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BMP-2, BMP-4, and PDGF-bb Stimulate Chemotactic Migration of Primary Human Mesenchymal Progenitor Cells

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Abstract For bone development, remodeling, and repair; the recruitment of mesenchymal progenitor cells (MPC) and their differentiation to osteoblasts is mandatory. The process of migration is believed to be regulated in part by growth factors stored within the bone matrix and released by bone resorption. In this study, primary human MPCs and to osteoblasts differentiated progenitor cells were examined for chemotaxis in response to human basic fibroblast growth factor (rhbFGF), human transforming growth factor beta 1 (rhTGF-β1), human platelet derived growth factor bb (rhPDGF-bb), human bone morphogenetic protein-2 (rhBMP-2), and recombinant bone morphogenetic protein-4 of *Xenopus laevis* (rxBMP-4) from 0.001 to 1.0 ng/ml each. The results of migration were expressed as a chemotactic index (CI). Migration of primary human progenitor cells was stimulated by rhBMP-2, rxBMP-4, and rhPDGF-bb in a dose-dependent manner. The increase of CI was up to 3.5-fold for rhBMP-2, 3.6-fold for rxBMP-4, and up to 22-fold for rhPDGF-bb, whereas rhTGF-β1 and rhbFGF did not stimulate cell migration in the concentration range tested. In contrast differentiated progenitor cells behave similar to primary human osteoblasts. RhBMP-2, rhPDGF-bb, and rhTGF-β1 stimulated the migration from 2.2 to 2.4-fold each, while rxBMP-4 and rhbFGF reached only a CI of 1.7–1.6. The effect of rhBMP-2, rxBMP-4, and rhPDGF-bb as chemoattractive proteins for primary human MPC, including the change in response to growth factors after differentiation suggests a functional role for recruitment of MPCs during bone development and remodeling, as well as fracture healing. J. Cell. Biochem. 87: 305–312, 2002.

Key words: chemotaxis; BMP-2; BMP-4; PDGF-bb; mesenchymal progenitor cells

A continuous source of osteoblast recruitment for bone growth, remodeling, and fracture repair is ensured by mesenchymal progenitor cells (MPC) which have been identified in bone marrow and other tissues [Caplan, 1991; Young et al., 1995]. Therefore, migration of bone forming cells is an important event during various physiological and pathological situations. The chemotactic response of osteoblasts and osteosarcoma cells to growth factors such as transforming growth factor beta 1 (TGF-β1), bone morphogenetic proteins (BMP), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) have been shown [Lind et al., 1996; Mayr-Wohlfart et al., 2001a].

TGF-β1 is known to regulate proliferation and expression of the differentiated phenotype of chondrocytes, osteoblasts, and osteoclasts [Lecanda et al., 1997]. Studies have shown that, during endochondral ossification, chondrocytes, and osteoblasts synthesize TGF-β1 [Joyce et al., 1990b]. Additional data suggest that a fracture activates the genes for TGF-β1, and, thus, the synthesis and release of TGF-β1 [Campbell et al., 1994]. Joyce et al. [1990a] could show that TGF-β1 appears in a hematoma within 24 h after fracture and persists for up to 10 days. TGF-β1 was seen intracellularly in mesenchymal cells and osteoblasts, and in the extracellular matrix. They concluded, that TGF-β1 is first released by platelets, and then synthesized by osteoblasts and chondrocytes within the callus during the entire healing process.

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BMP are part of the TGF-β1 superfamily and are known to be highly osteoinductive [Reddi, 1998]. Bostrom et al. [1995] could demonstrate the presence, location, and chronology of expression of BMP-2 and BMP-4 in fracture healing by immunohistochemistry. Showing the endogenous expression of BMP-2 and 4 they concluded that BMPs are important for the formation of intramembranous bone and are regulators of cell differentiation of mesenchymal cells into chondrocytes. Nakase et al. [1994] studied the temporal distribution of BMP-4 in fracture healing by RT-PCR. They could demonstrated that BMP-4 was only present in the early stages of fracture healing, and absent in control bones.

PDGF is a potent regulator of bone cells either alone or in combination with other factors. PDGF induces osteoblasts to undergo proliferation, chemotaxis, and matrix apposition [Cochran et al., 1993; Horner et al., 1996]. Nash et al. [1994] demonstrated that PDGF-bb increases the density and volume of callus formed in a rat tibial osteotomy model.

Fibroblast growth factors have mitogenic effects on fibroblasts and have been implicated in the regulation of chondrocyte and osteoblast function [Bostrom et al., 1999]. Nakamura et al. [1998] have demonstrated the osteogenic activity of bFGF in vivo. They suggest that bFGF accelerates both callus remodeling by increasing callus formation as a result of mitogenic effects on periostal cells and by stimulation of all stages of bone remodeling.

It is well known that these growth factors are secrete within the bone marrow environment, but until now, no information is available whether those growth factors also have an effect on the recruitment of MPC isolated from bone marrow or if these cells become responsive to chemoattractants at a later stage of differentiation. The aim of this study was to assess and quantify the chemotactic effects of rhBMP-2, rxBMP-4, rhPDGF-bb, rhbFGF, and rhTGF-β1 on primary human MPCs in comparison with in vitro differentiated MPC and primary human osteoblasts.

MATERIALS AND METHODS

Cell Culture

MPC were harvested from human bone marrow derived from routine surgical procedures (pelvic osteotomies) with informed consent from eight patients (age 8–16 years) and in accordance with the terms of the ethics committee of the University of Ulm.

MPC were isolated as described earlier [Deans and Moseley, 2000]. This isolation is specific for adherent MPC and maintains the progenitor phenotype [Bruder et al., 1998; Pittenger et al., 1999]. The MPC phenotype was proved by FACS analysis with CD9, CD90, CD105, and CD166 (positive), as well as CD14, CD34, and CD45 (negative), and by the potential to differentiate in osteoblasts, chondrocytes, or adipocytes. Usually over 95% of the cells were positive for all MPC specific and 100% negative for hemopoietic surface markers. Only MPC preparations lacking osteoblast specific gene expression were used for chemotaxis experiments.

After isolation the cells were cultured in a basal medium consisting of DMEM with 10% FCS, 1% glutamine, and 1% penicillin/streptomycin (all Biochrom Seromed, Berlin, Germany) at 37°C, 5% CO_2 in 95% humidity. In order to differentiate the MPC into osteoblasts, the basal medium was supplemented with 10^{-7} M dexamethasone, 50 μg/ml ascorbic acid, and 2.16 μg/ml β-glycerophosphate (Sigma, Germany) for 14 days. Medium was changed twice a week. The differentiation to osteoblasts was proved by "von Kossa"-staining, by enzymatic testing for alkaline phosphatase (*AP*) activity and by immunhistolgical staining with the MPC specific surface marker CD9, which is negative in osteoblasts and vanished during differentiation. In addition, only cell cultures expressing osteoblast specific genes were used for experiments with differentiated MPC.

Osteoblasts were harvested from cancellous human bone fragments derived from routine hip and knee replacements within surgical procedures. Donor material obtained from eight different patients (age 55–73 years) was plated after collagenase digestion for 2 h and cultured in DMEM with 10% FCS as described previously [Mayr-Wohlfart et al., 2001b]. Experiments were performed only in the first three cell passages as well as in the stage of cell maturation.

Growth Factors

Five different growth factors were used: recombinant human basic fibroblast growth factor (rhbFGF), recombinant human transforming growth factor beta 1 (rhTGF-β1), and

recombinant human platelet derived growth factor bb (rhPDGF-bb) were purchased from TEBU GmbH (Frankfurt, Germany), recombinant human bone morphogenetic protein-2 (rhBMP-2) was provided by the Theodor-Boveri-Institut of the University of Würzburg, Germany [Kübler et al., 1998] and recombinant xenopus bone morphogenetic protein-4 (rxBMP-4) was produced as described earlier [Mayr-Wohlfart et al., 2001b]. In initial experiments using concentrations of growth factors from 0.001 to 10 ng/ml no significant differences in the chemotactic response between 1 and 10 ng/ml was observed. Thus, for all growth factors concentrations of 0.001, 0.01, 0.1, and 1 ng/ml were tested in the chemotaxis assay.

Chemotaxis Assay

Chemotactic responses were measured by a modified Boyden chamber assay [Falk et al., 1980] using a 48-well microchemotaxis chamber (NeuroProbe, Inc., Baltimore, MD) with polycarbonate filters with 8 μm pores (Whatman Biometra, Göttingen, Germany) as described previously [Mayr-wohlfart et al., 2002]. The cells were trypsinized, counted, and partly used for RT-PCR as well as the chemotaxis assay. Growth factor dilutions in DMEM were filled into the lower wells and covered by the chemotaxis filter. Cells (1×10^4) in 50 μl DMEM were filled in the upper wells. After a 4 h incubation the filter was carefully removed, non-migrated cells on the upper side eliminated by rinsing with cold PBS and scraping over a rubber wiper. The remaining migrated cells on the lower side of the filter were fixed with 4% formaldehyde and stained with toluidine blue. In control experiments, the migrated cells on the filter were stained for CD9 (DAKO, Germany) in order to prove whether MPCs or other subpopulations respond to the tested factors. All growth factors were tested in triplicate for each concentration. Control wells with DMEM only in the bottom well were applied for each experiment. Conditioned medium of human fetal osteoblast-cultures was used as positive control in analogy to the use of conditioned medium from fetal cultures for fibroblast chemotaxis [Brenner et al., 2001].

The number of migrated cells in control and stimulated wells was counted for 12 random fields per well at 100 × magnification. Results were expressed as a chemotactic index (CI). This index was determined as the average number of migrated cells in stimulated wells divided by average number of migrated cells in control wells. A Zigmond–Hirsch checkerboard analysis was performed in triplicate wells to distinguish between concentration-dependent cell migration (chemotaxis) and random migration (chemokinesis). The analysis was performed after eliminating the concentration gradient by adding the chemoattractants to the upper chamber with the cell suspension [Zigmond and Hirsch, 1973].

RT-PCR for the Detection of Osteoblastic Marker Expression

The differentiation status of the MPCs, the differentiated progenitor cells, and primary osteoblasts was affirmed by RT-PCR for the expression of osteoblastic markers: runt-related transcription factor 2 (*RUNX2*); *AP*, osteocalcin (*OCN*); osteopontin (*OPN*); and osteonectin (*ON*; *SPARC*). Collagen type I alpha 1 (*COL1*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used to verify the PCR results as internal standards. Therefore, total RNA was isolated from 10^5 cells with the RNeasy® System and reverse transcription was done with Omniscript™ RT Kit (all QIAGEN®, Hilden, Germany) following the manufacturer's instructions. PCR-primers for *GAPDH*, *COL1*, *RUNX2*, *AP*, *OCN*, *OPN*, and *ON* were designed from sequences previously reported to GenBank® (National Institutes of Health genetic sequence database) using the Primer3 web site (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) with standard settings. Amplicon size was set to 180–250 bp for *GAPDH*, *COL1*, *RUNX2*, *AP*, *OCN* and 300–400 bp for *OPN* and *ON*. Temperature optimum was set to 60°C. In all cases the best primer pair was used. PCR-reactions were performed with a Robocycler® (Stratagene, Amsterdam, The Netherlands) using HotStarTaq™ Master Mix Kit (QIAGEN). PCR was performed under linear conditions using the cycle profile: initial incubation 15 min at 95°C, followed by 30 cycles annealing 45 s at 60°C, extension 45 s at 72°C, denaturation 60 s at 94°C, and terminating with 15 min at 72°C. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide, visualized and digitalized with an ImageMaster VDS system (Amersham Pharmacia Biotech, Freiburg, Germany).

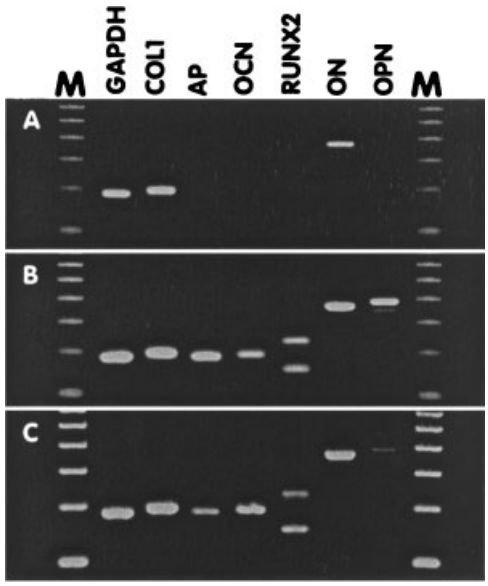

Fig. 1. Differences in the expression of osteoblastic marker genes by mesenchymal progenitor cells (MPC) (**A**), differentiated progenitor cells (**B**), and primary osteoblast (**C**) detected by RT-PCR analysis. M, 100 bp size marker; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COL1, collagen type I; AP, alkaline phosphatase; OCN, osteocalcin; RUNX2, runt-related transcription factor 2; ON, osteonectin; OPN, osteopontin.

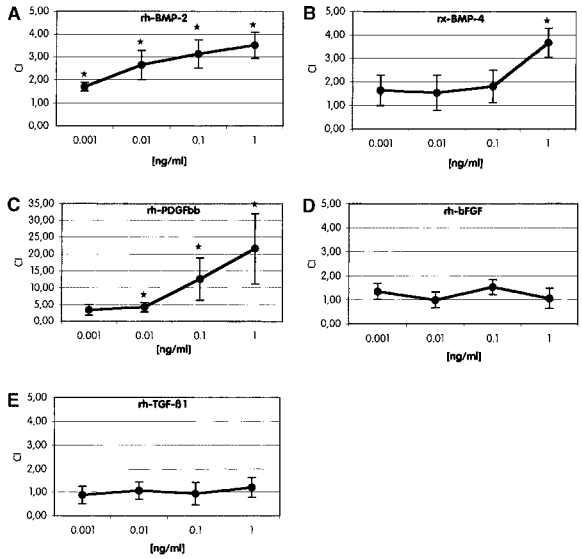

Fig. 2. Dose–response curve of chemotaxis to rhBMP-2 (**A**), rxBMP-4 (**B**), rhPDGF-bb (**C**), rhbFGF (**D**), and rhTGF-β (**E**). The chemotactic index (CI) (mean ± SD) of eight independent experiments, values measured in triplicate, is shown. *P* values from ANOVA and one-paired Wilcoxon tests less than $P = 0.05$ were considered significant and are marked with an asterisk (*).

Statistics

All experiments were performed in triplicate and the mean value was used for statistical analysis. Results are presented as mean ± standard deviation. The significance of differences between control and stimulation groups was determined using ANOVA and one-paired Wilcoxon tests. Values less than $P = 0.05$ were considered significant.

RESULTS

Chemotaxis of MPC

RhBMP-2, rxBMP-4, and rhPDGF-bb induced a chemotactic response of primary human MPC (Fig. 2A,B,C) but to a different degree and with somewhat different dose–response curves.

The stimulation of rhBMP-2 was significantly different from the control starting at 0.01 ng/ml and reached a maximum at 1.0 ng/ml with a CI of about 3.5-fold (± 0.15). After stimulation with rxBMP-4, a similar maximal CI of 3.6 (± 0.2) was observed, but a significant increase was delayed to the highest concentration applied. RhPDGF-bb stimulated migration with a different profile in a dose-dependent manner with a maximal CI of 22.4 ± 4 at 1 ng/ml (Fig. 2C). While 0.001 and 0.01 ng/ml stimulated migration with a CI of about 5, a concentration of 0.1 ng/ml raised the CI over 15-fold of the control experiment.

In contrast, rhTGF-β1 and rhbFGF did not show a stimulatory effect on cell migration in the concentration range from 0.01 to 1 ng/ml tested (Fig. 2D,E). Even raising the concentration up to 10 ng/ml for each growth factor in single experiments did not lead to a chemotactic response (data not shown).

Staining of the migrated cells on the chemotaxis membrane with anti-CD9 revealed that the vast majority was CD9 positive indicating that no subpopulation falsifies the result (data not shown).

The chemotactic response to the conditioned medium of human fetal osteoblast-cultures was used as a positive control, but the number of migrated cells could not be counted, because of the high response (> 1,000 migrated cells per well). In general, without addition of growth factors, 10–40 cells (0.1–0.4%) were counted because of chemokinesis. The addition of growth factors that induce chemotaxis raised the number of cells up to 2–4% with a maximum of 8% for 1 ng rhPDGFbb. For the other growth factors

tested, the maximum count of migrated cells was 1–2% cells, depending on the MPC donor and the growth factor concentration.

Chemotaxis of Differentiated MPCs and Primary Osteoblasts

Differentiated MPCs (dOB) responded in a similar way to primary human osteoblasts (pOB) as shown in Figure 3. The response to rhBMP-4 and most obvious to rhPDGF-bb declined from MPCs to the cells expressing an osteoblastic phenotype. In contrast, the chemotactic response to TGF-β1 increased slightly. Thus, in vitro differentiation of MPCs changed the responsiveness to the growth factors towards that of primary osteoblasts.

In these experiments, the differentiated bone cell phenotype was proven by the absence of CD9-marker in dOB (data not shown). Additionally, the MPCs, dOB, and pOB showed typical differences in the expression of the osteoblastic marker genes alkaline phosphatase (*AP*), *OCN*, *RUNX2*, and *OPN* as presented in Figure 1. Only osteonectin (*ON*) was detectable in all samples.

Checkerboard Analysis

The chemotactic responses of MPC and to osteoblasts differentiated cells were tested in a checkerboard analysis to determine whether cell migration was due to chemotaxis or chemokinesis. Checkerboard analysis in the tested concentration of 1 ng/ml showed that the stimulation of migration by rhBMP-2, rxBMP-4, and rhPDGF-bb for MPCs and rhBMP-2, rxBMP-4, rhPDGF-bb, rhTGF-β1, and rhbFGF for dOBs was present at a positive concentration gradient. This indicates that the cell migration seen in response to these growth factors was due to true chemotaxis, not chemokinesis (Table I).

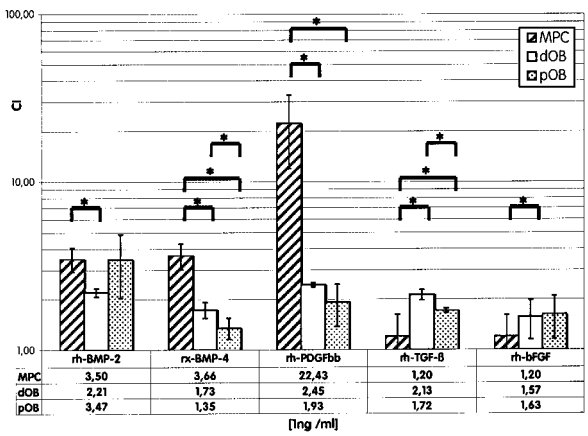

Fig. 3. Comparing migration response of MPC, differentiated progenitor cells (dOB), and primary osteoblasts (pOB) to the different growth factors in the concentration of 1 ng/ml. The CI (mean ± SD) of six independent experiments, values measured in triplicate, is shown. *P* values from Student's *t*-test less than $P = 0.05$ were considered significant and are marked with an asterisk (*).

DISCUSSION

In our study, we could demonstrate that human MPC from bone marrow have the ability to respond to several migration factors secreted by cells of the bone marrow environment and osteoblasts. This could be shown by the potent effect of the conditioned medium of fetal osteoblasts that served as a positive control. The detailed investigation of single factors revealed that the chemotactic response depends on the applied growth factor and also on the differentiation status of the cells.

Until today, chemotactic properties of PDGF-bb, bFGF, TGF-β1, and BMPs were only studied in rat and human osteoblasts [Lind et al., 1996; Godwin and Soltoff, 1997; Mayr-Wohlfart et al., 2001b]. Our study focused on the behavior of primary human MPC, which can differentiate into chondrocytes, adipocytes, osteoblasts, and other cells of mesenchymal origin. We tested migration of MPC in response to recombinant human bFGF, recombinant human TGF-β1, recombinant human PDGF-bb, recombinant human BMP-2, and recombinant xenopus BMP-4 and compared the results with MPCs after osteogenic differentiation in vitro as well as primary human osteoblasts. Our results show a positive chemotactic response of MPCs to human rhPDGF-bb, rxBMP-4, and rhBMP-2, but the lack of a directed migratory effect of rhTGF-β1 and rhbFGF. In vitro differentiation to osteoblast-like cells lead to a loss of response to rxBMP-4 and rhPDGF-bb, while the response to rhTGF-β1 and rhbFGF slightly increased. These data closely approached the values obtained for primary human osteoblasts published previously (CI = 2 for BMP2, CI = 2.3 for PDGFbb, CI = 2.4 for TGF-β1, CI = 1.6 for bFGF, CI = 1 for BMP4 [Pfeilschifter et al., 1990; Lind et al., 1995, 1996; Mayr-wohlfart et al., 2002].

We were able to demonstrate previously that rhBMP-2 and recombinant xenopus BMP-4 have potent stimulatory effects on human osteoblast

TABLE I. Checkerboard Analysis

Growth factor	Growth factor in upper well	Chemotactic index (CI) at 1 ng/ml in lower well			
		MPC		dOB	
		Mean	±SD	Mean	±SD
rhBMP-2	−	2.43	0.50	2.31	0.11
	+	1.17		1.08	
rxBMP-4	−	2.17	0.28	1.80	0.15
	+	1.27		0.85	
rhPDGF-bb	−	6.57	0.33	2.51	0.06
	+	1.02		1.36	
rhTGF-β1	−	0.75	0.20	1.94	0.11
	+	0.82		0.92	
rhbFGF	−	1.45	0.42	2.22	0.21
	+	1.69		0.80	

The table shows data from the checkerboard analysis. The concentration gradient between the wells (−) was eliminated by adding growth factors to the cell suspension in the top well (+). All growth factors were tested in the concentration of 1 ng/ml to induce the maximal stimulation of migration. In the table, the migration is expressed as CI. Data are means of at least triplicate wells with ±standard deviation.

differentiation [Mayr-Wohlfart et al., 2001b]. However, in spite of a high homology of the amino acid sequence of human rhBMP-2 and rxBMP-4 there is a remarkable difference in the chemoattractive properties for osteoblasts. Our observations on differentiated cells confirm the results of Lind et al. [1996], who compared human BMP-2, -4, and -6 and found chemotactic effects on differentiated human osteoblasts merely with BMP-2. Interestingly, we found that rxBMP-4 is also chemoattractive for undifferentiated MPC. Our results, therefore, indicate that the chemotactic response to BMP-4 is developmentally regulated in the osteoblastic lineage. It has been shown that BMP-2 and BMP-4 share the same receptor complex (BMPR-I and BMPR-II) for mediating various metabolic effects [Yamaji et al., 1994]. Since the effect of BMP-2 and rxBMP-4 differs in the migratory response of differentiated cells, the signaling pathway for the chemotactic response may differ from proliferation and differentiation.

PDGF was discovered in serum as the major mitogenic activity responsible for growth of cultured mesenchymal cells [Sporn and Roberts, 1990]. The mitogenic effect has also been found in human osteoblasts and various osteoblastic cell-lines [Abdennagy et al., 1992; Canalis et al., 1992]. PDGF was found as well to be a powerful chemotactic factor for mesenchymal cells, including osteoblasts from human and rat tissue [Hughes et al., 1992; Lind et al., 1995; Godwin and Soltoff, 1997]. These results were in part in line with our findings. While Godwin et al. reported a CI of 20 for osteoblasts at a concentration of 10 ng/ml PDGF-bb, Lind et al. [1995] reported a CI of 3.7 for the same concentration. Thus, although obtained with a 10-fold dosage the results of Lind et al. are compatible with our observations on in vitro differentiated cells and primary osteoblasts, while the maximum effects observed by Godwin et al. are comparable with the CI we obtained with MPCs, although at a much lower dosage.

TGF-β1 stimulates different cells of mesenchymal origin and preserves the amount and production of matrix components. In our study, rhTGF-β1 did not stimulate chemotaxis of MPCs. However, after differentiation, rhTGF-β1 led to a CI of 2.1 that was similar to the CI of primary human osteoblasts. Earlier Ballock et al. [1997] postulated that TGF-β1 only targets osteoblastic differentiated cells, and, therefore, we could regard our findings as a proof of that hypothesis.

It is known that bFGF, an angiogenic and proliferative growth factor produced by osteoblasts, increases cell proliferation of bone cells [Canalis et al., 1988]. In this study, we observed a borderline migratory response of primary human osteoblasts to bFGF while MPCs and in vitro differentiated MPCs did obviously not respond. Even raising the concentration up to 10 ng/ml did not alter the chemotactic behavior. In previous studies with osteoblasts Lind et al. [1995] found a maximum CI of 1.8–2.5 using a much higher concentration of 100 ng/ml.

In contrast, Midy and Plouet [1994] found no migration of fetal bovine osteoblasts stimulated with bFGF.

The differentiation of MPCs was proofed by PCR and by staining the migrated cells with surface specific markers for MPCs. Therefore, we could show that the migratory response was due to the differentiation status of the cells and was not falsified by subpopulations. The expression of *ON* coincides with previous reports that MPCs do express *ON* [Aubin, 1998a,b]. Maybe the expression of *ON* was increased as part of a shock response upon separation [Sauk et al., 1991; Kudo et al., 1994].

In conclusion, rhBMP-2, rxBMP-4, and rhPDGF-bb may contribute to the recruitment of MPCs in the context of bone formation, remodeling, and fracture healing. The results show that MPC are already targets for some chemoattractive factors and that the developmental stage has important influence on the migratory response.

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