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Monocyte Chemoattractant Protein-1 Induces Monocyte Recruitment That Is Associated With an Increase in Numbers of Osteoblasts

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Monocyte chemoattractant protein-1 (MCP-1) is a member of the chemokine family of cytokines. The principal function of MCP-1 is thought to be the stimulation of monocyte recruitment. Monocyte products are potential regulators of bone cell activity. Growth factors produced by monocytes may stimulate bone formation, while cytokines such as IL-1 and IL-6 can induce bone resorption. To determine whether MCP-1 enhances recruitment of monocytes during bone healing, studies were carried out in which MCP-1 was applied to osseous sites in vivo. Changes in monocyte number were determined by immunohistochemistry using the antibody ED-1 specific for peripheral monocytic cells. The effect of MCP-1 on osteoblast number was determined by counting the number of alkaline phosphatase positive cells in close proximity to bone. For comparison, osteoblast number was also determined following stimulation with platelet-derived growth factor (PDGF)-BB plus IGF-1 in vivo. Results indicate that MCP-1 stimulated a large increase in monocyte recruitment compared to vehicle alone. An increase in monocytes induced by MCP-1 was associated with an increase in the number of osteoblasts lining the bone surface, although not to the same magnitude as a positive control, PDGF-BB, and IGF-1. These results indicate that MCP-1 induces the recruitment of monocytes to bone and suggest that the recruitment is associated with an increase in osteoblast number. This is likely to occur via indirect mechanisms, because MCP-1 did not directly enhance DNA synthesis in osteoblastic cells in vitro. Thus, activated mononuclear phagocytes may play an important role in osseous wound healing by stimulating proliferation of osteoblastic cells, presumably through the elaboration of growth factors. (Bone 21:321–327; 1997)

Key Words: Chemokine; Monocyte chemoattractant protein-1; Platelet-derived growth factor; Monocyte; Osteoblast; Inflammation.

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Introduction

Bone resorption and formation are dynamic processes that occur in both normal and injured bone tissue. Regulation of these processes is mediated at the local level by cytokines and/or growth factors, which may be released from the bone matrix or activated by the process of bone resorption.[17,24] Mediators may also be secreted by resident cells, or by mononuclear cells that have migrated to sites of resorption and injury. Bone resorption is a prominent pathological feature of many inflammatory conditions, including periodontitis, osteomyelitis, malignancies such as multiple myeloma, and periapical granulomas resulting from dental infection. Monocytes are thought to play a significant role in regulating osseous metabolism based on the significant recruitment of monocytes that occurs at sites of bone injury and bone remodeling.[15] Activated mononuclear phagocytes can produce factors that stimulate either bone resorption or formation. These factors include IL-1β, TNF-α, IL-6, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and 1,25-dihydroxyvitamin-D_3.[20]

Recruitment of monocytes from the peripheral vasculature is induced by the local production of monocyte chemoattractants. This process involves the activation of adhesion molecules on both endothelial cells and monocytes, adhesion of monocytes to endothelium, transmigration of monocytes along the vasculature, diapedesis, and the migration of monocytes along interstitial tissue in response to a chemotactic gradient. Chemokines are thought to be important in the activation of leukocyte adhesion molecules and as chemoattractants in directed leukocyte migration.[7,11,16] Chemokines are small, inducible proinflammatory cytokines. Unlike classic leukocyte chemoattractants, chemokines stimulate chemotaxis of relatively specific leukocyte subsets. MCP-1 is a chemokine that stimulates chemotaxis in monocytes/macrophages, basophils, and a subset of memory T lymphocytes.[2,10,12,29] By contrast, polymorphonuclear leukocytes and the majority of peripheral blood lymphocytes lack MCP-1 receptors and, thus, do not respond to MCP-1.[29,31]

In vitro studies indicate that most normal nontransformed cells require stimulation for the induction of significant levels of MCP-1 expression.[7,11] MCP-1 production is induced in osteoblasts by inflammatory mediators; during osseous inflammation, its expression is temporally associated with the recruitment of monocytes in vivo.[23,32] Furthermore, MCP-1 is the principal monocyte chemoattractant produced by IL-1 stimulated normal human osteoblastic cells in vitro.[30] It is also the principal

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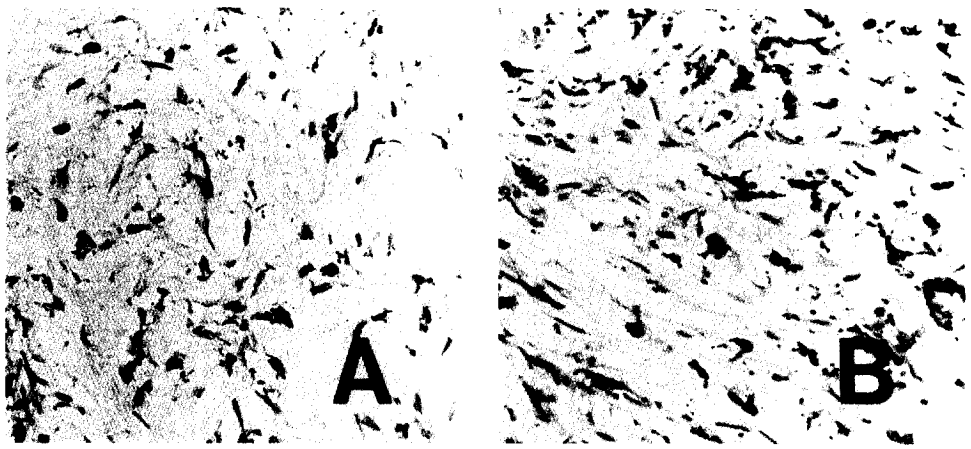

Figure 1. Recruitment of mononuclear phagocytes is enhanced by application of MCP-1. Osseous lesions were created by local application of KLH through the distal root canal of the mandibular first molar in sensitized rats. MCP-1 (280 ng) or vehicle alone was applied daily for 5 consecutive days. At 5 days after the last application, animals were sacrificed and histologic cryostat sections were prepared for immunohistochemistry using the ED-1 antibody to identify mononuclear phagocytes. Panel A, vehicle alone; Panel B, MCP-1. Each area shown is in close proximity to bone. No cells were immunostained with control IgG (data not shown). The area of bone can be found near the lower edge of each panel near the capitalized letters.

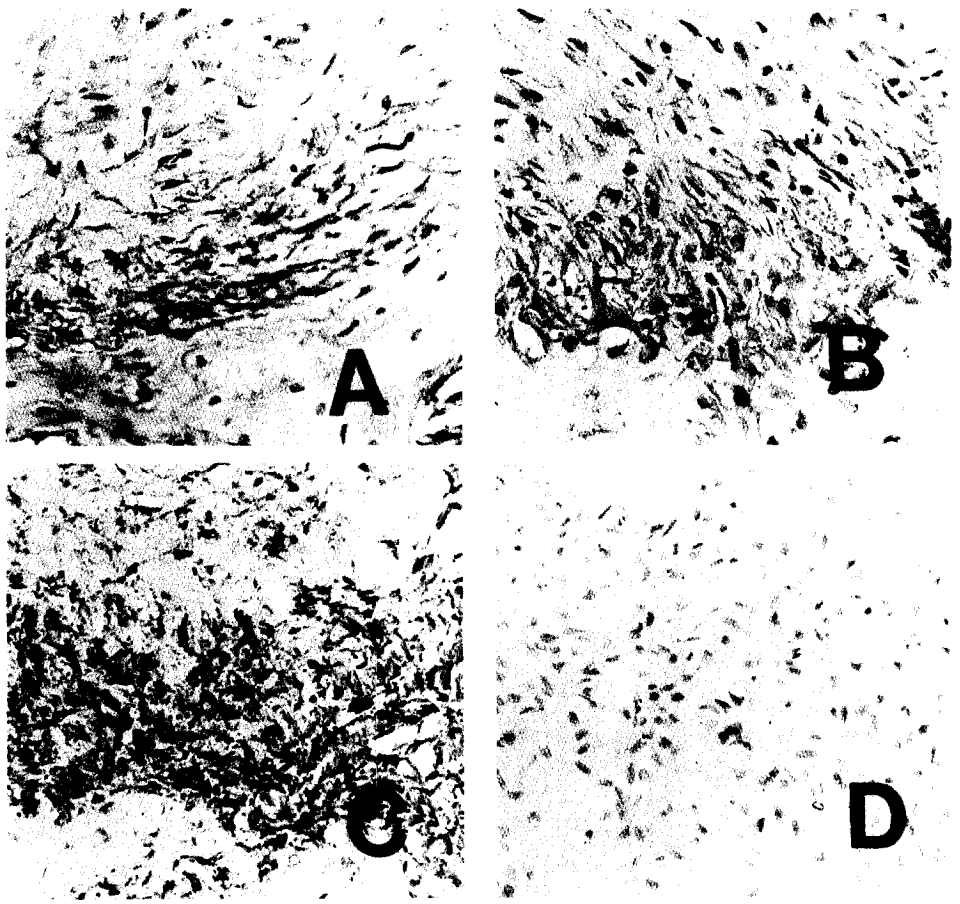

Figure 2. Osteoblast number is enhanced by application of MCP-1. Osseous lesions were created by local application of KLH through the distal root canal of the mandibular first molar in sensitized rats. MCP-1 (280 ng), PDGF-BB (400 ng) plus IGF-1 (1000 ng), or vehicle alone was applied daily for 5 consecutive days. At 5 and 28 days after the last application, animals were killed and histologic cryostat sections were prepared for immunohistochemistry using the 143-3 anti-alkaline phosphatase antibody (panels A, B, and C) to identify bone-lining osteoblastic cells or control ascites (panel D). Panels A and D, vehicle alone; panel B, MCP-1; panel C, PDGF-BB plus IGF-1. The area of bone can be found near the lower edge of each panel near the capitalized letters.

monocyte chemoattractant produced by osteosarcoma cells in vitro.[6]

Since bone resorption is typically linked to bone formation, and since monocytes produce a number of mitogenic factors capable of stimulating osteoblasts, we examined the capacity of exogenous MCP-1 to induce monocyte recruitment in inflamed bone and evaluated whether enhanced monocyte recruitment is associated with an increase in osteoblast number. The results indicate that MCP-1, when applied in close proximity to bone, enhances monocyte infiltration. Furthermore, the application of MCP-1 results in an increase in osteoblast number which is likely to occur by indirect mechanisms.

Materials and Methods

Chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Anti-alkaline phosphatase monoclonal antibody (clone 143-3) was generously supplied by Dr. Gideon Rodan.[18] Rat monocytes were identified with the mouse anti-rat antibody, ED-1, purchased from Sigma. Recombinant human PDGF-BB and IGF-1 were generously donated by the Institute of Molecular Biology (Worcester, MA). Human MCP-1 was isolated to homogeneity from human glioma cells as we have previously described.[9]

Immunization Protocol

Immunologically induced periapical lesions were produced following modification of a procedure first described by Torabinejad et al. in the cat.[28] 36 Sprague-Dawley male rats initially weighing 100–124 g were immunized with 1 mg keyhole limpet hemocyanin (KLH) in 1 mL complete Freund's adjuvant and PBS (1:1) using multiple intradermal dorsal injections. This was followed by a second immunization 2 weeks later using an identical procedure, except that incomplete Freund's adjuvant was used. 1 week after the second immunization, serum was collected in order to verify that a positive titer to KLH was established. By this time the average weight of the rats had increased to 300 g. Osseous lesions were then established by applying KLH through the root canal of the mandibular molars. To accomplish this, the pulp chamber of the tooth was exposed using surgical burs under sterile conditions in rats anesthetized with ketamine/xylazine/acepromazine, premixed as a cocktail (44.0/8.4/1.0 mg/cm^3, 0.1 cm^3 of cocktail per 100 g body weight) administered intramuscularly. The canals of the distal roots of the left and right first mandibular molars were cleaned and shaped by serial filing using #8 through #20 K type files (Brasselar USA, Savannah, GA). A microsyringe was introduced into the distal root and the solution of KLH (10 μg) was administered through the root canal. Subsequent immune complex formation following local application of KLH in preimmunized rats resulted in a periapical response that caused bone resorption, which was self-limiting and aseptic. This then provided an osseous environment in which to test the effect of MCP-1, described below. The occlusal access openings were then sealed with Cavit (ESPE, FRG). 1 week later, the canals were reopened and a second dose of KLH was administered.

Application of Biologic Mediators

One week after the second application of KLH, the root canal was reopened daily for 5 days, and the experimental factors were delivered through the canal with a microsyringe to the periapex. One molar tooth in each animal served as the experimental site into which factors were delivered. The contralateral molar served as negative control site and received vehicle without factors. The administration of factor was randomly made so that in some cases the left molar served as the experimental site, while in other cases the right molar was the experimental site. Each site received daily injection of 280 ng MCP-1 in 10 μl of sterile PBS, or the positive control for stimulation of osteoblasts, 400 ng PDGF-BB plus 1000 ng IGF-1 in 10 μL of sterile PBS, or the negative control, vehicle alone, 10 μL of sterile PBS, for 5 days. The amount of mediators added was based on similar amounts used by other investigators in vivo.[1,13] 5 days after the last application, half of the rats from each group were killed. The remaining rats were killed 28 days after factor administration.

Histologic Preparation

Anesthesia was established as described above and the thoracic cavity exposed. Following death, the animals were perfused through the left ventricle with 60 mL PBS, followed by 60 mL of 4% paraformaldehyde. The mandibles were dissected and placed in 4% paraformaldehyde for 4–6 h at 4°C. Decalcification was accomplished by incubation in EDTA (7%)/glycerol (15%), pH 7.1, and verified radiographically. Specimens were incubated overnight in 30% sucrose and then snap frozen in 2 methylbutane (−80°C). Each specimen was serial sectioned in a mesiodistal saggital plane, at a thickness of 5 μm, parallel to the long axis of the first mandibular molar. In order to visualize the apical third of the root and periapical lesion, serial sections were stained with hematoxylin and eosin.

Immunohistochemistry

Frozen serial sections (5–6 μm) were used for immunohistochemistry. Two antibodies were used: (1) anti-alkaline phosphatase monoclonal antibody 143-3 (1:3500 dilution), which identifies plasma membrane alkaline phosphatase,[18] a phenotypic marker for osteoblasts; and (2) the murine monoclonal antibody ED-1 (1:150 dilution), which identifies a plasma membrane marker on rat mononuclear phagocytes that recognizes peripheral monocytes and mature macrophages in tissue sections.[27] After application of primary antibodies or matched control antibodies, sections were washed in phosphate-buffered saline and treated with 0.3% H_2O_2 in methanol to suppress endogenous peroxidase activity. Visualization of primary antibody binding was achieved using avidin–biotin–horseradish peroxidase complex employing diaminobenzidine as the chromagen (Vector Laboratories, Inc., Burlington, CA). For the negative control, adjacent serial sections were incubated with isotype-matched ascites (for alkaline phosphatase control) or irrelevant hybridoma supernatant (for ED-1 control). Sections were counterstained with hematoxylin.

Analysis

Duplicate experiments were completed approximately 6 months apart. In Experimental Set One, immunohistochemical sections prepared from 3 or 4 animals from each treatment group were coded and examined blind at 500× magnification. In these sections, the distal root, periapical lesion, and surrounding peripheral bone could be visualized in the mesio-distal sagittal plane. Cell counts were made independently from the same field by using a microscope where two examiners could view a visual field simultaneously. In all cases, the cell counts obtained by two examiners were nearly identical. The data presented are the means of the measurements obtained by both examiners. In each field, cells were counted at the 3, 6, and 9 o'clock positions relative to the apex of the tooth. Approximately ten fields were counted for each section in the area of the osseous lesion. Osteoblasts were counted as alkaline phosphatase positive, bone-

associated cells. Mononuclear phagocytes were counted as ED-1 positive cells. In Experimental Set Two, immunohistochemical sections were prepared from five or six specimens per treatment group that were coded and examined blind using computer-assisted video image analysis. Each field was captured with a SONY RGB camera using the Image ProPlus video image analysis software package. The entire length of the bone surrounding the periapical lesion was analyzed. The number of osteoblasts was determined by counting the number of nuclei associated with alkaline phosphatase positive cells along the length of the bone surface. Thus, for each specimen in Set Two, the mean number of osteoblasts per linear unit of bone was determined, as was the mean number of mononuclear phagocytes per mm^2 in close proximity to bone. Statistical significance was determined by ANOVA with Tukey's method as a post-hoc test.

DNA Synthesis

Fetal rat calvarial osteoblastic cells were obtained by sequential collagenase digestion as described in Ref. 3. Primary cultures were incubated in DMEM supplemented with 10% newborn bovine serum and generally reached confluence within 3 days, at which time they were tested for ^{3}H-thymidine incorporation. Cells were incubated for 24 h in serum-free DMEM supplemented with 0.1% bovine serum albumin and then incubated in fresh serum free media supplemented with MCP-1 (0–100 ng/mL) or 10% NBS for 24 h. ^{3}H thymidine was added for the final 6 h and cells were assayed for incorporated ^{3}H thymidine, as we have previously described.[4]

Results

Localized bone resorption was initiated by antigenic challenge of KLH into the distal root of the mandibular first molar, followed by treatment with MCP-1 or vehicle alone (negative control), as described above. In **Figure 1**, brown immunostained cells represent mononuclear phagocytes identified with the ED-1 monoclonal antibody. The control site, which had undergone lesion induction followed by treatment with vehicle alone but had not received mediators, is shown in Figure 1A. When sites received MCP-1 (Figure 1B), there were more ED-1 positive cells compared to control sites receiving vehicle alone (Figure 1A). Sections were also incubated with matched control IgG and exhibited no positive immunostained cells, demonstrating that immunostaining does not occur as a result of nonspecific interactions between the section and antibody (data not shown). **Figure 2** represents frozen sections immunostained with an antibody to rat alkaline phosphatase. Figure 2A shows a site from an animal treated with vehicle alone following induction of osseous lesions. The darker, brown immunostained cells are osteoblasts identified with a monoclonal antibody to rat alkaline phosphatase, which were typically found as a band consisting of

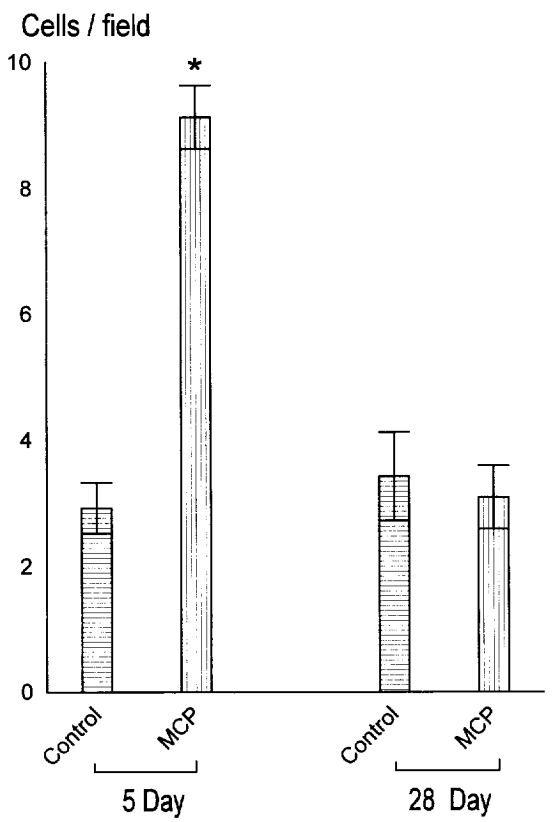

Figure 3. Quantitative analysis of MCP-1 enhanced mononuclear phagocyte recruitment in Experimental Set 1. Creation of osseous lesions and application of MCP-1 or vehicle alone (control) was performed as described in Figure 1. Mononuclear phagocytes were identified by ED-1 positive immunostaining and were counted under 500× magnification as described in *Materials and Methods*. * indicates statistically significant compared to the negative control ($p < 0.01$).

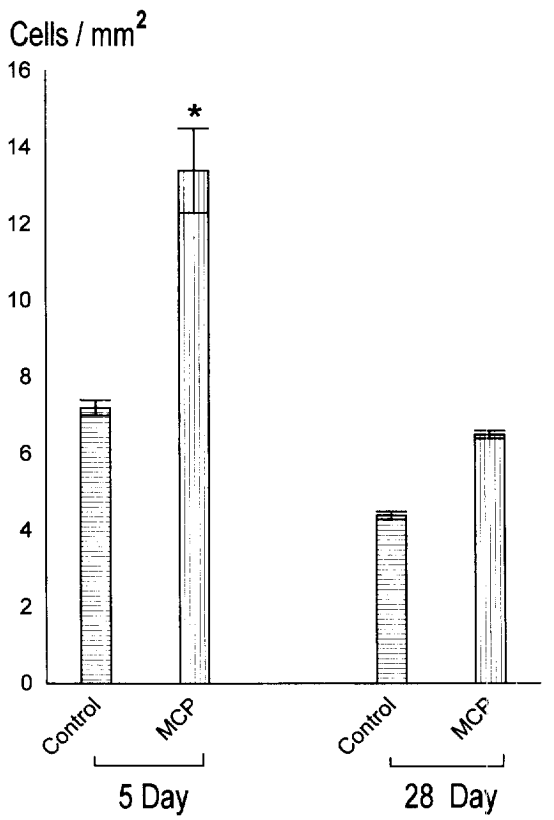

Figure 4. Quantitative analysis of MCP-1 enhanced mononuclear phagocyte recruitment in Experimental Set 2. Creation of osseous lesions and application of MCP-1 or vehicle alone (control) was performed as described in Figure 1. Mononuclear phagocytes were identified by ED-1 positive immunostaining and were counted with the use of an image analysis system as described in *Materials and Methods*. * indicates statistically significant compared to the negative control ($p < 0.01$).

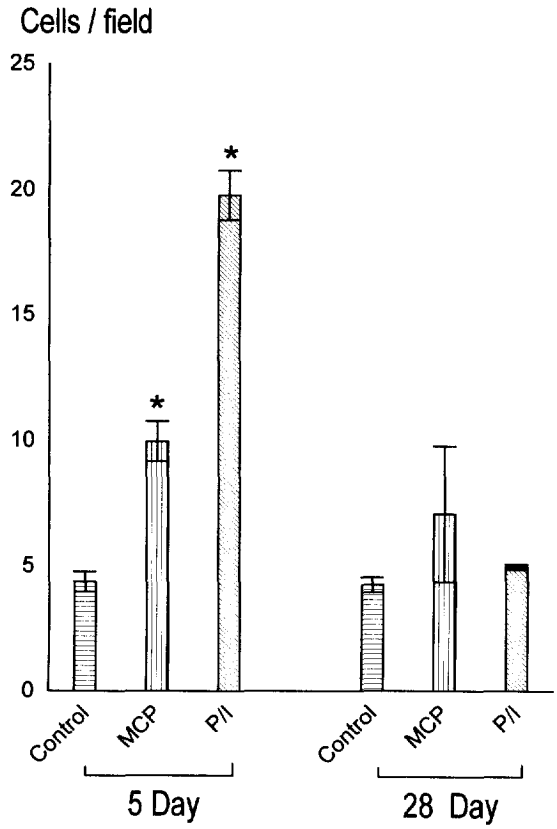

Figure 5. Quantitative analysis of osteoblasts following MCP-1 application in Experimental Set 1. Creation of osseous lesions and application of MCP-1; the positive control, PDGF-BB/IGF-1 (P/I), or the vehicle alone (control), was performed as described in Figure 2. Animals were killed 5 or 28 days following factor application. Osteoblasts were identified as alkaline-phosphatase immunostained cells adjacent to bone and were manually counted at 500× magnification. * indicates statistically significant compared to the negative control ($p < 0.01$).

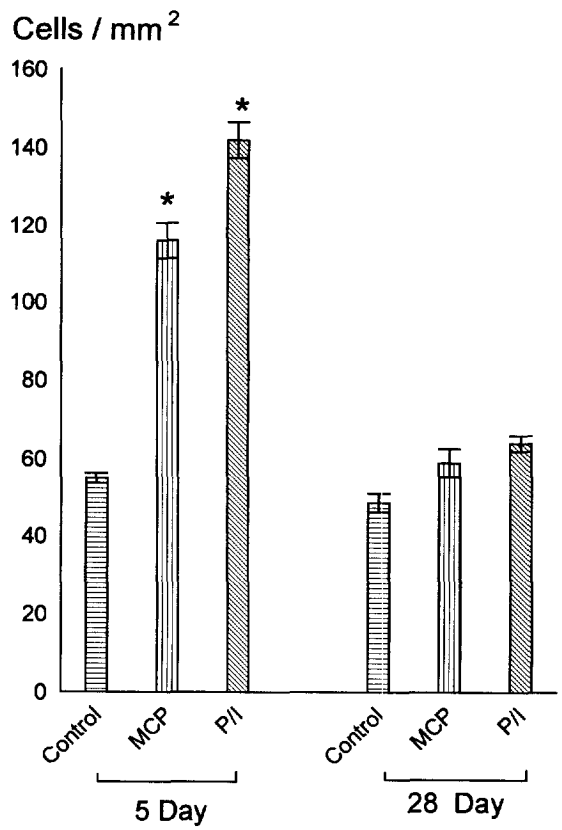

Figure 6. Quantitative analysis of osteoblasts following MCP-1 application in Experimental Set 2. Creation of osseous lesions and application of MCP-1: the positive control, PDGF-BB/IGF-1 (P/I), or the vehicle alone (control), was performed as described in Figure 2. Animals were killed 5 or 28 days following factor application. Osteoblasts were identified as alkaline-phosphatase immunostained cells adjacent to bone and were counted with the use of an image analysis system as described in *Materials and Methods*. * indicates statistically significant compared to the negative control ($p < 0.01$).

approximately 2- to 3-cell-thick layers along the bone surface. The lightly immunostained cells adjacent to osteoblasts are fibroblasts in the periodontal ligament which express low levels of alkaline phosphatase. Figure 2B represents a section from an animal treated with MCP-1, and has a greater number of immunostained osteoblastic cells than sites receiving vehicle alone, but fewer than sites receiving PDGF-BB plus IGF-1 (Figure 2C). In Figure 2D, lack of immunostaining with matched control ascites demonstrates that immunostaining present in Figures 2A–C does not result from nonspecific interactions of antibody with tissue sections.

Since monocytes are capable of producing mediators that stimulate osteoblast proliferation, we determined whether increased recruitment of monocytes following MCP-1 treatment was associated with an increase in osteoblast number. This was accomplished in two separate experiments carried out at separate times and analyzed by different examiners, which are described as Experimental Set 1 (see Figures 3 and 5) and Experimental Set 2 (see Figures 4 and 6). In Set 1, cells were manually counted at 500× magnification. Each microscopic field was positioned so that it was adjacent to bone when counting mononuclear phagocytes; the bone surface was placed at the widest diameter of the field when counting osteoblasts. The data are expressed as the number of cells per field. In Set 2, an image analysis system was utilized to count cells. In this set, data for mononuclear phagocytes are expressed as the number of cells per mm^2; for osteoblasts, as the number per mm of bone length. In **Figure 3**, the application of MCP-1 caused a threefold increase in the number of mononuclear phagocytes on day 5 ($p < 0.01$). 28 days after the last application of MCP-1, the number of monocytes returned to baseline levels for both the control and experimental groups. Similar results were obtained in the second set of experiments, as shown in Set 2 (**Figure 4**). 5 days following MCP-1 application, rats treated with MCP-1 exhibited a statistically significant twofold increase in the number of mononuclear phagocytes compared to control animals ($p < 0.01$) (Figure 4). However, this increase was transient since there was no significant enhancement 28 days following application of MCP-1.

The number of osteoblasts was then examined to determine whether it increased following application of MCP-1. In animals treated with MCP-1, there was approximately a twofold increase in osteoblast number in both Experiments 1 and 2, compared to control sites at the 5 day time point (**Figures 5** and **6**), both of which were statistically significant ($p < 0.01$). At the 28 day time point, MCP-1 did not induce a statistically significant increase in Set 1 (Figure 5) or in Set 2 (Figure 6), indicating that the MCP-1 does not cause a persistent increase in osteoblast number. To rule out the possibility that MCP-1 directly stimulated proliferation of osteoblastic cells, in vitro experiments were

Bone Vol. 21, No. 4
October 1997:321–327

Table 1. MCP-1 does not stimulate DNA synthesis in osteoblastic cells. Fetal rat calvarial osteoblastic cells were obtained by sequential collagenase digestion as described in *Materials and Methods*. Primary cultures were plated and tested for ^{3}H-thymidine incorporation. The data are representative of three experiments and are expressed as the counts/min ± the standard error of the mean.

	CPM	SEM
Control	28,379	1,437
CMCP-1 (1 ng/mL)	30,225	1,063
MCP-1 (10 ng/mL)	27,861	337
MCP-1 (100 ng/mL)	27,457	921
10% NBS	128,179	6,446

carried out. The results demonstrate that MCP-1 did not enhance thymidine incorporation in fetal rat calvarial cells at concentrations ranging from 1 to 100 ng/mL (**Table 1**), indicating that MCP-1 does not have a direct effect on the proliferation of these cells.

The effect of MCP-1 on osteoblasts was compared to that of a potent growth factor combination, PDGF-BB plus IGF-1 (Figures 5 and 6). PDGF-BB plus IGF-1 stimulated a fivefold increase in osteoblasts at the 5 day time point in Set 1 (Figure 5) and a 2.7 fold increase in Set 2 (Figure 6), compared to controls treated with vehicle alone. Increase enhanced by growth factor treatment was statistically significant in both experiments ($p < 0.01$). At the 28 day time point, the number of osteoblasts in the PDGF-BB/IGF-1 treatment groups had decreased and were not significantly different from the control ($p > 0.05$) (Figures 5 and 6).

Discussion

Molecular studies have provided insight into the regenerative process by identifying locally acting mediators that are expressed during healing. These mediators, termed growth factors, may stimulate a wide variety of cellular events including chemotaxis, proliferation, differentiation, and production of extracellular matrix proteins. We investigated whether application of MCP-1 in vivo induced an increase in the number of mononuclear phagocytes, and possibly through indirect mechanisms, an increase in the number osteoblasts. Treatment with MCP-1 stimulated up to a threefold increase in the number of monocytes. The effect was significant 5 days after treatment; at 28 days there was no significant increase, indicating that the effect is transient. Surprisingly, MCP-1 also induced a significant, twofold increase in osteoblast number compared to control sites. By comparison, application of the growth factor combination PDGF-BB and IGF-1 stimulated a three- to fivefold increase in the number of osteoblasts, compared to controls. This stimulation was reduced by day 28. Thus, the increase in the number of osteoblastic cells that resulted from both MCP-1 and PDGF stimulation was not long lasting. In osseous repair, there is also a transient increase in the number of osteoblastic cells. We suggest that there is an analogous increase in response to the mediators applied, which mimics that seen during wound healing. To our knowledge, the mechanisms responsible for the nature of this transient increase are unknown. We can speculate, however, that some of these cells may become trapped in newly forming bone or alternatively, there is a change in phenotypic expression over time leading to less intense alkaline phosphatase immunostaining.

The recruitment of mononuclear phagocytes is observed in different physiological and pathophysiological states. These cells play an important role in many processes through the elaboration of cytokines and growth factors. Whether the infiltrating monocytes participate in bone resorption or bone formation may depend on the specific growth factors and/or cytokines present at the local site. Our studies suggest that recruited mononuclear phagocytes could participate in bone formation by enhancing the number of osteoblasts. This is likely to be due to indirect mechanisms as we found that MCP-1 did not directly stimulate DNA synthesis in normal rat osteoblast-enriched cell populations. A potential explanation for this observation is that mononuclear phagocytes release growth factors that enhance proliferation of osteoblastic cells, as indicated by the results of Rifas et al.[25] This concept is further supported by our findings that the introduction of the growth factors PDGF-BB plus IGF-1 enhanced the number of osteoblasts in this model. These results are in agreement with observations that PDGF and IGF-1 can stimulate proliferation or chemotaxis of osteoblastic cells in monolayer culture, organ culture, or in vivo, and that PDGF is found to be expressed in early stages of wound healing by macrophages in close proximity to osteoblasts.[3,4,8,14,19,21,22,26] Thus, activated mononuclear phagocytes may play an important role in osseous wound healing by stimulating proliferation of osteoblastic cells, presumably through the elaboration of growth factors.

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