastic cells were incubated with physiologic concentrations of PTH, 1,25(OH)2D3, hydrocortisone, PDGF-BB, IL-18, TNF-α, IL-6, TGF-β, or vehicle control. Conditioned media was collected after 24 or 48h and MCP-1 was assayed by RIA. Values are means±SEM of triplicate samples. The minimum detectable level of MCP-1 was 5ng/ml. Values that are significantly different from the unstimulated control are indicated with a (*) for the 48h time point (p<0.01). Statistical significance was not determined for the 24h period because the value of the negative control was below the detection threshold. Table 1 is reproduced from with permission from the American Society for Bone and Mineral Research (J. Bone Min. Res. 9, 1123-1130, 1994).
types there is little or no MCP-1 expression, and that
eogenous stimulation is required for induction of MCP-
1. Zhu et al. (1994) studied the effect of several different
classes of biologic mediators on MCP-1 expression in
normal human osteoblastic cells (Table 1). Cells were
cultured in serum-free medium with or without factors
known to regulate osseous metabolism. Results showed
that after 24 hours, little MCP-1 expression (<5 ng/mL)
was found in medium conditioned by unstimulated
normal osteoblasts. However, physiologic concentrations
of MCP-1 were found in conditioned medium from cells
treated with IL-1β, TNF-α, IL-6, and TGF-β (Table 1).

These investigators also examined the effect of
osteotrophic mediators on MCP-1 mRNA levels in two
osteoblastic cell lines, SaOS-2 and MG-63 (Zhu et al.,
1994). Constitutive expression of MCP-1 was high in
MG-63 cells and low in SaOS-2 cells. Detectable levels
of MCP-1 mRNA were observed in both cell lines after
stimulation with TNF-α (20 ng/mL). The effect of
different mediators on MCP-1 mRNA levels was also
studied. In SaOS-2 cells, IL-1β and TNF-α induced a
dose-dependent increase in the MCP-1 mRNA up to 36-
fold and 28-fold, respectively. TGF-β and IL-6 induced
increases of approximately 3-fold. In MG-63 cells, the
following dose-dependent increases in the MCP-1
mRNA levels were seen: TNF-α, 20 fold; IL-1β, 15 fold;
TGF-β, 9 fold; and IL-6, 2 fold. For these cells the fold
stimulation of MCP-1 mRNA levels was generally
higher than the corresponding increase and protein
levels.

The above studies indicate that in osteoblasts, MCP-
1 is regulated by inflammatory cytokines rather than
hormones whose principal function is to regulate
calcium metabolism. Several cis regulatory elements and
trans-acting factors involved in MCP-1 regulation have
been identified in a variety of cell types (Hanazawa et
al., 1993). NF-κB binding sites appear to be involved in
the response to IL-1β and TNF-α (Ueda et al., 1994,
1997; Ping et al., 1996). Additional cis elements close to
these κB sites (Freter et al., 1995, 1996) and a 7 bp
response element in the 3′ untranslated region of the
MCP-1 promoter have also been shown to induce MCP-
1 transcription in 3T3 cells in response to PDGF (Freter
elements to IL-1 and TNF appear to lie at some distance
from the transcription initiation site. There is also a
cluster of regulatory elements in the proximal promoter
region, including response elements for the AP-1 trans-
acting factors, which appear to mediate TPA-induced
MCP-1 transcription (Shy et al., 1990; Li and
ebattuku, 1994).

Valente et al. investigated the regulation of MCP-1
expression by interferon-γ (Valente et al., 1998). IFN-γ is
a leukocyte-derived cytokine that has been shown to
inhibit osteoclast formation (Roodman, 1993) and yet
paradoxically, is an effective treatment for osteoporotic
patients who have thinned bones due to a failure to
form an adequate number of osteoclasts (Key et al.,
1995). A potential explanation of these apparently
contradictory effects may be due to the indirect effects
of IFN-γ on bone remodeling mediated through activation
of the monocytes. A number of studies have shown that
IFN-γ rapidly induces MCP-1 mRNA accumulation and
MCP-1 production in a variety of cell types (Barker et
al., 1991; Colotta et al., 1992; Schmouder et al., 1993;
Grandaliano et al., 1994). In osteoblastic cells, IFN-γ
stimulates MCP-1 transcription, but has no effect on
MCP-1 mRNA stability (Valente et al., 1998). The
transcriptional elements in the MCP-1 promoter that
mediate the response to IFN-γ were recently identified
using MCP-1 promoter/reporter gene constructs (Fig.
3A). MCP-1 transcription was positively regulated by a
gamma interferon activation site (GAS). However, an
adjacent 13 bp CT-rich sequence (GCTTCCCT
TTCCCT) was found to negatively regulate GAS-
mediated MCP-1 expression (Fig. 3B,C). This
negatively regulating cis element is present on the −227
promoter/reporter construct, but is absent on the −214
promoter/reporter construct. That the smaller (−214)
construct has more activity than the larger (−227)
construct indicates that a negative regulator has been
removed from the smaller construct. The smallest
construct (−198) lacks the positive gamma interferon
activation site (GAS), and hence, does not show IFN-γ
induced luciferase activity. We have termed the novel
negative regulatory element interferon response
inhibitory sequence (IRIS). Stat-1, a trans-acting factor,
was found to bind to the IRIS/GAS sequence. Thus,
at least part of IFN-γ induced MCP-1 transcription is
mediated by this trans-factor. These studies establish
that IFN-γ stimulates MCP-1 expression through a complex
cis-acting element, which has both positive and negative
components.

Physiologic consequences of MCP-1 expression in
vivo

The in vitro studies described above suggest that
MCP-1 expression by osteoblasts is linked to
inflammation. Thus, studies were performed to observe
in vivo expression of MCP-1 (Rahimi et al., 1995). An
inflammatory lesion was created in a murine mandible,
and cells expressing JE, the murine equivalent of human
MCP-1 (Rollins et al., 1989), were identified by
immunohistochemistry. We found that osteoblasts were
the principal cells expressing MCP-1 in inflamed bone.
There was little or no MCP-1 expression in non-
inflamed bone. Furthermore, there was both a spatial
and a temporal association with the recruitment of
mononuclear phagocytes in these lesions. The number
of MCP-1 positive cells was significantly correlated to
the number of monocytes/macrophages present (N=15,
r=0.69, p<0.01, Fig.4). These in vivo results strongly
suggest that MCP-1 is an important mediator involved in
the recruitment of monocytes/macrophages in inflamed
bone.

These studies were expanded to investigate
developmentally regulated expression of MCP-1 in
remodeling bone. They took advantage of the observation that developmentally regulated bone formation and bone resorption occur postnatally in erupting teeth. Bone resorption occurs above the tooth, along the path of resorption, while bone formation occurs at the opposite end (Marks and Schroeder, 1996). In one murine model, on days 5 and 8 postpartum, the greatest number of MCP-1 positive cells was observed in the occlusal area of the erupting first molar (Volejnikov et al., 1997). Bone lining cells consistent with osteoblasts were the principal cell type expressing MCP-1. There was significant correlation between the number of MCP-1 positive cells and monocyte recruitment. It was also shown that MCP-1 expression is correlated with monocyte recruitment to an area of bone formation. On the basis of these studies, we predict that monocytes recruited to the erupting side of the tooth participate in bone resorption by elaboration of bone resorbing cytokines, whereas those on the other side would be functionally distinct. These mononuclear phagocytes could promote bone formation through the production of growth factors.

To investigate the potential effect of MCP-1 on the regulation of bone in vivo, experiments were performed in which exogenous MCP-1 was applied, and the impact on monocyte recruitment and osteoblast number was assessed (Posner et al., 1997). At day 5 after MCP-1 application, a 3-fold increase in the number of mononuclear phagocytes was observed (Fig. 5). By day 28, the number of monocytes had returned to the baseline level, indicating that MCP-1 causes a transient increase in recruitment. Strikingly, MCP-1 application resulted in a significant 2-fold increase in the number of osteoblasts compared with controls treated with vehicle alone. To rule out the possibility that MCP-1 might be directly stimulate proliferation of osteoblastic cells, in vitro experiments were undertaken and demonstrated that it was not mitogenic (Posner et al., 1997). These results suggest that MCP-1 induces recruitment of mononuclear phagocytes, which in turn produce mitogenic factors that stimulate osteoblast proliferation.
Thus, MCP-1 expression may be associated with bone formation as suggested by the tooth eruption studies.

**Osteoblasts express a number of different chemokines which may be important in bone remodeling**

As discussed above, the osteoblastic cell line MG-63, has been used as a model to investigate chemokine expression. In addition to the production of MCP-1, two related chemokines, MCP-2 and MCP-3 were isolated as novel monocyte chemotactic agents from conditioned medium of MG-63 cells (Van Damme et al., 1992). MCP-2 and MCP-3 share with MCP-1 a target specificity for monocytes, activated T lymphocytes and basophils, but unlike MCP-1, they are also active for eosinophil leukocytes (Dahinden et al., 1994; Weber et al., 1995). A number of CXC chemokines (GCP-2, IL-8, GRO-α, GRO-γ, and IP-10) have also been purified from the conditioned medium of MG-63 cells (Proost et al., 1993). GCP-2, IL-8, GRO-α, and GRO-γ contain the characteristic ELR amino acid motif between the N-terminal and the first cysteine residue and are potent chemoattractants for neutrophils. Among the known CXC chemokine receptors, GCP-2, GRO-α and GRO-γ bind only CXCR2 whereas IL-8 binds both CXCR1 and CXCR2. The CXC chemokine IP-10, which was first identified by its induction in monocytic cells by IFN-γ, does not contain the ELR motif in the N-terminal domain, and appears to be a specific chemoattractant for activated T lymphocytes. IP-10 and another non-ELR CXC chemokine, MIG, share specificity for the recently identified receptor CXCR3 (Loetscher et al., 1996), which is selectively expressed on activated CD4+ and CD8+ T lymphocytes.

Osteoblasts from normal bone produce IL-8 constitutively, and this production can be enhanced with IL-1β or TNF-α stimulation (Chaudhary et al., 1992). IL-8 production has also been found in Pagetic osteoblast-like cells, osteosarcoma cells and osteoclast (Birch et al., 1993; Rothe et al., 1998). It has been suggested that IL-8 may contribute to bone remodeling under physiological or inflammatory conditions, because of its capacity to induce the signaling molecule, nitric oxide, by osteoclast-like cells (Suyner et al., 1996). IL-8 could potentially affect bone formation because of its activity as an angiogenic factor (Srieter et al., 1992). In addition, the expression of IL-8 may be important in bacterial infection of bone since IL-8 is a potent neutrophil activator and chemoattractant.

The CC chemokine, MIP-1α, has been shown to be produced by osteoblasts at bone remodeling sites (Kukita et al., 1997). Osteoblasts were frequently observed in the vicinity of these osteoblasts, suggesting that either osteoblast precursors were recruited by MIP-1α or osteoclastogenesis was stimulated by it. In support of the latter, it was shown that MIP-1α induced osteoclastogenesis on calcified matrices in vitro. However, it has also been reported that MIP-1α (and IL-8) stimulated osteoclast motility but inhibited bone resorption by rat osteoclasts, primarily through a reduction in the proportion of osteoclasts resorbing bone (Fuller et al., 1995). The authors suggested that the impact on bone resorption was indirect since, in their studies, these C-X-C chemokines had no direct effect on osteoclast formation or survival. Furthermore MIP-2, RANTES, MIP-1B, and MCP-1 did not have an effect on resorption (Fuller et al., 1995). These findings strongly suggest that MIP-1α is involved in the modulation of bone remodeling.

In summary, when osteoblastic cells are stimulated with inflammatory cytokines, they are able to produce CC chemokines, MCP-1, MCP-2, and MCP-3, which recruit and activate monocytes, subpopulations of lymphocytes, NK cells, basophils and eosinophils. Osteoblastic cells also produce CXC chemokines (GCP-2, IL-8, GRO-α, GRO-γ, and IP-10) that share in common the capacity to specifically recruit and/or activate neutrophils. Because chemokines are pro-inflammatory and are upregulated by proinflammatory cytokines, their actions on osteoclasts might represent mechanisms by which bone resorption is modulated by the inflammatory process when this occurs in bone. However, because chemokines are expressed at low levels constitutively by cells of osteoblastic lineage, they may also be components of the physiologic regulation of bone resorption.

**References**


MCP-1 expression in bone


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