[Frontiers in Bioscience 4, d571-580, July 1, 1999]

THE EXPRESSION OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 AND OTHER CHEMOKINES BY OSTEOBLASTS

Dana T. Graves [1], Yanling Jiang [2], and Anthony J. Valente [3]

[1] Department of Periodontology and Oral Biology, [2] Department of Endodontics, Boston University School of Dental Medicine, Boston, MA 02118, [3] Department of Medicine, University of Texas Health Science Center, San Antonio, Texas

TABLE OF CONTENTS

1. ABSTRACT

Chemokines are low molecular weight secretory proteins that function principally as stimulators of leukocyte recruitment. There are four defined chemokine subfamilies based on their primary structure, CXC, CC, C and CX_3C. Members of the CC chemokine subfamily, a such as monocyte chemoattractant protein 1 (MCP-1) are chemotactic for monocytes and other leukocyte subsets. Because monocytes produce factors that regulate bone formation or resorption, such as PDGF, IL-1 or TNF, chemokines that initiate their recruitment are likely to be important in regulating osseous metabolism. In the studies below, data is presented demonstrating mechanisms of MCP-1 expression in osteoblastic cells. These studies establish that MCP-1 is induced during osseous inflammation and in developmentally regulated bone remodelling, and is associated with enhanced monocyte recruitment when applied to osseous lesions.

2. INTRODUCTION

Chemokines are low molecular weight secretory proteins that function principally to stimulate leukocyte recruitment, and in some cases, leukocyte activation (1,2). They are secondary inflammatory mediators that are induced by primary mediators such as interleukin-1 and tumor necrosis factor. There are four defined chemokine subfamilies based on their primary structure, CXC, CC, C and CX_3C. The best characterized are the CC and CXC chemokines. Members of the CC chemokine subfamily, such as monocyte chemoattractant protein 1 (MCP-1), RANTES, MIP-beta and MIP-1alpha are chemotactic for monocytes, subsets of lymphocytes and natural killer cells, whereas CXC chemokines generally, but not exclusively, induce neutrophil chemotaxis.

3. EXPRESSION OF CHEMOKINES BY OSTEOBLASTIC CELLS

Bone remodeling involves the action of osteoclasts and osteoblasts. In a healthy young adults, bone resorption is linked to bone formation, so that the amount of bone loss is coupled with the formation of an equivalent amount of new bone (3). Since leukocytes produce factors capable of modulating both osteoclast and osteoblast activity, their recruitment is likely to represent a significant event in regulating osseous metabolism. Monocytes in particular have been identified as important regulators of bone activity since they produce bone resorptive factors such arachidonic acid metabolites, IL-1, TNF and others. They also produce growth factors which may contribute to the stimulation of bone formation. Thus, by inducing monocyte recruitment, chemokines could indirectly affect bone resorption or formation.

A number of different chemokines are expressed by either osteoblastic cells or osteoclasts. For example, the CC chemokine, MIP-1alpha is expressed by osteoblasts at bone remodeling sites (4). This expression is associated with bone remodelling as osteoclasts are frequently observed in the vicinity of these osteoblasts, suggesting that either osteoclast precursors are recruited by MIP-1alpha or osteoclastogenesis is stimulated by it. The latter is supported by recent findings that MIP-1α is an important factor in stimulating osteoclast differentiation *in vitro*, by stimulating formation of osteoclast precursors (5). Furthermore, MIP-1alpha (and IL-8) stimulates motility of osteoclastic cells (6). This does not appear to be a general effect of chemokines, since MIP-1beta, and MCP-1 do not have this effect on osteoclasts (6).

When osteoblastic cells are stimulated with inflammatory mediators *in vitro*, they produce CC chemokines such as MCP-1, MCP-2, and MCP-3 (7), which can recruit and activate monocytes, and selectively stimulate subpopulations of lymphocytes, NK cells, basophils and eosinophils (1,2). Osteoblastic cells also produce CXC chemokines (GCP-2, IL-8, GRO-alpha, GRO-gamma, and IP-10) that share in common the capacity to specifically recruit and/or activate neutrophils (8). Osteoblasts and osteoclasts produce IL-8 constitutively.

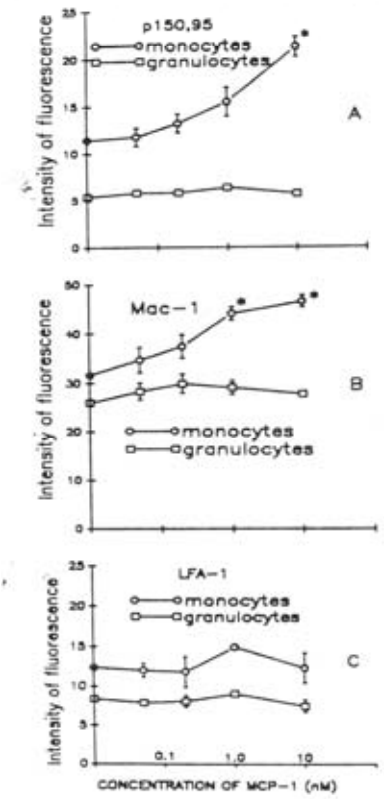

Figure 1. MCP induces CD11/CD18 expression. Expression of p150.95 (CD11a) (A), Mac-1 (CD11b) (B), and LFA-1 (CD11c) (C) was measured by indirect immunofluorescence flow cytometry as described in (25). Circles represent values obtained with specific antibodies and squares represent values obtained with matched control immunoglobulin. Asterisks indicate statistical significance compared with unstimulated control ($p<0.01$). Reproduced with permission from: The *J Immunol*148: 2423-2428, 1992 (The American Association of Immunologists).

Figure 2. Attachment of MCP-1-stimulated Monocytes To Laminin-Coated Surfaces Involves B_2 Integrins. Monocytes were preincubated with 50 µg//ml of control rat immunoglobulin, A; beta-1 antibody (Mab 13), B; control immunoglobulin, C; or beta-2-antibody (TS1/18), D. Monocyte attachment assays were carried out, and cell attachment was determined as described in (69). Photomicrographs were taken using a x20 objective. Arrowheads, attached monocyte. Bar, 50 um. Figure 2 is reproduced with permission from: Amer. Journal of Physiology (The American Physiological Society).

This production is enhanced by IL-1beta or TNF-alpha stimulation (9,10). IL-8 production has been found in Pagetic osteoblast-like cells and osteosarcoma cells (11). Human osteoclastic cells grown in culture produce high levels of IL-8 when stimulated by the pro-inflammatory factors, IL-l and TNF (10). The expression of IL-8 may be important in bacterial infection of bone since IL-8 is a potent neutrophil activator and chemoattractant.

Chemokine expression may play an important role in inflammatory cell recruitment in cartilage and bone destruction that occurs in rheumatic disease. Interleukin-8 (IL-8), MIP-1alpha and MCP-l have been implicated in rheumatoid arthritis (RA), while MIP-1alpha has been detected in synovial arthritis (12,13). Osteoblastic cells cultured from patients with rheumatoid arthritis or osteoarthritis exhibit strong induction of MCP-1, MIP-1alpha and IL-8 (14). Chemokine production has also been implicated in failures of total hip replacement implants. When histologic sections of failed hip replacements are analyzed, polyethylene debris is surrounded by macrophages at the bone-implant interface (15). Expression of chemokine mRNAs is also observed at these sites. Thus, particles released from wear of hip prostheses may induce the production of cytokines and chemokines leading to the recruitment of inflammatory cells. The production of bone-resorptive cytokines by these cells could then lead to loosening of the implant.

4. MONOCYTE CHEMOATTRACTANT PROTEIN-1

MCP-1, a monomeric polypeptide, is typically secreted in two predominant forms with molecular weights of 9 and 13 kD. Lectin blots indicate that disaccharide galactose-b-3D-N-acetyl galactosamine is present on the 13 kD MCP-1 isoform but not the 9 kD isoform. MCP-1 induces recruitment of monocytes, a subset of T lymphocytes, eosinophils, and basophils (1,2). Leukocyte recruitment requires coordinated activity involving the expression of adhesion molecules on endothelial cells and leukocytes. Chemotactic signals induce the activation of integrins on monocytes, particularly integrins of the β_2 family, which interact with counter-receptors on vascular endothelial cells. MCP-1 has been shown to both up-regulate the expression of β_2 integrin on monocyte cell surfaces (figure 1) and the activation of these molecules. In addition, β_2 integrins are involved in the adhesion of monocytes to extracellular matrix proteins in response to chemokines such as MCP-1 (figure 2). After diapedesis, monocytes migrate through connective tissue by binding to extracellular matrix proteins such as collagen, fibronectin and laminin. Monocyte migration across these proteins in response to MCP-1 also is dependent upon β_2 integrins (figure 3).

Significant expression of MCP-1 in normal cells usually requires stimulation by pro-inflammatory agents, while it appears to be constitutively produced by different human tumors (16-19). MCP-1 expression may account for much of the monocyte chemotactic activity produced by tumor cells and contribute to the presence of tumor-

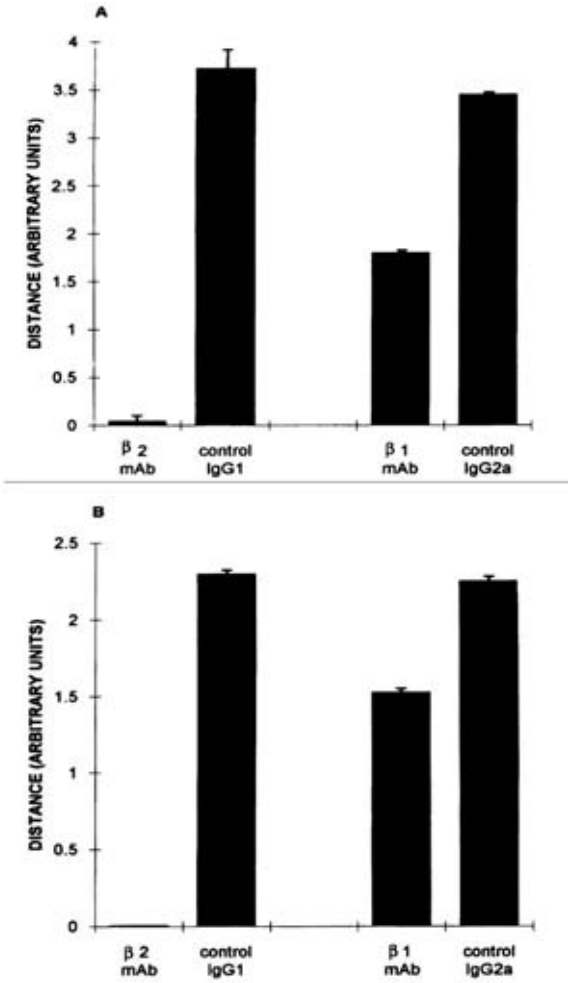

Figure 3. Differential Effect of mAb TO beta-1 and beta-2 Integrins On Monocyte Migration On Laminin or Fibronectin. The role of integrins in monocyte migration was measured using the sub-agarose migration assays as we described in (70). Blockage of monocyte migration with mAb's directed to beta-1 and beta-2 integrins (mAb13 & L130 respectively) on (A) laminin and (B) fibronectin. Each data point represents the mean ± S.E.M. of quadruplicate samples. Reproduced with permission from: Amer. Journal of Physiology 267: C1112-C1118, 1994 (The American Physiological Society).

associated macrophages. MCP-1 was first purified by Valente and co-workers (20) and subsequently sequenced independently by two different groups (21,22). The murine form of MCP-1, JE, was initially identified as an immediate early gene induced in response to platelet-derived growth factor (23). The structure and properties of murine and human MCP-1 have recently been reviewed by Rollins (24). 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 (1,2,24). 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 (25). In addition, this chemokine elicits a respiratory burst at high concentrations, which leads to the generation of oxygen radicals (26). MCP-1 is also a potent inducer of histamine release from basophils and has been implicated as an important mediator in allergic inflammation (27). The principal effect of injecting MCP-1 *in vivo* is the recruitment of monocytes (21).

In early studies, MCP-1 was shown to bind to freshly isolated monocytes through high affinity cell surface binding sites (28,29). Subsequently, a MCP-1 receptor (CCR2) was cloned (30) 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. Both forms of the receptor mediate ligand-dependent Ca^{2+}-mobilization, cellular migration and inhibition of adenyl cyclase. However, differences in the C-terminal appear to result in specificity for Gα-protein subtypes, suggesting that there are functional differences in the CCR2 subtypes (31). The CC chemokine receptors identified so far all demonstrate multiple ligand specificities. In addition to MCP-1, CCR2 binds the related chemokines, MCP-2, MCP-3 and MCP-4 (2,24). 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 markedly impaired as is the ability of macrophages to clear infection by the intracellular bacteria Listeria monocytogenes (32,33). Furthermore, disruption of the MCP-1 gene also leads to impairment in the monocyte recruitment process and shares features with the CCR2 knockout model (34). Thus despite multiple ligand specificity, some functions of the chemokines and chemokine receptors may remain unique and essential.

Although chemokines were first described based upon their capacity to stimulate recruitment or activation of leukocytes, it has become evident that they possess other functions, including regulating events in bone marrow. For example, MCP-1 in conjunction with TGF-beta, inhibits cycling of early progenitor cells in the bone marrow (35). In support of this, antibody to MCP-1 antagonizes the inhibitory effect that long-term cultures of stromal cells have on progenitor cell proliferation.

5. MCP-1 EXPRESSION BY OSTEOBLASTIC CELLS *IN VIVO*

Our laboratories have recently focused on the expression of MCP-1 and bone cells. These studies have elucidated mechanisms for the regulated expression of MCP-1, which may be generally applicable to other cell types. The significance of these studies stems from the likelihood that MCP-1 expression (as well as that of other

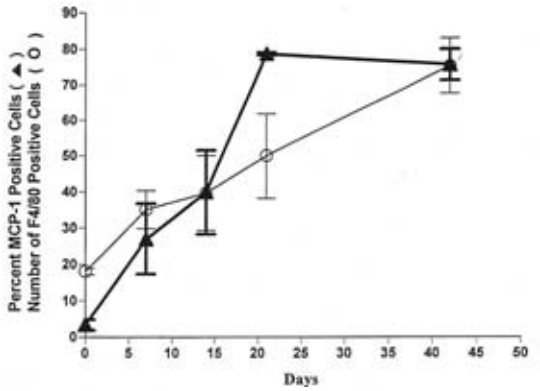

Figure 4. MCP-1 Expression Is Temporally Correlated With The Recruitment Of Mononuclear Phagocytes**.** To statistically correlate the expression of MCP-1 and the recruitment of monocytes/macrophages in bone, osseous lesions were induced in BALB/c mice. Experimental animals were sacrificed at 0 (no exposure), 7, 14, 21, or 42 days after induction of inflammation. Immunohistochemistry was carried out using a polyclonal antiserum to murine MCP-1 (closed triangles) and adjacent sections were incubated with F4/80 monoclonal antibody to detect mononuclear phagocytes) (open circles). The graph shows the percentage of MCP-1 positive cells lining bone and the number of F4/80 positive cells in the same field. The X-axis represents the number of days following osseous induction with 0 (zero) representing control animals. Bars represent the SEM. Statistical analysis of MCP-1 positive cells lining bone and F4/80 positive cells showed them to be significantly correlated (N = 15, r = 0.69, p <= 0.01). Reproduced with permission from: *Endocrinology* 136: 2752-2759, 1995.

chemokines) affects osseous metabolism. Because of its role in stimulating monocyte recruitment, we investigated MCP-1 expression by osteoblasts under conditions where osseous inflammation is induced (36). In these experiments, inflammatory lesions were created in the murine mandible, and cells expressing MCP-1 were identified by immunohistochemistry. Osteoblasts were the principal cells expressing MCP-1 in inflamed bone, while MCP-1 expression was virtually non-existent in non-inflamed normal bone. Moreover, there was both a spatial and a temporal association with the recruitment of mononuclear phagocytes and MCP-1 production in these osteolytic lesions (figure 4). Correlation analysis revealed that the number of MCP-1 positive cells was significantly associated with the number of monocytes/macrophages present ($r = 0.69$, $p < 0.01$). These *in vivo* results strongly suggest that MCP-1 is an important mediator involved in the recruitment of monocytes/macrophages to inflamed bone. *In vitro* studies support the notion that MCP-1 is one of the principal CC chemokines induced in osteoblasts in response to bacterial stimulation (37).

The above approach was expanded to investigate developmentally regulated expression of MCP-1 in remodeling bone (38). These studies took advantage of the observation that developmentally regulated bone remodelling occurs postnatally in erupting teeth. Bone resorption occurs above the tooth, along the path of resorption, while bone formation occurs at the opposite end (39). In a 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 (figure 5). 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. An association between the formation of osteoclasts and MCP-1 expression was also noted, suggesting that the recruited monocytes may act to enhance osteoclastogenesis. In the area of bone formation, MCP-1 production coincided with monocyte recruitment. This was unexpected since monocyte recruitment had previously been correlated with resorption but not bone formation. It is possible, though not proven, that monocytes recruited to the erupting side of the tooth participate in bone resorption by elaborating bone resorbing cytokines, whereas those recruited to sites of bone formation would be functionally distinct. The latter could promote bone formation through the production of growth factors. Que and Wise have similarly described a relationship between tooth eruption and MCP-1 expression (40). They reported that mediators which promote bone-remodelling, such as IL-1, TGF-beta and CSF-1, enhance the expression of the MCP-1 in areas of tooth eruption *in vivo* and in cultured cells associated with tooth eruption *in vitro* (41). Thus, expression of MCP-1 may be critical for recruiting monocytes to initiate the cellular events of developmentally regulated bone remodelling which occurs during tooth eruption.

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 (42). At day 5 following MCP-1 application, a 3-fold increase in the number of mononuclear phagocytes was observed (figure 6). By day 28, the number of monocytes had returned to the baseline level, indicating that MCP-1 caused a transient increase in recruitment. Furthermore, 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 directly stimulate proliferation of osteoblastic cells, *in vitro* experiments were undertaken and demonstrated that it was not mitogenic for osteoblastic cells (42). It is possible that MCP-1 induces recruitment of mononuclear phagocytes, which in turn produce mitogenic factors that stimulate osteoblast proliferation. These results are consistent with reports that monocyte/macrophage products are mitogenic for osteoblastic cells *in vitro* (43). Thus, MCP-1 expression may be indirectly associated with bone formation as suggested by the tooth eruption studies.

6. REGULATION OF MCP-1 EXPRESSION

It is thought that monocytes play an important role in regulating osseous metabolism. Significant monocyte recruitment occurs at sites of bone injury and remodeling. As mentioned above, monocytes affect bone through the production of factors that can stimulate bone

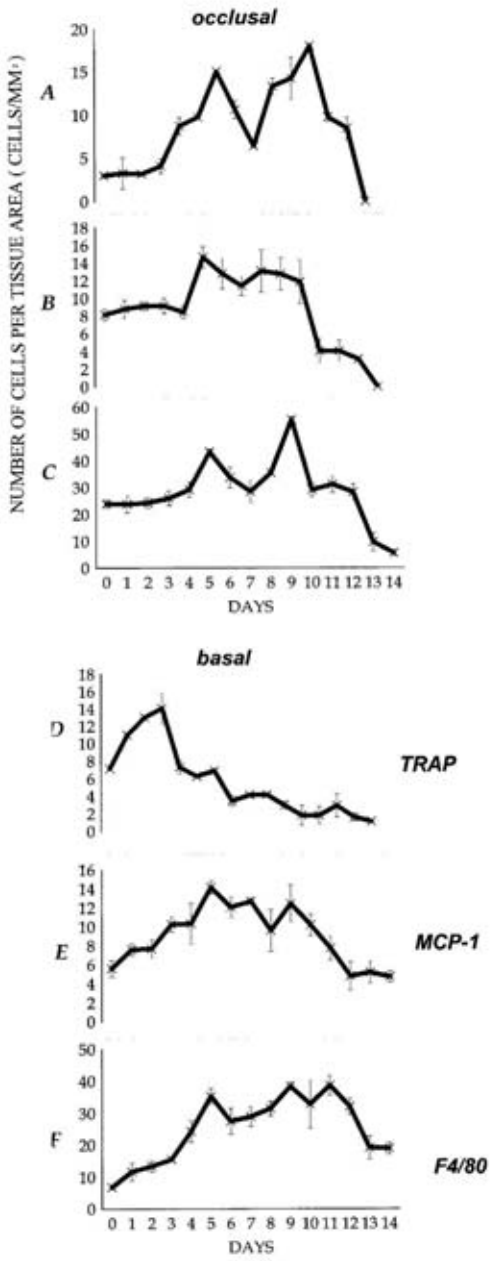

Figure 5: Quantitation of MCP-1 Positive Cells, Monocytes And Osteoclasts During Tooth Eruption. Mice were sacrificed from birth to day 14 postpartum. Adjacent serial sections of the mandibular first molars were examined to assess changes in MCP-1 expression,. Monocyte recruitment and osteoclastogenesis during developmentally regulated tooth eruption. MCP-1 immunopositive osteoblastic cells, F4/80 positive mononuclear phagocytes, and bone-lining TRAP positive osteoclasts were counted using an image analysis system as described in (38). A,B,C: occlusal area (bone resorption); D,E,F: basal area (bone formation). Reproduced with permission from (American Journal of Pathology) 150 #5 May (1997).

resorption or formation. Our laboratory and that of Van Damme and colleagues were the first to demonstrate that MCP-1 could be expressed by osteoblastic cells *in vitro* (17,18). The studies described below have addressed the regulation of MCP-1 expression in osteoblastic cell lines and normal osteoblastic cells.

The cell line, MG-63, has been used as an osteoblastic cell "model" for chemokine studies. In addition to MCP-1, MCP-2 and MCP-3 were isolated as novel monocyte chemoattractants from conditioned medium of MG-63 cells (7). 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 eosinophils (44,45). In most non-transformed cells, chemokines are induced by inflammatory mediators. For example, TNF-alpha induces murine MCP-1 expression via activation of fos and jun in an osteoblastic cell line (46,47). The CXC chemokine, IP-10, is also expressed by osteoblastic cells and its expression is regulated by cytokines such as IFN-gamma, IL-1alpha, and TNF-alpha (48). A number of other CXC chemokines (GCP-2, IL-8, GRO-alpha, and GROamma) have also been isolated from the conditioned medium of MG-63 cells (8). GCP-2, IL-8, GRO-alpha and GRO-gamma contain the characteristic ELR amino acid motif found in CXC chemokines between the N-terminus and the first cysteine residue and are potent chemoattractants for neutrophils (49).

Williams and colleagues tested normal human osteoblastic cells to identify monocyte chemoattractants produced by stimulated osteoblasts (50). Results showed that IL-1 stimulation induced normal human osteoblastic cells to produce monocyte chemotactic activity. Without IL-1 stimulation, osteoblast-conditioned medium had no effect on monocyte chemotaxis. The addition of IL-1 induced the expression of monocyte chemotactic activity in the medium within 6 hours. 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 reports that IL-1 alone has little or no chemotactic activity for monocytes. Evidence that MCP-1 antiserum inhibits virtually all IL-1 stimulated monocyte chemotactic activity produced by normal human osteoblastic cells suggested that MCP-1 is an important osteoblast-produced chemokine (figure 7).

In vitro studies have shown that for most normal cell types there is little or no MCP-1 expression and exogenous stimulation is required for induction of MCP-1. Zhu *et al* studied the effects 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. After 24 hours, little MCP-1 expression (< 5 ng/mL) was found in medium conditioned by unstimulated cells or in cells that were stimulated with non-inflammatory calciotropic mediators (PTH, Vit D, hydrocortisone). However, physiologic concentrations of MCP-1 were found in conditioned medium from cells treated with IL-1beta, TNF-alpha, IL-6, and TGF-beta.

Table 1. Secretion of MCP-1 by normal human osteoblasts

Addition	MCP-1 (ng/ml)	
	24h	48h
Control	<5	14 ± 0.3
PTH (10 nM)	<5	9 ± 1.4
$1,25(OH)_2D_3$ (20 nM)	<5	10 ± 1.2
Hydrocortisone (140 nM)	<5	11 ± 0.8
PDGF-BB (15 ng/ml)	<5	14 ± 1.5
TGF-beta (2 ng/ml)	7 ± 1.1	11 ± 0
IL-6 (20 nM)	19 ± 0	23 ± 1.3
TNF-alpha (20 ng/ml)	48 ± 2.6	93 ± 7.6*
IL-1beta (50 u/ml)	63 ±1.7	84 ±7.0*

Serum-free cultures of normal human osteoblastic cells were incubated with physiologic concentrations of PTH, $1,25(OH)_2D_3$, hydrocortisone, PDGF-BB, IL-1beta, TNF-alpha, IL-6, TGF-beta, or vehicle control. Conditioned media was collected after 24 or 48h and MCP-1 was assayed by RIA. Values are mean ± 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 48 h time pint ($p<0.01$). Statistical significance was not determined for the 24 h period because the value of the negative control was below the detection threshold. Table 1 is reproduced from with permission from: J. Bone Min. Res. (1994); 9: 1123-1130 with permission of the American Society for Bone and Mineral Research.

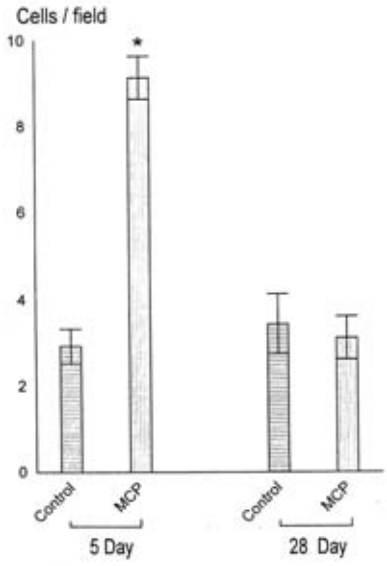

Figure 6: MCP-1 Application *In vivo* Is Associated With An Increase in Osteoblast Number. Creation of osseous lesions and application of MCP-1 or the vehicle alone (control), was performed as described in (42). Animals were sacrificed five or twenty eight days following application. Monocytes/macrophages were counted as F4/80 immunopositive cells and counted with the use of an image analysis system. * indicates statistically significant compared to the negative control ($p<0.01$). Reproduced with permission from: Bone, 21:321-327, 1997 (Elsevier Science Ltd).

These investigators also examined the effect of osteotrophic mediators on MCP-1 mRNA levels in two osteoblastic cell lines, SaOS-2 and MG-6 (51). Constitutive expression of MCP-1 was very 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-alpha (20 ng/mL). Similar to results obtained with normal cells, in the SaOS-2 cells, IL-1beta and TNF-alpha induced dose-dependent increases in the MCP-1 mRNA level of 36-fold and 28-fold, respectively. TGF-beta and IL-6 induced increases of approximately 3-fold each. In MG-63 cells, the following dose-dependent increases in the MCP-1 mRNA levels were seen: TNF-alpha, 20 fold; IL-1beta, 15 fold; TGF-beta, 9 fold; and IL-6, 2 fold.

The above studies indicate that MCP-1 is regulated by inflammatory cytokines. Several cis regulatory elements and trans-acting factors involved in this regulation have been identified in a variety of cell types. NF-kappaB binding sites appear to be involved in response to IL-1β and TNF-alpha, (52-54). Additional cis elements close to these kappaB sites (55,56) and a 7 bp response element in the 3' untranslated region of the MCP-1 promoter have also been shown to mediate MCP-1 transcription in 3T3 cells in response to PDGF (55-57). Interestingly, the response 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 (58,59).

Valente *et al* investigated the regulation of MCP-1 expression by interferon-gamma (60). IFN-gamma is a leukocyte-derived cytokine that has been shown to inhibit osteoclast formation (61) and yet paradoxically, it has been shown to be an effective treatment for osteopetrotic patients who suffer from an inadequate amount of osteoclasts (62). A number of studies have shown that IFN-gamma rapidly induces MCP-1 mRNA accumulation and MCP-1 in a variety of cell types (63-66). In some cell types MCP-1 is regulated at the post-transcriptional level (67). To assess the level at which MCP-1 mRNA levels were enhanced by IFN-γ, nuclear run on experiments were carried out in MG-63 osteoblastic cells (figure 8). Although the basal levels of MCP-1 mRNA are high in MG-63 cells, IFN-gamma stimulation resulted in a marked increase in MCP-1 transcription. In the same report, it was also shown that IFN-γ did not enhance MCP-1 mRNA stability. Thus, MCP-1 expression in IFN-gamma-stimulated osteoblastic cells is regulated at the transcriptional level.

IFN-gamma has been shown to induce transcription in several genes through a transcriptional element termed the GAS (gamma interferon activated site). The consensus sequence (TTNCNNNAA) has been defined for GAS. A GAS element exists within the proximal promoter region of MCP-1 at -214 relative to the ATG start codon (figure 9A.). The core sequence of this element (TTCCTGGAA) resembles closely the symmetrical dyad sequence defined as the binding site for the Stat family of transcription factors (with the exception of Stat2).

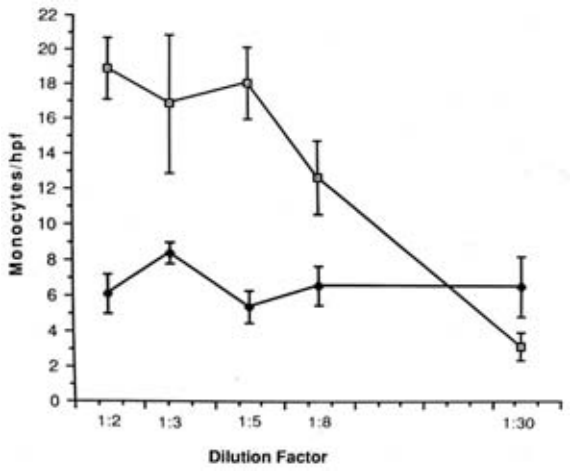

Figure 7. Antibody to MCP-1 Inhibits Monocyte Chemotactic Activity Produced by Stimulated Osteoblasts. Normal human osteoblasts were stimulated with IL-1βετα and the conditioned media was collected and diluted with plain media as indicated. Each dilution was preincubated with MCP-1 antiserum (closed circles) or control IgG (open circles). Samples were then tested for recruitment of peripheral blood monocytes using a modified Boyden chamber as indicated in (50). Reproduced with permission from: Amer. Journal of Physiology, 263:C194-C199, 1992 (The American Physiological Society).

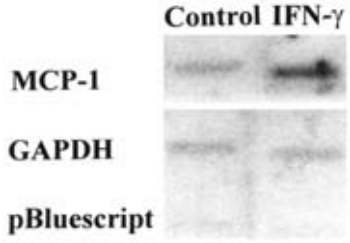

Figure 8. Nuclear Run-on Analysis of MCP-1 Transcription. MG-63 cells were incubated with or without (100 U/ml) IFN-gamma for 6 h. Nuclei from control or IFN-gamma-stimulated cells were isolated, and ^{32}P-labeled nuclear run-on products were hybridized to denatured plasmid DNAs slot-blotted on nylon membrane. Bands represent hybridization with MCP-1 (row 1), GAPDH (row 2), and pBluescript vector without insert (row 3). Reproduced with permission from: Amer. Journal of Physiology, 263:C194-C199, 1992 (The American Physiological Society).

Adjacent and 5' to this site in the MCP-1 promoter is a CT-rich element (GCTT CCCT TTCC TAC) which shows some homology to the consensus sequence for the interferon-stimulated response element (ISRE) (68), that has been shown to mediate transcriptional responses to both IFN-γ and IFN-α/β. The importance of this site is suggested by the high degree of conservation between species (figure 9B).

Transfection studies were carried out with a deletion series of MCP-1 promoter/luciferase plasmids to identify and characterize the IFN-γ-responsive elements (figure 10). When the largest construct tested was examined, construct 'a' (-2910/+21), IFN-γ stimulated a 1.7 fold increase in luciferase activity compared to unstimulated controls. Construct 'a' included the κB and other regulatory elements located in the distal enhancer region that have recently been shown to regulate MCP-1 transcription in response to IL-1β and TNF-α (52). In construct 'b' (-248/+21) and 'c' (-227/+21) the functional κB site and the majority of the 5' flanking sequence were eliminated. This IFN-gamma induced a 3 fold increase in luciferase activity in these constructs, indicating that they distal enhancer elements in the MCP-1 promoter are not required for IFN-gamma induced transcription in these cells. Since construct C contained a GAS element and an adjacent CT rich element, construct 'd' (-214/+21) was prepared so that it terminated at the 5' end of the GAS element and eliminated the adjacent CT element. Unexpectedly, this construct produced the greatest increase in the reporter gene activity observed in these experiments (≥10 fold). Thus the CT element appears to inhibit the response to IFN-gamma-mediated by the GAS element. When the GAS element was deleted (construct 'e', -198/+21), responsiveness to IFN-gamma was lost completely. Therefore these experiments indicate that in the MG-63 osteoblastic cell line, the rapid IFN-gamma-induced increase in transcription of MCP-1 appears to be mediated entirely through the GAS element located at position -214 to -198, whereas the adjacent CT element appears to negatively regulate this GAS-mediated response. We have termed this CT element the interferon response inhibitory sequence (IRIS), which describes its observed functional activity.

7. SUMMARY

The above studies demonstrate that chemokines are expressed by osteoblastic and osteoclastic cells. More specifically, the chemokine MCP-1 is produced by osteoblastic cells *in vitro* and *in vivo*, and this expression occurs in inflamed bone or is induced by inflammatory mediators. Furthermore, the application of MCP-1 in osseous lesions enhances that degree of monocyte recruitment. Because chemokines are both proinflammatory and upregulated by proinflammatory cytokines, their actions might contribute to bone resorption associated with inflammatory osteolytic lesions. However, they may also be components of the physiologic regulation of bone resorption since some chemokines are expressed at low levels constitutively by cells of osteoblastic lineage.

8. REFERENCES

1. Graves D, Y. Jiang: Chemokines, a family of chemotactic cytokines. *Crit Rev Oral Biol Med* 6, 81-176 (1995)

2. Baggiolini M., B. Dewald & B. Moser: Human chemokines: an update. *Annu Rev Immunol* 15, 675-705 (1997)

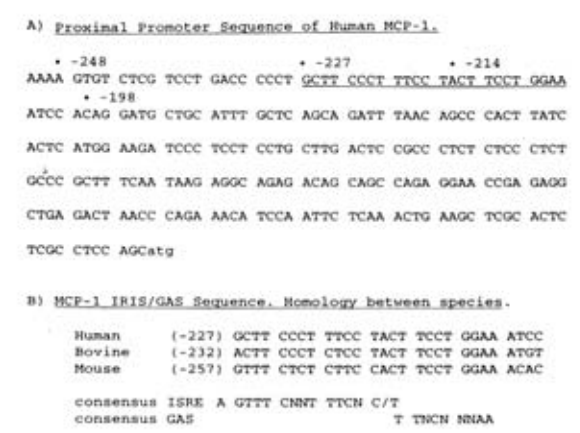

Figure 9. Nucleotide sequence of the proximal 5' flanking region of the MCP-1 gene. A: Sequence from the National Center for Biotechnology (NCBI) GeneBank accession # D26087. Numbers refer to nucleotide positions relative to the translation start site. The IFN-gamma response element is underlined. B) Sequences from bovine, rat and murine MCP-1 showing homology with the human MCP-1 IFN-gamma response element. Numbers refer to position of the 5' nucleotide relative to the translation start site. Sequences taken from the GeneBank at NCBI.

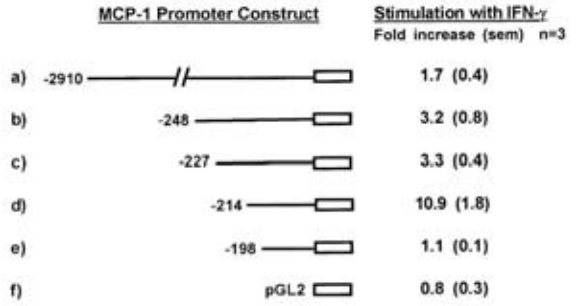

Figure 10. Identification of the IFN-gamma Responsive Region In The Human MCP-1 Promoter. A schematic diagram indicating the location of the deletion constructs in the MCP-1 gene 5' flanking region and the resulting responsiveness to IFN-gamma in transfected MG-63 cells. Cells were transfected with the MCP-1/luciferase constructs and the CMV-Renilla plasmid (transfection efficiency control) as described in *Materials and Methods*, incubated for 3 days, then stimulated with either 1000 U/ml human recombinant IFN-γ or with medium (unstimulated control) for 4 h. Luciferase activity was determined and normalized to renilla activity. Results are expressed as fold stimulation of luciferase activity.

3. Vaananen HK,:. Mechanism of bone turnover. *Ann Med* 25, 353-359 (1993)

4. Kukita T, H. Nomiyama, Y. Ohmoto, A. Kukita , T. Shuto, T. Hotokebuchi, Y. Sugioka , R. Miura & T. Iijima: Macrophage inflammatory protein-1 alpha (LD78) expressed in human bone marrow: its role in regulation of hematopoiesis and osteoclast recruitment. *Lab Invest* 76, 399-406 (1997)

5. Scheven BA, J.S. Milne, I. Hunter, S.P. Robins: Macrophage-inflammatory protein-1alpha regulates preosteoclast differentiation *in vitro*. *Biochem Biophys Res Commun* 254, 773-8 (1999)

6. Fuller K, J. Owens & T. Chambers: Macrophage inflammatory protein-1 alpha and IL-8 stimulate the motility but suppress the resorption of isolated rat osteoclasts. *J Immunol*154, 6065-72 (1995)

7. Van Damme J, P. Proost, J. Lenaerts & G. Opdenaker: Structural and functional identification of two human tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J Exp Med* 176, 59-65 (1992)

8. Proost P, C. De Wikf-Peeters, R. Conings, G. Opdenakker, A. Billiau & J. Van Damme: Identification of a novel granulocyte chemotactic protein (GCP-2) from human tumor cells. *In vitro* and *in vivo* comparison with natural forms of GRO, IP-10, an d IL-8. *J Immunol* 150, 1000 (1993).

9. Chaudhary L, T. Spelsberg & B. Riggs: (1992). Production of various cytokines by normal human osteoblast-like cells in response to interleukin-1 beta and tumor necrosis factor-alpha: lack of regulation by 17 beta-estradiol. *Endocrinology* 130, 2528-2534 (1992)

10. Rothe L, P. Collin-Osdoby, Y. Chen T. Sunyer L. Chaudhary, A. Tsay, S. Goldring, L. Avioli, P. Osdoby: Human osteoclasts and osteoclast-like cells synthesize and release high basal and inflammatory stimulated levels of the potent chemokine interleukin-8. *Endocrinology* 139, 4353-4363 (1998)

11. Birch M, A Ginty, C. Walsh, W. Fraser, J. Gallagher & G. Bilbe: (1993). PCR detection of cytokines in normal human and pagetic osteoblast-like cells*J Bone Min Res* 8, 1155-1162 (1993)

12. Koch, A.E., S.L Kunkel, L.A. Harlow, D.D. Mazarakis, G.K. Haines, M.D. Burdick, R.M. Pope & R.M. Strieter: Macrophage inflammatory protein-i alpha. A novel chemotactic cytokine for macrophages in rheumatoid arthritis. *J Clin Invest* 93, 921-928 (1994)

13. AI-Mughales J, T.H. Blyth, J.A. Hunter & P.C. Wilkinson: The chemoattractant activity of rheumatoid synovial fluid for human lymphocytes is due to multiple cytokines. *Clin Exp Immunol* 106, 230-236 (1996)

14. Lisignoli G, S. Toneguzzi C. Pozzi, A. Piacentini, M. Riccio, A. Ferruzzi, G. Gualtieri, A. Facchini: Proinflammatory cytokines and chemokine production and expression by human osteoblasts isolated from patients with rheumatoid arthritis and osteoarthritis. *J Rheumatol* 26, 791-799 (1999)

15. Ishiguro N, T. Kojima, T. Ito, S. Saga, H. Anma, K. Kurokouchi, Y. Iwahori, T. Iwase, H. Iwata: Macrophage activation and migration in interface tissue around loosening total hip arthroplasty components. *J Biomed Mater Res* 35, 399-406 (1997)

16. Bottazzi B, N. Polentarutti, R. Acero, A. Balsari, D. Boraschi, P. Ghezzi, M. Salmona & A. Mantovani: Regulation of the macrophage content of neoplasms by chemoattractants. *Science* 220, 210-212 (1983)

17. Graves D, Y. Jiang, M. Williamson & A. Valente: Identification of monocyte chemotactic activity produced by malignant cells. *Science* 245, 1490-149 (1989).

18. Van Damme J, B. Decock, J. Lenaerts, R. Conings, R. Bertini, A. Mantovani & A. Billiau:. Identification by sequence analysis of chemotactic factors for monocytes produced by normal and transformed cells stimulated with virus, double-stranded RNA or cytokine. *Eur J Immunol* 19, 2367-2373 (1989)
19. Zhang L, A. Khayat, H. Cheng & D. Graves: The pattern of monocyte recruitment in tumors is modulated by mcp-1 expression and influences the rate of tumor growth. *Lab Invest*6, 579-590 (1997)
20. Valente A., D. Graves, C. Vialle-Valentin., R. Delgado & C. Schwartz: Purification of a monocyte chemotactic factor secreted by nonhuman primate vascular cells in culture. *Biochemistry* 27, 4162-4168 (1988)
21. Yoshimura T, E. Robinson, S. Tanaka, E. Apella, J. Kuratsu, E. Leonard : Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *J Exp Me d*169, 1449-1459. (1989)
22. Furutani Y, H. Nomura, M Notake, Y. Oyamada, T. Fukui, M Yamada, C. Larsen, J. Oppenheim & K. Matsushima: Cloning and sequencing of the cDNA for human monocyte chemotactic and activating factor (MCAF). *Biochem Biophys Comm*159, 249-255 (1989)
23. Cochran B, A. Reffel & C. Stiles: Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell* 33, 939 (1983)
24. Rollins B,: Chemokines. Blood 90, 909-928 (1997)
25. Jiang Y, D. Beller, G. Frendl & D. Graves: Monocyte chemoattractant protein-1 (MCP-1) regulates adhesion molecule expression and cytokine production in human monocytes. *J Immunol* 148, 2423-2428 (1992)
26. Rollins B, A. Walz & M. Baggiolini: Recombinant human MCP-1/JE induces chemotaxis calcium flux and the respiratory burst in human monocytes. *Blood* 78, 1112-1116 (1991)
27. Bischoff S, M. Krieger, T. Brunner & C. Dahinder: Monocyte chemotactic protein 1 is a potent activator of human basophils. *J Exp Med* 175, 1271-1275 (1992)
28. Yoshimura T, & E. Leonard: Identification of high affinity receptors for human monocyte chemoattractant protein-1 on human monocytes. *J Immunol*145, 292-297 (1990)
29. A.Valente., M. Rozek., C. Schwartz & D.T. Graves: Characterization of monocyte chemotactic protein 1 binding to human monocytes. *Biochem Biochys Res Commun* 176, 309-314 (1991)
30. Charo I, S. Myers, A. Herman, C. Franci, A. Connolly & S. Coughlin: Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc Natl Acad Sci U S A* 91, 2752-2756 (1994)
31. Kuang Y, Y. Wu, H. Jiang & D. Wu: Selective G protein coupling by C-C chemokine receptors. *J Biol Chem* 271, 3975-3978 (1996)
32. Kurihara T, G. Warr, J. Loy & R. Bravo: Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J Exp Med* 186, 1757-1762 (1997)
33. Kuziel W, S. Morgan, T. Dawson, S.Griffin, O. Smithies, K. Ley & N. Maeda: Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2 *Proc Natl Acad Sci U S A* 94, 12053-12058 (1997)
34. Lu B, B. Rutledge, L. Gu., J. Fiorillo, N. Lukacs, S. Kunkel, R North, C. Gerard & B. Rollins: Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Me d*187, 601-608 (1998)
35. Cashman JD, C.J. Eaves, A.H. Sarris, A.C. Eaves: MCP-1, not MIP-1alpha, is the endogenous chemokine that cooperates with TGF-beta to inhibit the cycling of primitive normal but not leukemic (CML) progenitors in long-term human marrow cultures. *Blood* 92, 2338-44 (1998)
36. Rahimi P, C. Wang, P. Stashenko, S. Lee, J. Lorenzo & D. Graves: MCP-1 expression and monocyte recruitment in osseous inflammation. *Endocrinology* 136, 2752-2759 (1995)
37. Jiang Y, & D.T. Graves: Periodontal Pathogens Stimulate CC-Chemokine Production By Mononuclear And Bone-derived Cells. *J Periondontol*, in press.
38. Volejnikov S, M. Laskari, S. Marks & D. Graves: Monocyte recruitment and expression of monocyte chemoattractant protein-1 are developmentally regulated in remodeling bone in the mouse. *Am J Pathol* 150, 1711-1721 (1997)
39. Marks S.J., & H. Schroeder: Tooth eruption: theories and facts. *Anat Record* 245, 374-93 (1996)
40. Que B.G, & G.E. Wise: Colony-stimulating factor-1 and monocyte chemotactic protein-1 chemotaxis for monocytes in the rat dental follicle. *Arch Oral Biol* 42, 855-860 (1997)
41. Que B.G., & G.E. Wise: Tooth eruption molecules enhance MCP-1 gene expression in the dental follicle of the rat. *Dev Dyn*. 21, 346-351 (1998)
42. Posner L, T. Miligkos, J.Gilles, D. Carnes, D. Taddeo & D. Graves: Monocyte chemoattractant protein-1 induces monocyte recruitment that is associated with an increase in numbers of osteoblasts. *Bone* 21, 321-327 (1997)
43. Rifas L, S.L. Cheng, V. Shen, W.A.. Peck: Monokines produced by macrophages stimulate the growth of osteoblasts. *Conn Tiss Res* 23, 163-178 (1989)
44. Dahinden C., T. Geiser, T. Brunner, V. von Tscharner, D. Caput, P. Ferrara, A. Minty & M. Baggiolini: Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine. *J Exp Med* 179, 751-756 (1994).
45. Weber M., M. Uguccioni, B. Ochensberger, M. Baggiolini, I. Clark-Lewis & C. Dahinden: Monocyte chemotactic protein MCP-2 activates human basophil and eosinophil leukocytes similar to MCP-3. *J Immunol* 154, 4166-4172 (1995)
46. Hanazawa S, A. Akeshita, S. Amano, T. Semba, T. Nirazuka, H. Katoh & S. Kitano: Tumor necrosis factor-alpha induces expression of monocyte chemoattractant JE via fos and jun genes in clonal osteoblastic MC3T3-E1 cells. *J Biol Chem* 268, 9526-9532 (1993)
47. Takeshita A, S. Hanazawa, S. Amano, T. Matumoto & S. Kitano: IL-1 induces expression of monocyte chemoattractant JE in clonal mouse osteoblastic cell line MC3T3-E1. *J Immunol*. 150, 1554-1558 (1993)
48. Ohmori Y, & T. Hamilton: Cell type and stimulus specific regulation of chemokine gene expression. *Biochem Biophys Res Commun*198, 590-596 (1994)

49. Ahuja SK, P.M. Murphy: The CXC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. *J Biol Chem* 271, 20545-20550 (1996)
50. Williams S.R., Y. Jiang, D. Cochran, G. Dorsam, & D.T. Graves: Regulated expression of monocyte chemoattractant protein-1 in normal human osteoblastic cells. *Am J Physiol*263, C194-C199 (1992)
51. Zhu J, A.Valente, J. Lorenzo, D. Carnes & D. Graves: Expression of monocyte chemoattractant protein 1 in human osteoblastic cells stimulated by proinflammatory mediators. *J Bone Min Res*999, 1123-1130 (1994)
52. Ueda A, K. Okuda, S. Ohno, A. Shirai, T. Igarashi, K. Matsunaga, J. Fukushima, S. Kawamoto, Y. Ishigatsubo & T. Okubo: NF-kB and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *J Immunol* 153, 2052-2063 (1994)
53. Ping D, P.L. Jones & J.M. Boss: TNF regulates the *in vivo* occupancy of both distal and proximal regulatory regions of the MCP-1/JE gene. *Immunity* 4, 455 (1996)
54. Ueda A., Y. Ishigatsubo, T. Okuba & T. Yoshimura: Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kB sites and NF-kB/Rel subunit specificity. *J Biol Chem* 272, 31092 (1997)
55. Freter R.R., J.A. Alberta, A.L. Hwang, G.Y. Wrentmore & C.D. Stiles: Platelet-derived growth factor induction of the immediate-early gene MCP-1 is mediated by NF-kB and a 90-kDa phosphoprotein coactivator. *J Biol Chem* 271, 17417 (1996)
56. Freter R.R., J.C. Irminger, J.A Porter, S.D. Jones & C.D. Stiles: A new platelet-derived growth factor-regulated genomic element which binds a serine/threonine phosphoprotein mediates induction of the slow immediate-early gene MCP-1. *Mol Cell Biol* 15, 315 (1995)
57. Freter R.R., J.C. Irminger, J.A. Porter, S.D. Jones & D. S.C. A novel 7-nucleotide motif located in 3' untranslated sequences of the immediate-early gene set mediates platelet-derived growth factor induction of the JE gene. *Mol Cell Biol* 12, 5288 (1992)
58. Shyy Y-J., Y-S. Li & P.E. Kolattukudy: Structure of human monocyte chemotactic protein gene and its regulation by TPA. *Biochem Biophys Res Commun* 169, 346 (1990)
59. Li Y-S, & P. Kolattukudy: Function role of the cis-acting elements in human monocyte chemotactic protein-1 gene in the regulation of its expression by phorbol ester in human glioblastoma cells. Molecular. *Cell Biol* 141, 121-128 (1994)
60. Valente A.J., J. Xie, M.A Abramova, U.O. Wenze, H.E. Abboud, & D.T. Graves: A complex element regulates IFN-gamma-stimulated monocyte chemoattractant protein-1 gene transcription. *J Immunol* 161, 3719-3728 (1998)
61. Roodman D.G.,: Role of cytokines in the regulation of bone resorption. *Calcified Tissue Intl* 53, (suppl 1):S94 (1993)
62. Key L.L.Jr., R.M Rodriguiz, S.M Willi, N.M Wright, H.C. Hatcher, D.R. Eyre, J.K Cure, P.P. Griffin & W.L. Ries: Long-term treatment of osteopetrosis with recombinant human interferon gamma. *New Engl J Med*. 332, 1594. (1995)
63. Barker J., M. Jones, C. Swenson, V. Sarma, R. Mitra, P. Ward, K. Johnson, J. Fantone, V. Dixit & B. Nickoloff: Monocyte chemotaxis and activating factor production by keratinocytes in response to IFN. *J Immunol* 146, 1192. (1991)
64. Colotta C.F., A. Borre, J.M. Wang, M. Tattanelli, F. Maddalena., N. Polentarutti, G. Peri & A. Mantovani: Expression of a monocyte chemotactic cytokine by human mononuclear phagocytes. *J Immuno l*148, 760-765 (1992)
65. Schmouder R.L., R.M. Strieter & S.L. Kunkel: Interferon-gamma regulation of human renal cortical epithelial cell-derived monocyte chemotactic peptide-1. *Kidney Intl* 44, 43 (1993)
66. Grandaliano G., A. Valente, M. Rozek & H. Abboud: Gamma interferon stimulates monocyte chemotactic protein (MCP-1) in human mesangial cells. *J Lab Clin Med* 123, 282-289 (1994)
67. Bosco, M. C., G. L. Gusella, I. Espinoza-Delgado, D. L. Longo, & L. Varesio: Interferon-gamma upregulates interleukin-8 gene expression in human monocytic cells by a posttranscriptional mechanism. *Blood* 83, 537 1994
68. Levy D.E., D.S. Kessler, R. Pine, N. Reich & J.E. Darnell Jr: Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control. *Genes & Dev* 2, 383 (1988)
69. Jiang Y, J.F. Zhu, F.W Luscinskas, D.T. Graves: MCP-1-stimulated monocyte attachment to laminin is mediated by beta 2-integrins. *Am J Physiol* 267, C1112-C1118 (1994)
70. Penberthy T.W., Y. Jiang, F.W. Luscinskas, D.T. Graves: MCP-1-stimulated monocytes preferentially utilize beta 2-integrins to migrate on laminin and fibronectin. *Am J Physiol* 269, C60-C68 (1995)

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

Abbreviations: MCP-1 monocyte chemoattractant protein 1, RANTES regulated upon activation normal T lymphocyte expressed and secreted, MIP-alpha, monocyte inflammatory protein 1 alpha, MIP-beta, monocyte inflammatory protein 1 beta, IL-1 interleukin 1, TNF tumor necrosis factor, IL-8 interleukin 8, NK cells natural killer cells, GCP-2 granulocyte chemotactic protein-2 , GRO-α growth-related protein , GRO-gamma, growth-related protein , IP-10 interferon (IFN)-inducible protein 10, TGF-beta, transforming growth factor beta, PDGF platelet derived growth factor, TPA 12-O-tetradecanoyl phorbol-13-acetate, IFN-gamma interferon-gamma, GAS gamma interferon activated site, IRIS interferon response inhibitory sequence

Send correspondence to: Dr Dana T. Graves, Department of Periodontology and Oral Biology, Boston University School of Dental Medicine, W-202, 700 Albany St., Boston, MA, 02118 Tel: 617-638-8547, Fax: 617-638-4924, E-mail: dgraves@acs.bu.edu

Received 5/27/99 Accepted 6/14/99