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Role of bone morphogenetic protein 2 in the crosstalk between endothelial progenitor cells and mesenchymal stem cells

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Abstract. In recent studies, we and others have demonstrated that bone morphogenetic protein-2 (BMP-2) promotes vascularization, inhibits hypoxic cell death of cancer cells and may be involved in tumor angiogenesis. The activation of circulating endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) represents a crucial factor in the process of postnatal neovascularization. BMP-2 protein expression has been detected in several tumor tissues and BMP receptors are expressed in EPCs and MSCs. We therefore analysed the influence of recombinant human (rh) BMP-2 on the function of human EPCs and human bone marrow derived MSCs. Treatment of EPCs isolated from peripheral blood with rhBMP-2 did not induce any significant changes in EPC viability but induced a dose-dependent activation of chemotaxis. Incubation of human MSCs isolated from bone marrow aspirates with rhBMP-2 revealed no significant effect on MSC proliferation. Incubation of EPCs with supernatants of MSCs significantly increased the cell viability compared to controls cultivated with endothelial cell medium. Protein and mRNA expression of the vascular endothelial growth factor (VEGF) family member, placental growth factor (PlGF), which is known to be involved in the expansion and recruitment of EPCs, was induced in MSCs after treatment with rhBMP-2. We conclude that tumor-associated BMP-2 secretion might promote tumor angiogenesis by chemotactic effects on EPCs circulating in the peripheral blood and by increased secretion of paracrine angiogenic growth factors including PlGF in MSCs of the tumor stroma.

Introduction

Bone morphogenetic proteins (BMPs) are members of a large family of TGF-ß-related growth factors which use similar signal transduction pathways that involve transmembrane serine threonine kinase receptors and Smad proteins. They are crucial factors for the regulation of physiologic angiogenesis during embryonic development and bone formation (1). Previously, we and others have demonstrated that activation of the BMP pathway by BMP-2 can also promote tumor growth and angiogenesis in breast and lung cancer (2-5).

Emerging evidence indicates that bone marrow-derived EPCs can contribute to tumor vascularization (6). The Id (inhibitors of differentiation) basic-helix-loop-helix transcription factors which play a vital role in angiogenesis have been identified as one of the main targets of the BMP-2 signaling pathway (7). The importance of Id transcription factors for tumor angiogenesis has been demonstrated in Id1/Id3 double-null mice that failed to support tumor growth and metastasis from xenografts due to poor vascularization (8). The transplantation of wild-type bone marrow or VEGF-mobilized stem cells restored tumor angiogenesis and growth. The angiogenic defect in Id-mutant mice was due to impaired mobilization of ECPs and impaired proliferation and incorporation of VEGF receptor 1-positive cells (9).

MSCs are pluripotent adult stem cells residing within the bone marrow microenvironment and in many sites of the human body including the artery and vein wall (10,11). Recent results suggest that intravenous administration of MSCs improves cardiac function after acute myocardial infarction partly through the enhancement of angiogenesis in the ischemic myocardium (12). Interestingly, injection of MSCs has recently been shown to lead to incorporation of MSCs in the tumor stroma (13) indicating a potential role for MSCs in tumor vascularization. This is supported by findings of a very recent study that demonstrated a favourable effect on tumor growth *in vivo* when MSCs were mixed with tumor cells and transplanted subcutaneously into BALB/c-nu/nu mice. The tumor cells exhibited elevated capability of proliferation, angiogenesis and metastasis (14). BMP-2 plays an important role in the regulation of differentiation and function of mesenchymal stem cells (15). During fracture healing, BMP-2 is involved in the formation and vascularization of new bone (16,17).

Because of this background we analysed the influence of BMP-2 on the function of human EPCs and bone marrow derived MSCs. The purpose of our study was to support the hypothesis that BMP-2 is involved in the crosstalk between EPCs circulating in the peripheral blood and MSCs of the tumor stroma during the process of tumor angiogenesis.

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Materials and methods

Cell culture. Mononuclear cells were isolated by density-gradient centrifugation with Biocoll from 20 ml of peripheral blood. Immediately after isolation, $4x10^6$ mononuclear cells were plated on 24-well culture dishes coated with human gelatin and maintained in endothelial basal medium (EBM, CellSystems) supplemented with EGM SingleQuots and 20% FCS. After 4 days in culture, nonadherent cells were removed by a thorough washing with PBS. To detect the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbo-cyanine-labeled acetylated low-density lipoprotein (DiLDL), cells were incubated with 2.4 μg/ml DiLDL at 37˚C for 1 h. Cells were then fixed with 2% paraformaldehyde for 10 min, and lectin staining was performed by incubation with fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin I (lectin, 10 μg/ml) for 1 h. After the staining, samples were viewed with an inverted fluorescent microscope. Dual-stained cells positive for both lectin and DiLDL were judged to be EPCs. Isolated EPCs were detached using 1 mmol/l EDTA in PBS (pH 7.4), harvested by centrifugation and resuspended in 500 μl of EBM.

Primary human MSCs were established from bone marrow aspirations under local anesthesia from healthy volunteer donors. Mononuclear cells (MNCs) were isolated by Percoll gradient centrifugation and plated at a density of $1x10^6$ cells/ml in 175 cm^2-polystyrene flasks in Dulbecco's minimum essential medium (DMEM) with a selected batch of 10% fetal bovine serum (FBS). Cells were allowed to adhere for 72 h followed by the removal of nonadherent cells and media changes every 3-4 days. Adherent cells were removed with 0.05% trypsin-EDTA and replated (passaged) at a density of $1x10^6$/175 cm^2. Multiple individual donor preparations were performed and second or third passage MSCs were used for individual experiments. All MSC preparations were morphologically identical and uniformly expressed SH2 and SH3 markers.

Recombinant BMP-2 and the BMP inhibitor noggin were a gift from Peter Hortschansky, Jena, Germany. All other reagents were supplied by Sigma, unless stated otherwise.

Cell viability assay. The CellTiter-Blue® cell viability assay (Promega) was used for monitoring cell viability. The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Non-viable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal. According to the protocol the assay procedure involved adding single reagents directly to cells cultured in serum-supplemented medium. After an incubation step, data were recorded using a plate-reading spectrophotometer.

MSC proliferation assay. MSCs ($2x10^4$) were cultured in medium and treated with or without 20 ng/ml VEGF, 200 ng/ml BMP-2, or 20 ng/ml VEGF combined with 200 ng/ml BMP-2. Cell number was counted in a Neubauer chamber on day 1 and 3.

RT-PCR. Total RNA from cell cultures was prepared using the RNeasy kit (Qiagen AG). Cells ($0.5x10^6$) were lyzed in guanidine isocyanate solution and stored at -200˚C until used. RNA was extracted with the RNAeasy kit (Qiagen) and 0.05 μg was reversely transcribed into cDNA with random hexamer primers. cDNA aliquots were amplified in triplicate in a thermocycler for 30-40 cycles of PCR using specific primers designed to have a Tm of 56-60˚C and 0.2 U of Taq polymerase. The amplified products were analyzed by 1% agarose gels, and ratios of the amounts of amplified products compared to those of glyceraldehyde phosphate dehydrogenase (GAPDH) amplified in 25-28 cycles were measured. The specific primers used were: PlGF sense, 5'-AGGATCCGGG AACGGCTCGTCAG-3', antisense primer, 5'-TCTCGAG TCATTACCTCCGGGGAACA-3'; ß-actin sense, 5'-CGGG AAATCGTGCGTGACAT-3', antisense, 5'-GAACTTTGG GGGATGCTCGC-3'.

Immunoassay. A sandwich immunoassay was used to measure the concentration of PlGF protein in cell culture

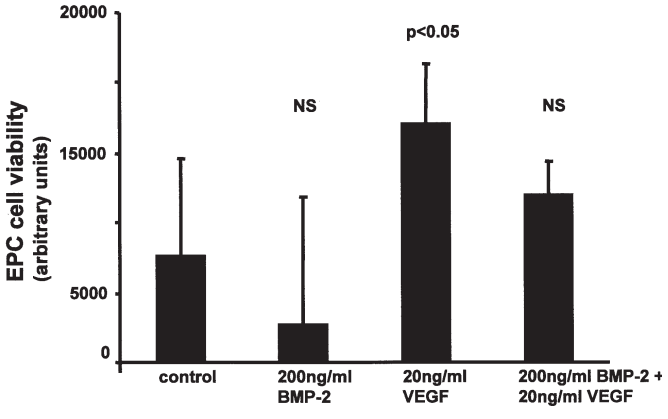

Figure 1. BMP-2 has no significant influence on EPC viability. A cell viability assay based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin) was used for analysing cell viability. EPCs were cultured over 24 h in EBM alone or treated with 200 ng/ml rhBMP-2, 20 ng/ml VEGF or a combination of rhBMP-2 and VEGF. Data represents the average of 6 independent experiments (n=6); bars are means + SD.

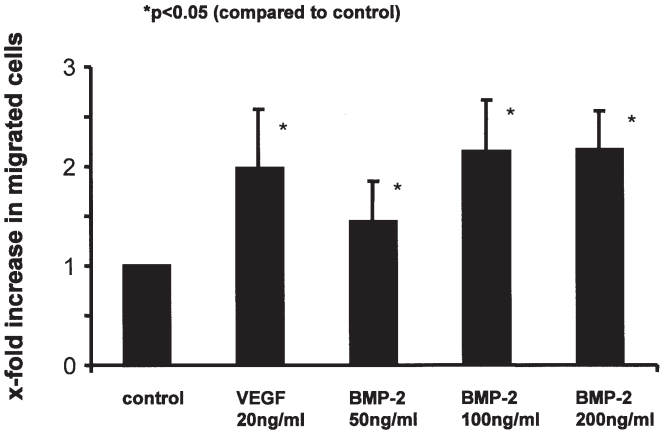

Figure 2. BMP-2 induces EPC chemotaxis. In transwell assays, rhBMP-2 was applied to the lower chamber with a concentration range of 50-200 ng/ml in EBM. Incubation with an additional 20 ng/ml VEGF served as a positive control and incubation with EBM alone as a negative control. EPCs were seeded onto the semipermeable membrane in the upper chamber. After 24 h of incubation the number of EPCs which migrated through the semi-permeable membrane was counted. Data represent the average of 5 independent experiments (n=5); bars are means + SD.

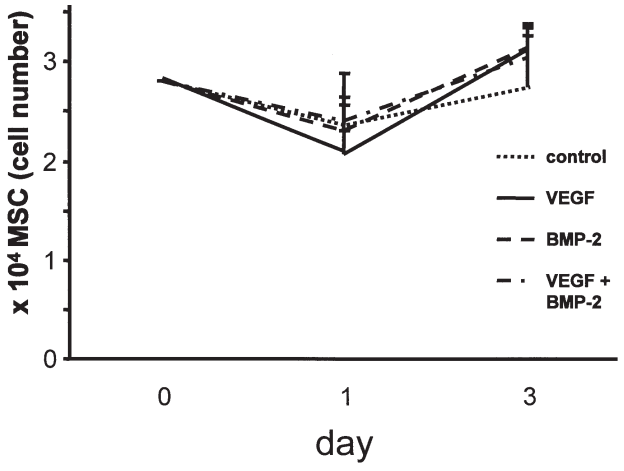

Figure 3. BMP-2 has no significant effect on MSC proliferation. Human MSCs were cultured in a medium and treated with or without 20 ng/ml VEGF, 50-200 ng/ml BMP-2, or 20 ng/ml VEGF combined with 200 ng/ml BMP-2. Cell number was counted in a Neubauer chamber on day 1 and 3. Data represent the average of 6 independent experiments (n=6); bars are means + SD.

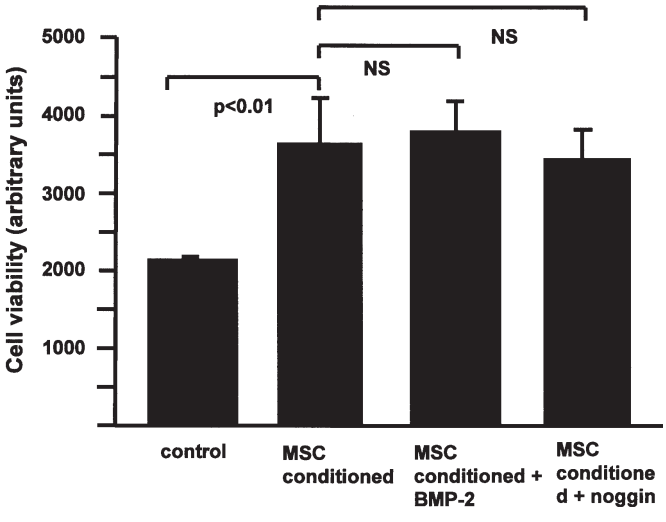

Figure 4. MSC-conditioned medium enhances EPC viability. EPCs were incubated for 24 h with supernatants collected from human MSCs which had been cultivated with DMEM and 10% FCS. Analysis of cell viability with an assay based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin) revealed a significant stimulation of EPC viability compared to medium controls. Additional treatment with 200 ng/ml BMP-2 or with 400 ng/ml of the BMP-2 inhibitor noggin had no further effect on EPC viability. Data represent the average of 6 independent experiments (n=6); bars are means + SD.

supernatant using the anti-hPlGF monoclonal antibody MAB264 (R&D Systems) and the polyclonal biotinylated anti-hPlGF antibody BAF264 (R&D Systems).

Migration assay. Isolated EPCs were detached using 1 mM EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 μl EBM and counted. Then, 2x10^4 EPCs were placed in the upper chamber of a modified Boyden chamber. The chamber was inserted in a 24-well culture dish containing EBM$^{+/-}$ human recombinant VEGF (20 ng/ml) and/or human recombinant BMP-2 (50, 100 or 200 ng/ml). After 24 h of incubation at 37˚C, the lower side of the filter was washed with PBS and fixed with 2% formaldehyde. For quantification, cell nuclei were stained with 4',6-diamidine-2-phenylidole dihydrochloride (DAPI). Cells migrating into the lower chamber were counted manually in three randomly selected microscopic fields.

EPCs were placed in the upper chamber of a modified Boyden chamber. The chamber was inserted in a 24-well culture dish containing EBM$^{+/-}$ human recombinant VEGF (20 ng/ml) or human recombinant BMP-2 (50, 100 or 200 ng/ml). After 24 h of incubation at 37˚C, the lower side of the filter was washed with PBS and fixed with 2% formaldehyde. For quantification, cell nuclei were stained with 4',6-diamidine-2-phenylidole dihydrochloride (DAPI). Cells migrating into the lower chamber were counted manually in three randomly selected microscopic fields.

Statistical analysis. Comparisons between groups were made applying the Wilcoxon matched pairs test.

Results

BMP-2 and EPC viability. A cell viability assay based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin) was used for analysing cell viability. Incubation with 200 ng/ml rhBMP-2 over 24 h revealed no significant influence on EPC viability compared to EBM protocols. For positive control EPCs were cultured with 20 ng/ml VEGF and the viability of EPCs was significantly stimulated. Additional treatment with rhBMP-2 did not induce a significant change in cell viability compared to VEGF alone (Fig. 1).

BMP-2 and EPC chemotaxis. In transwell assays, rhBMP-2 was applied to the lower chamber with a concentration range of 50-200 ng/ml in EBM. Incubation with the addition of 20 ng/ml VEGF served as a positive control and incubation with EBM alone as a negative control. EPCs were seeded onto the semi-permeable membrane in the upper chamber. After 24 h of incubation the number of EPCs which migrated through the semipermeable membrane was counted. We found that rhBMP-2 acted as a chemoattractant on EPCs. The addition of rhBMP-2 led to an increase in cell migration compared to cells without rhBMP-2. A maximum effect was observed with 100 ng/ml rhBMP-2, which was comparable to the incubation with 20 ng/ml VEGF (Fig. 2).

BMP-2 and MSC proliferation. MSCs were cultured in medium and treated with or without 20 ng/ml VEGF, 200 ng/ml BMP-2, or 20 ng/ml VEGF combined with 200 ng/ml BMP-2. MSC proliferation was not significantly enhanced with treatment of 20 ng/ml VEGF or 200 ng/ml BMP-2 compared to medium controls on day 3 (Fig. 3). Combined treatment with VEGF and BMP-2 showed no additive effect on MSC proliferation.

MSC conditioned medium and EPC viability. Cultured MSCs have been shown to secrete large amounts of the angiogenic

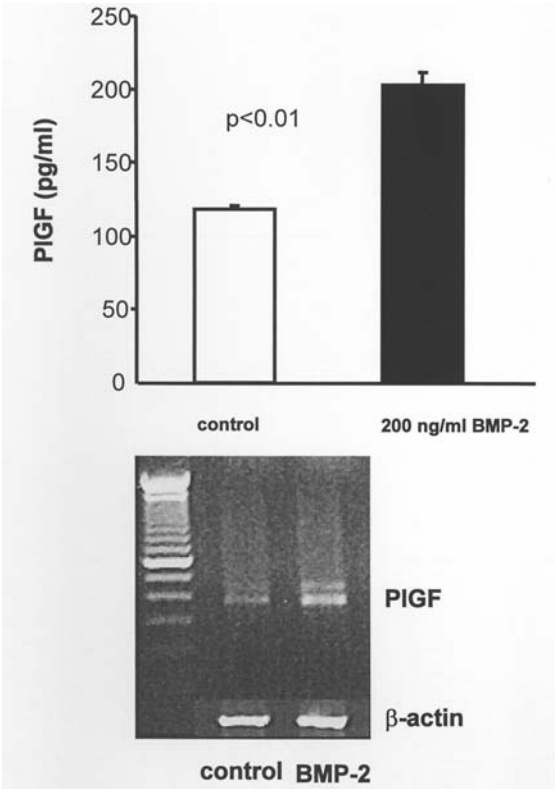

Figure 5. BMP-2 induces PlGF mRNA protein expression in human MSCs. Human MSCs were incubated for 24 h in serum-free DMEM with or without 200 ng/ml rhBMP-2. Supernatants were collected and a sandwich immunoassay was used to measure the concentration of PlGF protein. Data represent the average of 5 independent experiments (n=5); bars are means + SD. Total RNA from MSCs was isolated for the analysis of PlGF and ß-actin mRNA expression with RT-PCR.

and mitogenic factors including vascular endothelial growth factor, adrenomedullin, and insulin-like growth factor-1. Therefore, we incubated EPCs for 24 h with supernatants collected from MSCs which had been cultivated with DMEM and 10% FCS. Analysis of cell viability revealed a significant stimulation of EPC viability compared to medium controls (Fig. 4). Additional treatment with 200 ng/ml BMP-2 or with 400 ng/ml of the BMP-2 inhibitor noggin had no further effect on EPC viability.

BMP-2 and PlGF expression in MSCs. PlGF is a cytokine that is known to be involved in the recruitment of EPCs. Data from large-scale gene expression profiling using high-density cDNA microarrays in primary human MSCs found that PlGF was induced early after treatment with BMP-2. In agreement with microarray data, we confirmed that PlGF protein and mRNA expression was significantly induced by 24 h of incubation with 200 ng/ml BMP-2 compared to control medium (Fig. 5).

Discussion

EPCs reportedly differentiate into endothelial cells and participate in angiogenesis, including neovascularization at sites of neoplastic development (18). Recently, it has been shown that MSCs are incorporated in the tumor stroma (13) and stimulate angiogenesis in ischemic tissue, but the exact mechanism remains controversial. Previously, we and others demonstrated that activation of BMP-2 expression in tumor tissue can promote tumor vessel formation in breast and lung cancer and inhibits hypoxic cell death in tumor cells (3,5,19). In the present study, we report results that support a possible role of BMP-2 in the crosstalk between EPCs and MSCs during the process of tumor angiogenesis.

As expected from our previously published studies on mature endothelial cells, BMP-2 had no significant direct effect on the EPC viability (3). However, BMP-2 induced a dose-dependent activation of EPC chemotaxis. In order to understand the impact of BMP-2 on migratory activities of target cells, it is important to know that BMPs are well established chemoattracting and migratory factors for many cell types, e.g. monocytes, osteoblasts and mesenchymal progenitor cells (20-22). Since almost all cells are able to produce BMPs, the difference in concentration of the BMP is responsible for this effect and explains the movement of EPCs towards the BMP-2 source in our assays. Therefore, an elevated expression of BMP-2 by the tumor cells might be responsible for the recruitment of EPCs from capillaries of the tumor environment.

Neovascularization is critical for bone formation and bone fracture healing (23). Recently, it has been shown that paracrine functions of VEGF secreted by MSCs are involved in blood vessel formation during the healing process of damaged bone (24). BMP-2 plays an important role in the regulation of differentiation and functions of MSCs and might also influence their angiogenic activity. In our study BMP-2 and VEGF exhibited no significant effect on MSC proliferation with no additive effect by combined treatment. Therefore, it seems to be unlikely that BMP-2 stimulated MSC proliferation is a crucial factor for the process of BMP-2-induced tumor angiogenesis. This was supported by our findings that MSC conditioned medium potently stimulated EPC viability while additional treatment of the MSCs with BMP-2 or with the BMP-2 inhibitor noggin had no further stimulatory effect on EPC viability. Our results favor a BMP-2-dependent increase in the expression of angiogenic growth factors by MSCs. In this respect PlGF, a known gene of the VEGF family, has been identified in a large-scale gene expression profiling using high-density cDNA microarrays in primary human MSCs as an early-regulated gene whose induction was detected after 2 h of treatment with BMP-2 (25). In our study we confirmed that 24 h of treatment with BMP-2 induced protein and mRNA expression of PlGF in bone marrow-derived human MSCs. Originally, PlGF was reported to be expressed in umbilical vein endothelial cells and placenta, suggesting a predominant role in placental angiogenesis (26). However, it was discovered that in the adult organism, PlGF plays an important role in the recruitment of endothelial and hematopoietic stem cells (HSC) in pathological conditions such as angiogenesis during ischemia, inflammation, wound healing, and cancer (27,28), whereas in normal adult tissues PlGF is not or only very weakly expressed. It is tempting to speculate that during tumor angiogenesis MSCs in the tumor stroma might be stimulated by paracrine BMP-2 expression of cancer cells and/or macrophages to produce PlGF which then recruits and expands endothelial progenitors to support tumor vascularization.

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together the results of this study support the s that tumor-associated BMP-2 secretion promotes tumor angiogenesis by chemotactic effects on circulating EPCs and by increased expression of paracrine angiogenic growth factors including PlGF in MSCs of the tumor stroma.

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