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Osteogenin and recombinant bone morphogenetic protein 2B are chemotactic for human monocytes and stimulate transforming growth factor β_1 mRNA expression

(bone induction/cartilage/developmental cascade/bone repair)

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ABSTRACT **Subcutaneous implantation of demineralized bone matrix initiates a sequence of developmental events, which culminate in endochondral bone formation. During early stages of development of matrix-induced implants, ED1, Ia-positive monocytes–macrophages were observed, suggesting that in the initial phases of the endochondral bone formation cascade, the bone-inductive protein osteogenin and related bone morphogenetic proteins (BMPs) might serve as potent chemoattractants to recruit circulating monocytes. In this investigation, we demonstrate that at concentrations of 10–100 fg/ml (0.3–3 fM), native bovine osteogenin and recombinant human BMP-2B (rhBMP-2B) induce the directed migration of human blood monocytes *in vitro*. This chemotactic response was associated with expression of BMP binding sites (receptors) on monocytes. About 750 receptors per cell were detected with an apparent dissociation constant of 200 pM. Both osteogenin and rhBMP-2B at higher concentrations (0.1–30 ng/ml) stimulated mRNA expression for an additional regulatory molecule, type β_1 transforming growth factor (TGF-β_1) in human monocytes. TGF-β_1, in turn, is known to induce a cascade of events leading to matrix generation. Monocytes stimulated by TGF-β are known to secrete a number of chemotactic and mitogenic cytokines that recruit endothelial and mesenchymal cells and promote their synthesis of collagen and associated matrix constituents. TGF-β_1 in concert with these other cytokines and matrix components regulates chemotaxis, mesenchymal proliferation, differentiation, angiogenesis, and controlled synthesis of extracellular matrix. Our results demonstrate that osteogenin and related BMPs through their profound effects on monocyte recruitment and cytokine synthesis may promote additional successive steps in the endochondral bone formation cascade.**

Bone has considerable potential for repair and regeneration, and the stages of fracture repair recapitulate the sequential developmental stages of embryonic endochondral bone formation. A cellular cascade that mimics embryonic endochondral bone development can be induced locally by implantation of demineralized bone matrix in extraskeletal sites (1–3). The cellular and biochemical events of this matrix-induced endochondral bone-forming cascade have been well characterized (3). Upon implantation of demineralized bone matrix, there is a transient immigration of polymorphonuclear leukocytes on day 1. This is followed by migration and attachment of mesenchymal cells to the matrix by day 3. These mesenchymal progenitor cells proliferate and differentiate into chondroblasts and chondrocytes on days 6–7. Vascularization of the implant occurs concomitantly with calcification of the hypertrophic cartilage matrix on day 9, and the cartilage is resorbed and replaced by new bone on days 10–12. Bone remodeling (days 12–18) and differentiation of hematopoietic cells result in formation of an ossicle complete with all associated marrow elements by day 21.

Although the precise molecular mechanisms involved in this biological cascade are unknown, it has been shown that the osteoinductive factor(s) can be extracted from the bone matrix and reconstituted with inactive residual collagenous matrix to restore full bone inductive activity (4). The bone inductive protein that initiates this cascade, osteogenin, has been isolated from bone and purified, and the amino acid sequence of several tryptic peptides has been determined (5). The genes for osteogenin and related bone morphogenetic proteins (BMPs) have been cloned and expressed (6–9). Recombinant BMP-2A, -2B, and osteogenin (BMP-3) individually induce endochondral bone differentiation *in vivo* (7, 8). Osteogenin and related members of the bone morphogenetic proteins are members of the type β transforming growth factor (TGF-β) superfamily (6). TGF-β is a multifunctional regulator of cellular growth and differentiation in developing systems (10, 11), and it is a prominent component of the extracellular matrix of bone (12–14). *In vitro* studies have suggested a role for TGF-β in cartilage and bone formation where TGF-β induces the synthesis of cartilage-specific proteoglycans and type II collagen by mesenchymal cells (15) and stimulates proliferation and collagen synthesis by osteoblasts (16). The regulatory importance of TGF-β *in vivo* has been shown by immunohistochemical studies, which demonstrated that TGF-β is synthesized by chondrocytes and osteoblasts during embryogenesis and in the *in vivo* model of endochondral ossification (10, 17). Moreover, direct injection of TGF-β over the periostea of parietal bones (18) or into the subperiosteal region of femurs (19) in newborn rats increases bone formation *in vivo*.

Chondrogenesis and osteogenesis are complex, multistep processes that involve interactions of multiple cell types, growth factors, and extracellular matrix. The precise mechanisms of action of osteogenin and BMPs and their cellular targets are not known. In this communication, we demonstrate that human monocytes respond chemotactically to both native osteogenin and recombinant human BMP-2B (rhBMP-2B) and at higher concentrations are stimulated to increase expression of TGF-β_1 mRNA. This increase in TGF-β_1 mRNA expression in response to osteogenin and BMP-2B may be an early step in setting into motion the ensuing cascade of events in bone induction.

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Abbreviations: BMP, bone morphogenetic protein; TGF, transforming growth factor; rh, recombinant human; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Osteogenin and Recombinant BMP-2B. Osteogenin was purified as described (5) and the amount of protein was quantitated by amino acid analysis. rhBMP-2B was cloned, expressed, and purified from conditioned medium as described (8). Briefly, a hybrid of the proregion of BMP-2A fused to the mature region of BMP-2B (BMP2A/2B) was constructed in the mammalian expression plasmid pRK5 to enhance secretion of biologically active BMP-2B. Human embryonic kidney cells (cell line 293) were transfected with this expression vector and enhanced levels of mature, biologically active BMP-2B were secreted into the medium. Mature rhBMP-2B was purified from the conditioned medium by heparin Sepharose, Mono Q, and C4 chromatography as described (8).

***In Vivo* Bioassay.** Osteogenin and rhBMP-2B (100–300 ng) were routinely tested for bone induction *in vivo* by reconstitution with 25 mg of guanidine-extracted collagenous residue of rat demineralized bone matrix as described (5).

Identification of Cell-Surface Antigens. In an effort to identify early responding cells, implants composed of osteogenin or BMP-2B reconstituted with guanidine-extracted rat residue were removed on days 1–3, snap frozen in embedding medium (O.C.T. compound; Miles), sectioned at −23°C (8 μm), and stained with toluidine blue or hematoxylin and eosin or prepared for immunoperoxidase staining. T lymphocytes, monocytes–macrophages, and Ia^+ cells were identified by using cell-specific monoclonal antibodies OX19, ED1, and OX6, respectively (Bioproducts for Science, Indianapolis) with an immunoperoxidase staining technique (ABC Vectastain Kit; Vector Laboratories) as described (20). Control sections were incubated without the primary antibody or with a nonrelated antibody.

Monocyte Isolation. Mononuclear leukocytes were isolated by Ficoll/Paque density-gradient centrifugation (21) from heparinized peripheral blood of healthy normal volunteers undergoing leukapheresis at the National Institutes of Health Blood Bank. To obtain highly purified populations of monocytes, the mononuclear leukocytes were separated by counterflow centrifugal elutriation as described (21, 22). Monocytes obtained by this procedure have been shown to be >90% pure as judged by dual fluorescence microfluorometry with specific cell-surface markers. The monocytes were washed; suspended in Dulbecco's modified Eagle's medium (DMEM; Quality Biologicals, Gaithersburg, MD) containing 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine; and quantitated by Coulter Counter analysis.

Chemotaxis Assay. Counterflow centrifugal elutriation-purified monocytes were suspended at 1.1×10^6 monocytes per ml in Gey's balanced salt solution (GBSS; National Institutes of Health Media Unit) containing 2% bovine serum albumin, antibiotics, and 0.015 M Hepes. The chemotaxis assay was carried out in 48-well microchamber plates (Neuroprobe, Rockville, MD) with 5-μm pore polycarbonate filters as described (22). Osteogenin, rhBMP-2B, and rhTGF-β_1 (generous gift of G. Hammonds, Genentech) were assayed at the indicated concentrations. The synthetic peptide fMet-Leu-Phe (Peninsula Laboratories) was the positive control and GBSS was the negative control. After a 90-min incubation at 37°C, the polycarbonate filters were removed, fixed, stained with Diff-Quik (American Scientific Products, Stone Mountain, GA), and quantitated with an Optomax image analyzer (Optomax, Hollis, NH). Chemotactic activity is defined as the mean (±SEM) number of monocytes that migrated through the 5-μm pores in three standard fields for each of triplicate filters.

Binding of ^{125}I-Labeled BMP-2B. Purified monocytes were incubated for 2 hr at 37°C in serum-free medium to remove any bound ligand. Recombinant BMP-2B was iodinated to a specific activity of 2–3 μCi/pmol (1 Ci = 37 GBq) by a modified chloramine-T method (23) as described (24). The cells were washed and resuspended in binding buffer: DMEM with 0.1% bovine serum albumin (Sigma) and 25 mM Hepes (pH 7.4) at a concentration of 20×10^6 cells per ml. Aliquots of the cell suspension (100 μl) were incubated with 1–500 pM ^{125}I-labeled BMP-2B in a final vol of 200 μl for 2 hr at 4°C with constant agitation. Nonspecific binding was determined in the presence of a 200-fold excess of unlabeled BMP-2B. After the binding incubation, cells were pelleted and resuspended in 200 μl of ice-cold binding buffer and centrifuged through a silicon/paraffin oil cushion. The radioactivity associated with the resulting pellet was determined with a Beckman γ counter to determine the amount of bound ligand.

RNA Isolation and Northern Blot Analysis for mRNA Expression. Total RNA was isolated from 15×10^6 monocytes incubated in suspension in the presence or absence of purified bovine osteogenin, recombinant BMP-2B, or lipopolysaccharide (LPS, *Escherichia coli* 055:B5; Sigma) by the acid guanidinium thiocyanate/phenol/chloroform extraction procedure (25). For Northern blots, 6 μg of total RNA was fractionated on 1.0% agarose gels containing formaldehyde and transferred to nitrocellulose filters. Blots were prehybridized for 4 hr at 42°C in 5× Denhardt's solution before ^{32}P-labeled nick-translated cDNA probes were added. Hybridization was carried out for 16–18 hr at 42°C. Probes included the cDNA probe for human TGF-β_1 (26), human interleukin 1β cDNA (27), tumor necrosis factor α cDNA (28), and the cDNA for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (29). After hybridization the filters were washed in 2× standard saline citrate (SSC) containing 0.1% $NaDodSO_4$ twice at 25°C for 30 min and once at 60°C in 0.1× SSC containing 0.1% $NaDodSO_4$ for 30 min. Autoradiograms were made by exposure to Kodak XAR-5 film with intensifier screens at −70°C with exposure times ranging from 4 to 48 hr. Relative expression levels were determined by densitometric evaluation of autoradiograms.

RESULTS

Identification of Cellular Infiltrates. Within 48 hr after implantation of collagenous matrix reconstituted with osteogenin, an infiltrate of mononuclear cells and fibroblast-like cells became apparent around the implant (Fig. 1*A*). To characterize these cells, frozen sections from day 2 osteogenin-induced bone-forming implants were stained with cell type-specific monoclonal antibodies by immunoperoxidase. Staining with an antibody that recognizes monocytes–macrophages (ED1) indicated that numerous cells of the monocyte lineage infiltrated the implant area and were in direct contact with the implanted particles (Fig. 1*B*). In addition, the presence of Ia^+ mononuclear cells (Fig. 1*C*) indicated that the monocytes had been activated. The lack of OX19 staining (Fig. 1*D*) suggested that T lymphocytes were not an important constituent of the osteogenin-induced infiltrate.

Chemotactic Activity of Osteogenin and Recombinant BMP-2B. Based on the morphological and immunocytochemical evidence that monocytes were the primary cells recruited to the site of implantation, purified bovine osteogenin and a related rhBMP-2B were evaluated for their ability to induce monocyte chemotaxis *in vitro*. Both osteogenin and rhBMP-2B stimulated the migration of human monocytes in a dose-dependent manner. While the response varied with individual donors, the peak response was found to occur at 10–100 fg/ml (0.3–3 fM) (Fig. 2). Zigmond–Hirsch checkerboard analysis (30) revealed that osteogenin and BMP-2B affected the migration of mononuclear cells only when present at higher concentrations in the lower compartment of the chemotaxis chamber. This indicates that the mononuclear cell response to these proteins is predominantly one of chemotaxis rather than chemokinesis. The response was

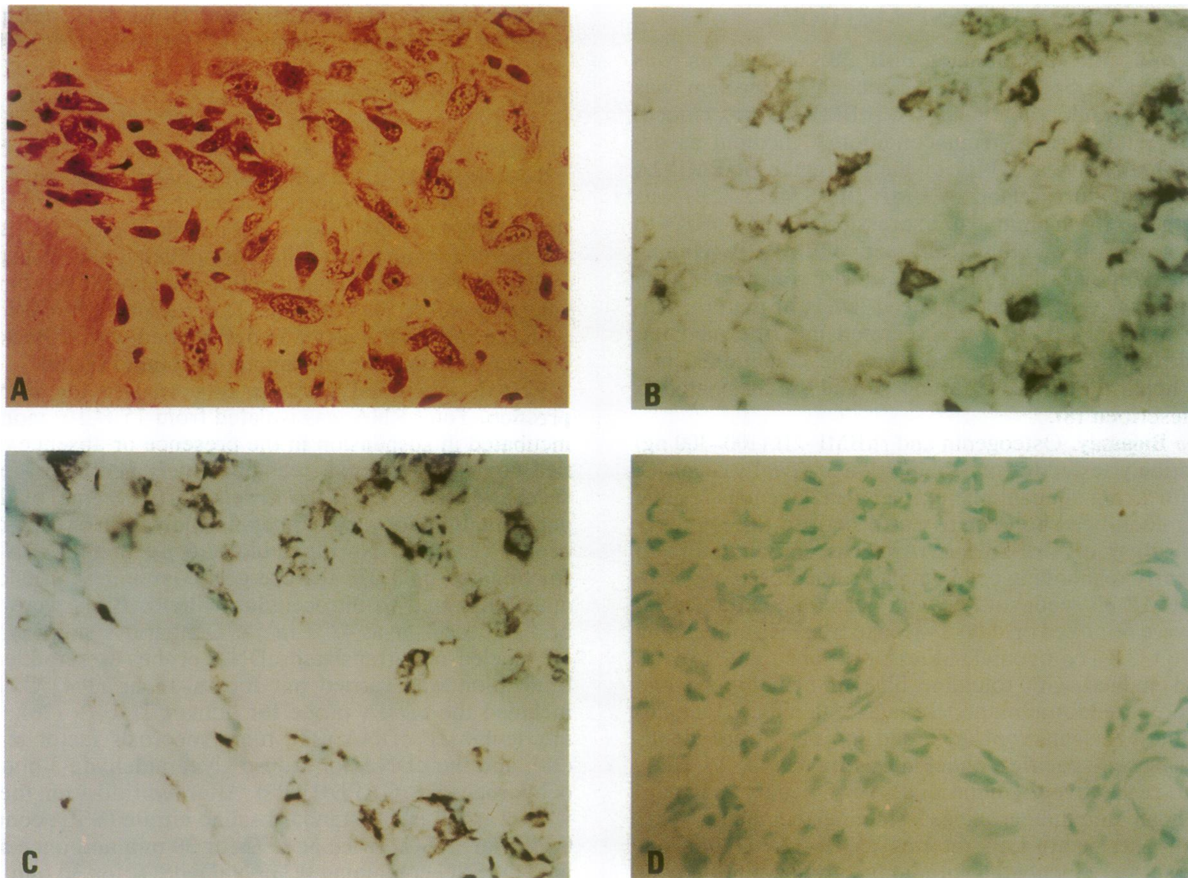

FIG. 1. Characterization of cellular infiltrates. Frozen sections from day 2 implants were stained with hematoxylin and eosin (*A*) or by an immunoperoxidase technique with monoclonal antibodies directed at surface antigens on monocyte–macrophages (ED1) (*B*), to Ia antigens (OX6) (*C*), and on T lymphocytes (OX19) (*D*). Note the occurrence of $ED1^+$ monocytic cells and Ia^+ activated cells and the paucity of T cells. (×140.)

found to be specific for monocytes since purified lymphocytes did not respond chemotactically to either osteogenin or recombinant BMP-2B (data not shown).

BMP-2B Receptors on Monocytes. Directed migration of monocytes depends on the interaction of cell-surface receptors with specific chemotactic ligands. We therefore determined whether peripheral blood monocytes possess receptors for these bone morphogenetic proteins. We examined monocytes for the presence of BMP-2B receptors. Incubation of purified monocytes with increasing concentrations of biologically active ^{125}I-labeled BMP-2B demonstrated saturable binding for BMP-2B (Fig. 3). Scatchard analysis (Fig. 3 *Inset*) of the binding data revealed a high-affinity binding site with an apparent dissociation constant of 200 pM. Freshly isolated human monocytes possess ≈750 binding sites per cell.

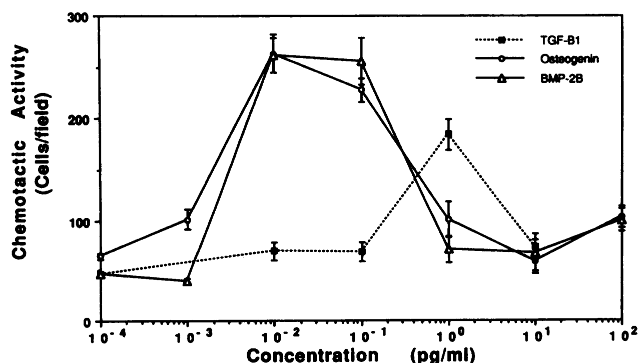

FIG. 2. Monocyte chemotactic activity of osteogenin and BMP-2B. Purified human monocytes were assayed for their ability to respond to these bone inductive proteins at the indicated concentrations. Recombinant TGF-β_1 (previously shown to be chemotactic for human monocytes in the 4–40 fM range) was included as a positive control. Data shown are from a representative experiment and represent the mean number of monocytes ± SEM that migrated in three fields for triplicate filters (9 determinations per point). fMet-Leu-Phe at 10 nM was the positive control (261 ± 19) and GBSS was the negative control (12 ± 2).

Osteogenin and BMP-2B-Induced Expression of TGF-β mRNA. Stimulated monocytes are known to secrete a number of chemotactic and growth factors that recruit endothelial and mesenchymal cells and promote their synthesis of collagen and other matrix constituents (31, 32). Preliminary experiments designed to examine the expression of other cytokines and growth factors produced by monocytes in response to osteogenin and BMP-2B treatment revealed no change in the level of expression of certain cytokines, most notably interleukin 1β and type α tumor necrosis factor (data not shown). However, purified monocytes exposed to BMP-2B *in vitro* for 30 min to 2 hr at 0.1–30 ng/ml were found to have elevated levels of TGF-β_1 mRNA. The induction of TGF-β_1 mRNA was maximal at 1 hr and was concentration dependent with augmented expression starting at 0.1 ng/ml (Fig. 4). Constitutively expressed GAPDH levels were not altered by recombinant BMP-2B.

DISCUSSION

The data presented here indicate that the osteoinductive factors, native bovine osteogenin, and rhBMP-2B are potent chemoattractants for human blood monocytes (peak effect at 0.3–3 fM). rhBMP-2B has 49% identity in the C-terminal mature domain as compared to native osteogenin obtained from bovine bone. It is noteworthy that recombinant

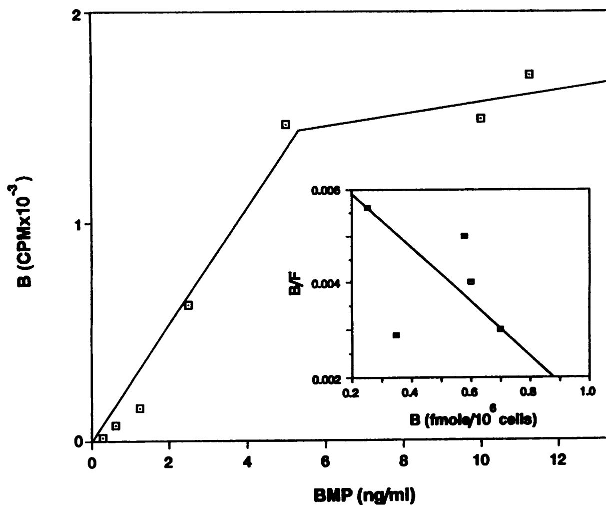

FIG. 3. Binding of ^{125}I-labeled BMP-2B to peripheral blood monocytes. Purified monocytes were evaluated for receptor expression of BMP-2B. Data shown are representative of three separate experiments demonstrating specific binding of ^{125}I-labeled BMP-2B to freshly isolated monocytes. Each data point represents the mean of triplicate determinations. (*Inset*) Scatchard analysis of the binding curves revealed a high-affinity binding site with an apparent dissociation constant of 200 pM. Freshly isolated human monocytes possess ≈750 binding sites per cell. B, bound; F, free.

BMP-2B and native osteogenin are equipotent in human monocyte chemotaxis. The potent chemotactic effect of these bone morphogenetic proteins on monocytes suggests that these proteins may play a role in the recruitment and activation of monocytes. TGF-β_1 was previously shown to be the most potent chemotactic substance for monocytes (33). The present results demonstrate that these bone morphogenetic proteins may be even more potent than TGF-β_1 in inducing monocyte migration.

The observation that cells of the monocyte lineage and Ia$^+$ cells infiltrate into the area of implanted matrix while there is a paucity of T cells is consistent with a recent report by Henricson *et al.* (34). Using a demineralized bone matrix implant system to study bone formation at a heterotopic site, Henricson showed Ia$^+$ cells on the periphery of the matrix at very early time points. At later stages of bone formation (7–10 days), the Ia$^+$-expressing cells disappeared and were replaced by chondrocytes and osteoblasts (34).

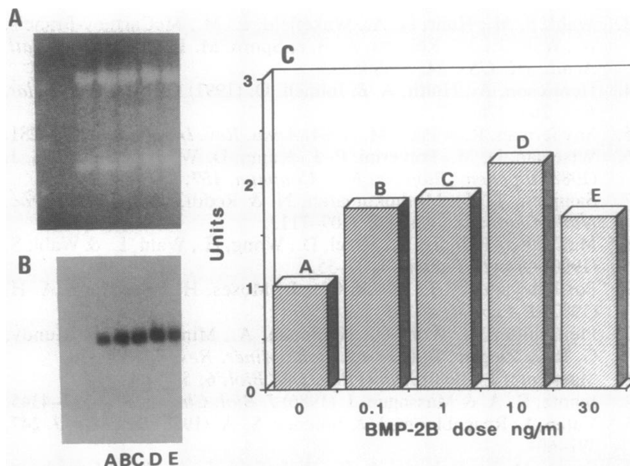

FIG. 4. Induction of TGF-β_1 gene expression by BMP-2B. RNA was isolated from purified monocytes after 1 hr of incubation with medium alone (control) or BMP-2B (0.1–30 ng/ml). After extraction, the mRNA was analyzed by RNA blot hybridization using a ^{32}P-labeled human cDNA probe for TGF-β_1 or murine GAPDH. (*A*) Ethidium bromide staining of rRNA to demonstrate equivalent loading. (*B*) Autoradiogram of the resulting blot for TGF-β_1 mRNA. (*C*) Densitometric scanning of the TGF-β_1 blot relative to the GAPDH blot revealed a stimulation of TGF-β_1 mRNA levels by BMP-2B.

The identification of BMP-2B receptors on freshly isolated peripheral blood monocytes supports the notion that monocyte chemotaxis is dependent on the interaction of a chemotactic ligand with a specific cell-surface receptor (22, 35). The discrepancy between the dose range for monocyte chemotaxis and the saturation curve for the putative receptor suggests that occupancy of a few receptors is sufficient to induce chemotaxis of monocytes. Alternatively, it is possible that BMP-2B acts on a subpopulation of monocytes that have a higher affinity to elicit the chemotactic response. There is a precedence in the TGF-β_1 literature for the existence of monocyte subpopulations (36). These observations are also consistent with other *in vitro* studies which have shown that TGF-β_1 induces cellular chemotaxis at concentrations much lower than those required to elicit other responses (33). In the present investigation, it is likely that a lower threshold dose of BMP-2B elicits chemotaxis and a higher dose is required to induce TGF-β_1 expression with attendant pleiotropic responses.

The observation that osteogenin or BMP-2B at higher concentrations stimulates the expression of TGF-β_1 mRNA in monocytes has important implications for the mechanism underlying the bone formation cascade. Although it is unlikely that this increased production of TGF-β_1 is the penultimate modulator of osteogenin or BMP-2B-induced bone formation, it may play a role in setting into motion the early events in the cascade. It is noteworthy that TGF-β_1 alone is not osteoinductive in the *in vivo* bioassay (37). However, TGF-β_1 at picomolar concentrations has been reported to induce expression of genes for type α tumor necrosis factor, interleukin 1, basic fibroblast growth factor, and c-sis (platelet-derived growth factor) in monocytes (33, 36, 38), all of which have important regulatory roles in angiogenesis and in connective tissue formation. TGF-β_1 is also a potent chemoattractant for monocytes (33, 36), fibroblasts (39), and osteoblast-like cells (40). TGF-β_1 has important chemotactic and anabolic actions on fibroblasts (41), where it stimulates the production of critical components of the extracellular matrix such as collagen and fibronectin as well as the receptors for these matrix proteins (42, 43). Furthermore, it inhibits the production and action of proteolytic enzymes that degrade the extracellular matrix components (44, 45).

Fig. 5 summarizes the current working hypothesis for BMP-induced chemotaxis of monocytes and attendant release of cytokines with its possible role in bone formation. It is possible that BMPs act synergistically with TGF-β_1 to promote the bone inductive cascade. We postulate that osteogenin and other BMPs have early effects in bone induction by stimulating the chemotaxis of monocytes to the area. Once attracted, the monocytes are stimulated to produce a number of chemotactic and mitogenic cytokines and growth factors that recruit mesenchymal cells and promote their synthesis of collagen and other extracellular matrix constituents and regulate angiogenesis. It is well known that vascular invasion is a prerequisite for bone formation (46). Previous work has demonstrated that both osteogenin and TGF-β_1 bind avidly to type IV collagen (47, 48). It is possible that type IV collagen and other matrix components around the endothelial cells of the invading capillaries may bind growth and differentiation factors and present them locally in an immobilized form to responding mesenchymal cells and osteoprogenitors to initiate osteogenesis. *In vitro* osteogenin stimulates expression of the markers of the osteogenic phenotype in periosteal cells, osteoblasts, and bone marrow stromal cells (49). The net result of these interactions between mesenchymal

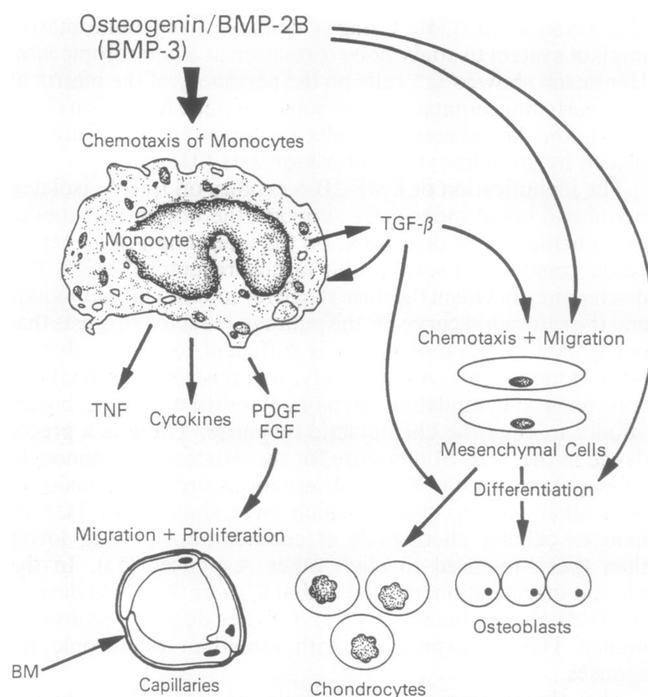

FIG. 5. Role of osteogenin in bone induction cascade: A model. Osteogenin and BMP-2B stimulate chemotaxis of monocytes and increase TGF-β_1 mRNA levels. Osteogenin, BMP-2B, and TGF-β_1 stimulate the chemotaxis and migration of mesenchymal cells. In addition, TGF-β_1 is a chemoattractant for fibroblasts and stimulates transcription and translation of matrix components such as collagen types I and III and fibronectin. Activated monocytes release cytokines and growth factors such as basic fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), which stimulate angiogenesis and capillary invasion. It is well known that vascular invasion is a prerequisite for bone formation. These bone inductive proteins are then envisioned to be sequestered by the matrix, where they may subsequently regulate the differentiation of chondrocytes and osteoblasts. TNF, tumor necrosis factor; BM, basement membrane.

cells, growth factors, cytokines, and extracellular matrices is the cascade of bone induction. In conclusion, this investigation provides evidence that osteogenin and related BMPs are chemotactic *in vitro* for monocytes in addition to being potent inducers of differentiation of bone *in vivo*.

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