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Myofibroblast progenitor cells are increased in number in patients with type 1 diabetes and express less bone morphogenetic protein 6: a novel clue to adverse tissue remodelling?

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Abstract *Aims/hypothesis:* Growth factor imbalance and endothelial progenitor cell dysfunction are well-known elements of the inappropriate response to injury in human and experimental diabetes. We hypothesised that in diabetes the outgrowth of myofibroblast progenitor cells (MFPCs) is also altered and that this relates to aberrant gene expression of growth factors involving members of the TGF- β /bone morphogenetic protein (BMP) superfamily. *Subjects and methods:* MFPCs were cultured from peripheral blood mononuclear cells of patients with type 1 diabetes and control subjects. Microarray analysis, quantitative PCR and ELISA were used to identify differentially regulated TGF- β /BMP superfamily genes in diabetes- and control-derived MFPC. Possible effects of BMP6 on TGF- β -induced gene expression were examined in cultured renal fibroblasts (TK173 cells). *Results:* Blood from diabetic patients yielded higher numbers of MFPCs than blood from control subjects (1.6-fold increase, $p < 0.05$), involving increased proliferation and decreased apoptosis. BMP6 mRNA and protein were downregulated in MFPCs derived from patients with

diabetes (3.9- and 1.8-fold decrease, respectively, $p < 0.05$). Furthermore, an inverse correlation was observed between BMP6 mRNA level and the number of MFPCs in patients with diabetes ($r = -0.85$, $p < 0.05$). In TK173 cells, BMP6 antagonised the TGF- β -induced expression of the genes encoding plasminogen activator inhibitor-1 and connective tissue growth factor (70 and 50% reduction, respectively). *Conclusions/interpretation:* Considering the importance of BMP6 in processes such as angiogenesis and its novel anti-TGF- β effects, we propose that the excess numbers of BMP6-deficient MFPCs may favour adverse tissue remodelling in patients with diabetes, both numerically and by inappropriate orchestration of their microenvironment.

Keywords BMP6 · Gene expression profiling · Growth factor · Progenitor cells · TGF- β · Type 1 diabetes mellitus

Abbreviations α SMA: α -smooth muscle actin · acLDL: acetylated LDL · BMP: bone morphogenetic protein · BrdU: bromodeoxyuridine · col I: collagen type I · cPC: circulating progenitor cell · CTGF: connective tissue growth factor · EPC: endothelial progenitor cell · GDF9: growth differentiation factor 9 · HRP: horseradish peroxidase · MFPC: myofibroblast progenitor cell · PAI-1: plasminogen activator inhibitor 1 · PBMC: peripheral blood mononuclear cell

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Introduction

Bone marrow-derived circulating progenitor cells (cPCs) have not only been shown to be important contributors to the repair of damaged tissue [1, 2] but are also involved in the pathological processes of tissue remodelling, such as atherosclerosis, fibrosis and chronic transplant vasculopathy [3–5]. In several of these conditions, myofibroblasts or smooth muscle cells driving adverse tissue remodelling originate largely from cPCs [6]. Recently it was demonstrated that, besides endothelial cells, smooth muscle cells can also be cultured from peripheral blood [7, 8].

Moreover, it was shown that the number of colony-forming units in endothelial progenitor cell (EPC) cultures correlated with cardiovascular function [9] and that the development of fibrocytes, defined as collagen-secreting cells present in peripheral blood, was upregulated in patients with burn wounds and that a positive correlation existed between fibrocyte numbers and serum TGF- β levels [10].

In diabetes the tissue response to injury is compromised, as evidenced by the development of excessive renal and cardiovascular damage and the ensuing matrix accumulation. Thus far, dysfunction of cPCs in patients with diabetes has been examined only at the EPC level, showing decreased outgrowth of diabetes-derived EPC and impaired angiogenic activity [11, 12]. Outgrowth of myofibroblast progenitor cells (MFPCs) in patients with diabetes has not yet been investigated.

It seems likely that cPC dysfunction in diabetes relates, at least in part, to imbalance of growth factor signalling. In particular, several authors have demonstrated that TGF- β , a profibrotic cytokine and potent stimulator of myofibroblast differentiation, is elevated in patients with diabetes and that it functions as a crucial factor in the progression of diabetic nephropathy [13, 14]. Levels of plasminogen activator inhibitor-1 (PAI-1) and connective tissue growth factor (CTGF), both key mediators of profibrotic TGF- β activity, are also significantly increased in tissue and plasma of experimental and human diabetic nephropathy [15–20]. Furthermore, Korpinen et al. have demonstrated increased secretion of TGF- β by 2-day cultured peripheral blood mononuclear cells (PBMCs) from patients with type 1 diabetes and showed an association of these TGF- β levels with the occurrence of diabetic nephropathy [21]. In contrast, renal expression of another member of the TGF- β superfamily, the antifibrotic bone morphogenetic protein 7 (BMP7), is decreased during the course of experimental diabetic nephropathy [22], and *in vitro* studies with human mesangial cells have revealed that a high glucose concentration induces the expression of BMP antagonists CTGF and gremlin [23, 24]. The relevance of TGF- β /BMP signalling imbalance is underscored by a similar reduction in matrix accumulation and even the reversal of experimental diabetic nephropathy by the exogenous administration of BMP7 or inhibition of TGF- β [25, 26].

We hypothesised that, in diabetes, the TGF- β /BMP signalling imbalance is related to aberrant programming of cPCs, leading to increased outgrowth and dysregulation of MFPCs with consequent fibroplasia and scar formation instead of functional repair. Therefore, we determined the number of MFPCs that could be cultured from peripheral blood of patients with diabetes and control subjects, and we set out to evaluate the possible role of TGF- β /BMP superfamily members in functional and numerical differences between diabetes- and control-derived MFPCs.

Subjects and methods

Subjects

Six patients with type 1 diabetes, who visited the outpatient department of Vascular Medicine of the University Medical Centre in Utrecht, were entered into the study. The patients, three males and three females with a mean age of 37.4 ± 7.0 years (ranging from 32 to 48 years), were all free of medication except for insulin therapy. The mean duration of diabetes was 21.7 ± 10.8 years. Patient blood glucose levels were relatively well regulated (mean HbA_{1c} of $7.1 \pm 0.3\%$) and patients did not show any signs of organ complications due to diabetes. As controls, we selected six healthy age- and sex-matched subjects (mean age 37.6 ± 7.2 years). All subjects gave informed consent. The study was performed according to the principles of the Declaration of Helsinki.

Culture of myofibroblast progenitor cells

MFPCs were isolated and cultured according to protocols used by others for the culture of fibrocytes, *i.e.* collagen-secreting cells cultured from peripheral blood [10, 27]. Briefly, 20–40 ml peripheral blood samples of patients and control subjects were collected in tubes containing EDTA. Total PBMCs were isolated by density gradient centrifugation on HistoPaque 1077 (Sigma, St Louis, MO, USA). PBMCs were seeded on six-well plates coated with $0.5 \mu\text{g/ml}$ human fibronectin (Sigma) in PBS at a density of 5×10^6 cells per well and cultured in low-glucose (1 g/l) DMEM supplemented with 20% fetal bovine serum, L-glutamine (2 mmol/l), penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified incubator (37°C, 5% CO₂, 95% air). After 3 days, non-adherent cells were removed by a single aspiration and medium was changed. Following another 4 days of culture, the adherent cells were harvested by incubation with trypsin/EDTA in PBS and gentle scraping with a rubber policeman. The number of cells was determined using a haemocytometer.

Evaluation of MFPC phenotype

MFPC phenotype was evaluated by indirect immunofluorescence staining for collagen type I (col I) and α -smooth muscle actin (α SMA). Adherent cells from two healthy control subjects and two patients with type 1 diabetes were cultured on fibronectin-coated eight-well glass chamber slides (Lab-Tek; Nunc, Naperville, IL, USA) at a density of 1×10^6 cells per well for 3, 7 and 17 days. Cells were fixed in ice-cold methanol/acetone and washed in PBS/0.05% Tween 20, followed by incubation with a monoclonal anti-col I antibody (C2456; Sigma) at a dilution of 1:50 or a monoclonal anti- α SMA antibody (A2547, Sigma) at a dilution of 1:400. Binding of primary antibodies was detected by a tyramide signal amplification fluorescein technique, using horseradish peroxidase (HRP)-conjugated

rabbit anti-mouse Ig (Dako, Glostrup, Denmark) followed by incubation with Fluorophore Tyramide Amplification Reagent (Perkin-Elmer, Boston, MA, USA). The specificity of immunofluorescence staining was demonstrated by incubation with isotype-matched IgG control antibodies. Cells were counterstained with propidium iodide, mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and visualised by confocal laser scanning microscopy.

To confirm that the cultured MFPCs were different from EPCs, adherent cells were also incubated with 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labelled acetylated LDL (DiI-acLDL; Molecular Probes, Leiden, The Netherlands) for 1 h, and after fixation were incubated with fluorescein isothiocyanate (FITC)-labelled *Ulex europaeus* agglutinin I (*Ulex* lectin; Sigma) for 1 h. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and cells were visualised by fluorescence microscopy.

Analysis of MFPC proliferation and apoptosis

To measure cumulative MFPC proliferation in control subjects and diabetic patients, bromodeoxyuridine (BrdU; Sigma) was added to chamber slides of three control- and three diabetes-derived MFPC cultures at the time of seeding and each time when medium was changed ($t=3, 7, 10$ and 14 days). After $3, 7$ and 17 days, cells were fixed in ice-cold ethanol and incubated with proteinase K. Fixed cells were treated with 4 N HCl , blocked with PBS/2% BSA/1% Triton X-100 and incubated with mouse-anti-BrdU (BD Biosciences, San Jose, CA, USA) at a dilution of $1:40$. BrdU-positive cells were identified by incubation with HRP-conjugated rabbit-anti-mouse immunoglobulin (Dako) and enzyme activity of HRP was detected using 3,3'-diaminobenzidine (Sigma). Similarly, apoptotic activity of MFPCs was analysed by immunocytochemical staining with a rabbit anti-active caspase 3 antibody (BD Biosciences) at a dilution of $1:100$. Both cumulative proliferation and apoptotic activity were assessed by counting positive stained nuclei.

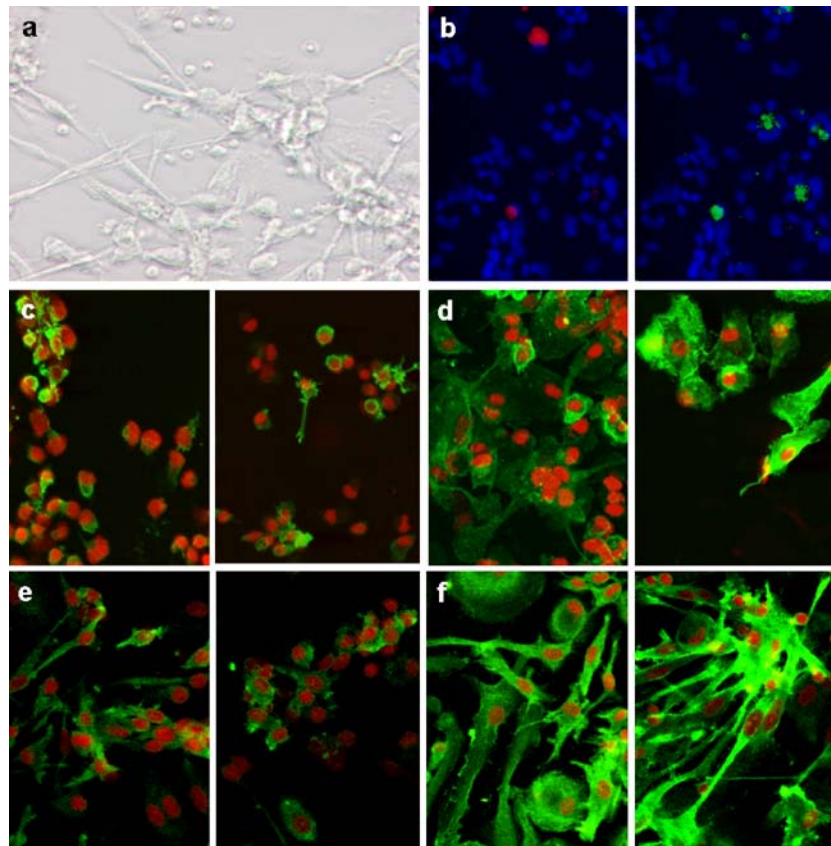


Fig. 1 Outgrowth of MFPCs from peripheral blood mononuclear cells. Mononuclear cells were isolated from peripheral blood and seeded on fibronectin-coated wells. After 7 days of culture, approximately 70% of the adherent cells had obtained a fibroblast-like morphology (**a**). **b** Fluorescence microscopy of MFPCs with DAPI as nuclear stain (*blue*) illustrates that only a small number of adherent cells were positive for uptake of DiI-labelled acetylated LDL (*red*, *left panel*) and binding of FITC-*Ulex* lectin (*green*, *right panel*). **c** Confocal laser scanning microscopy of control-derived MFPCs with propidium iodide as nuclear stain (*red*) demonstrated

that approximately 70% of adherent cells stained positive for collagen I (*left panel*) and α SMA (*right panel*) after 7 days. **d** After 17 days of culture, virtually all control-derived adherent cells showed a myofibroblast phenotype (collagen I shown in *left panel*, α SMA shown in *right panel*). **e** In diabetes-derived MFPC cultures, approximately 90% of cells were positive for collagen I (*left panel*) and α SMA (*right panel*) after 7 days. **f** After 17 days of culture, all diabetes-derived adherent cells showed a myofibroblast phenotype (collagen I shown in *left panel*, α SMA shown in *right panel*)

Comparative gene expression profiling of MFPCs

To determine gene expression profiles of MFPCs from patients with diabetes compared with those of MFPCs from healthy individuals, total RNA was extracted from 7-day cultured MFPCs using RNeasy columns (Qiagen, Venlo, the Netherlands). The quantity and quality of the RNA samples were determined using a spectrophotometer (Shimadzu Biotech, Kyoto, Japan) with a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the Eukaryote Total RNA Nano assay. Total RNA (300 ng) from diabetic patients and control subjects was pooled for each group in equal amounts for each subject. RNA pools were subsequently amplified using the MessageAmp Aminoallyl kit (Ambion, Austin, TX, USA) and labelled with Cy3 and Cy5 (Amersham Biosciences, Amersham, UK). Duplicate dye-swap microarray hybridisations (i.e. a total of four hybridisations) with 300 ng of probe per sample were performed on 19 K human 70-mer oligochips (Genomics Laboratory, Utrecht, The Netherlands). Microarray slides were scanned using a DNA microarray scanner (Model G2565BA; Agilent Technologies) at 100% laser intensities and 30% photomultiplier tube sensitivity. Stored

images were analysed using Imagene (Biodiscovery, Marina Del Rey, CA, USA) software. Data were normalised using the Lowess algorithm. Only spots with a signal intensity exceeding background +2 SD were considered. Log₂-transformed signal intensities between channels were normalised to have an identical log₂ median. For the present report, genes were considered significantly regulated when in both experiments log₂-transformed ratios were >0.7 or <-0.7 (equivalent to 1.62-fold up- and downregulation).

Quantitative PCR

BMP6, *BMP7* and *GDF9* (the gene encoding growth differentiation factor 9) mRNAs were assessed in individual samples from diabetic patients and control subjects by quantitative real-time PCR using Assays-on-Demand Gene Expression Products with predesigned probe and primers (Applied Biosystems, Foster City, CA, USA). Hydroxymethylbilane synthase and TATA-box binding protein were used as internal references.

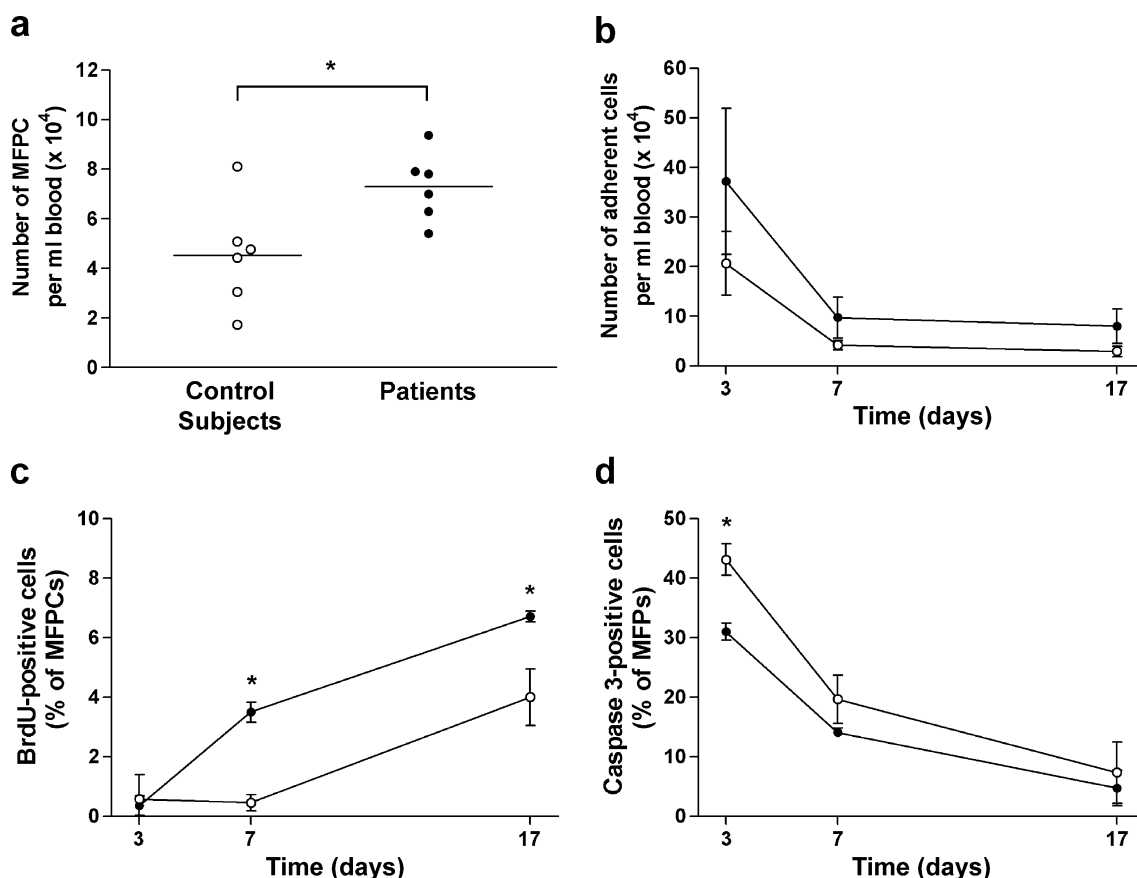


Fig. 2 The number of outgrowing MFPCs in patients with type 1 diabetes (*filled circles*) and healthy control subjects (*empty circles*). **a** At day 7 of culture, the number of MFPCs per ml blood was significantly higher in six patients with diabetes than in six age- and sex-matched control subjects. **b** The time course (days 3, 7 and 17) of six additional cultures showed consistently higher numbers of

MFPCs in diabetic patients ($n=3$) than in control subjects. **c** Cumulative proliferation of MFPCs was significantly higher in diabetes-derived cultures. **d** Apoptotic activity was significantly lower in diabetes-derived cultures. * $p<0.05$ for diabetes- vs control-derived cultures

Individual mRNA expression is given as \log_2 -transformed relative quantities adjusted to mean mRNA expression of the control group. (Relative $Q = 2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = \Delta Ct[\text{sample}] - \Delta Ct[\text{mean control}]$, $\Delta Ct = Ct[\text{target}] - Ct[\text{reference}]$.)

BMP6 and BMP7 protein measurement

BMP6 and BMP7 protein levels were measured in conditioned media of three control- and three diabetes-derived MFPC cultures after 3, 7 and 17 days by commercial sandwich ELISA using a paired set of anti-human BMP6 and BMP7 antibodies, respectively (R&D Systems, Abingdon, UK). Standards were diluted in DMEM containing 20% fetal bovine serum. The detection limit of both assays was 50 pg/ml.

Culture of human renal fibroblasts

To study the effects of BMP on TGF- β activity, we used the SV40-transformed human renal fibroblast cell line TK173 [28]. TK173 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin (Invitrogen). For gene expression experiments, cells were seeded at a density of 2×10^5 cells per well in six-well plates. After overnight culturing, cells were serum-starved for 12 h and stimulated with and without 1.5 ng/ml TGF- β 1, in the presence or absence of 50 ng/ml BMP6 and 200 ng/ml BMP7 (all purchased from R&D Systems). Effective concentrations of BMP6 and BMP7 were determined by readout of luciferase activity in TK173 cells transfected with a specific BMP response element construct coupled to a luciferase reporter. The observed EC50 values were similar to those provided in the data sheet by the manufacturer. After 4 h, total RNA was extracted, cDNA was synthesised and gene expression of PAI-1 and CTGF was determined by quantitative PCR using Assays-on-Demand Gene Expression Products (Applied Biosystems), as described above.

Statistical analysis

Differences in MFPC number, proliferation, apoptosis, BMP6 secretion, and mRNA expression were evaluated with Student's *t*-test or ANOVA with Bonferroni correction for multiple comparisons. Correlation between relative BMP6 mRNA expression and the number of MFPCs was assessed by linear regression. For all comparisons, a value of $p < 0.05$ was considered to be significant (two-tailed).

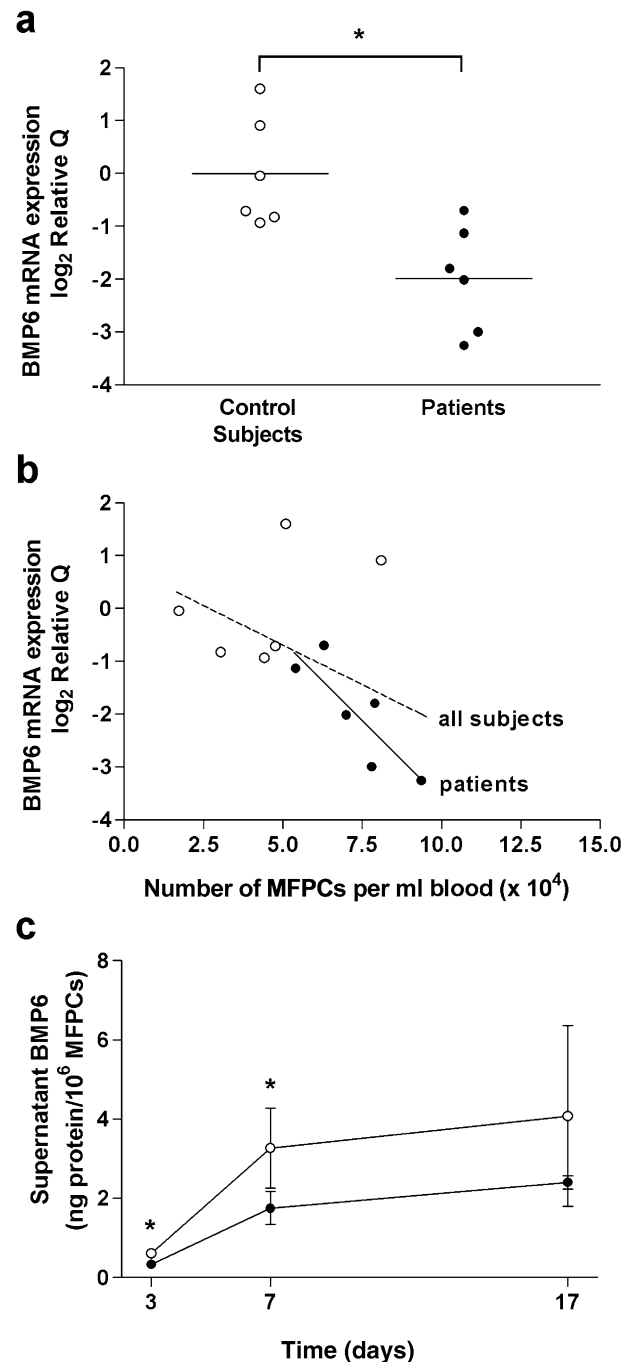


Fig. 3 **a** BMP6 mRNA expression in individual patients (filled circles) and control subjects (empty circles) was assessed by quantitative PCR and expressed as \log_2 -transformed relative quantities adjusted to the mean BMP6 mRNA expression of the control group (Relative Q). In patients with diabetes, \log_2 Relative Q was significantly lower than in control subjects. **b** Separate analysis of data from diabetes-derived MFPCs alone revealed a significant inverse correlation between BMP6 mRNA level and the number of outgrowing MFPCs (solid line; $r = -0.85$, $p < 0.05$). When these parameters were analysed in diabetic patients and control subjects together, a similar trend was observed, but this did not reach statistical significance (dashed line; $r = -0.48$, $p = 0.11$). **c** BMP6 in MFPC-conditioned medium was assessed by sandwich ELISA. Supernatant from diabetes-derived MFPCs contained significantly less BMP6 per 10^6 MFPCs than supernatant from control-derived MFPCs. Results are expressed as means \pm SD. * $p < 0.05$ for diabetes- vs control-derived MFPCs

Results

A subset of PBMCs cultured on fibronectin develops into myfibroblasts upon in vitro culture

In both control- and diabetes-derived cultures, adherent cells at day 3 were all negative for col I and α SMA, which are markers of the myfibroblast phenotype (data not shown). After 7 days of culture, most of the adherent cells had obtained a spindle-shaped, fibroblast-like morphology (Fig. 1a). In contrast to EPC cultures, which are known to be virtually all positive for uptake of DiI-acLDL and binding of *Ulex* lectin, MFPC cultures contained only a small number (<5%) of acLDL⁺-*Ulex* lectin⁺ cells (Fig. 1b). At day 7, approximately 70% of the control-derived adherent cells showed positive staining for col I and α SMA (Fig. 1c). After culture for an additional 10 days, virtually all adherent cells stained strongly positive for both col I and α SMA (Fig. 1d). In diabetes-derived MFPC cultures, cells at day 7 were more elongated than those in control-derived MFPC cultures, and approximately 90% were positive for col I and α SMA staining, compared with 70% in control-derived MFPC cultures (Fig. 1e). After 17 days of culture, all diabetes-derived adherent cells showed a myfibroblast phenotype (Fig. 1f).

The number of MFPCs is increased in patients with diabetes compared with control subjects

At day 7 of culture, the number of outgrowing MFPCs per ml blood was significantly higher in patients with type 1 diabetes than in normoglycaemic control subjects ($7.3 \pm 1.4 \times 10^4$ vs

$4.5 \pm 2.2 \times 10^4$, $p=0.007$; Fig. 2a). This difference was also significant if the number of outgrowing MFPCs was expressed relative to the number of initially seeded PBMC ($7.5 \pm 1.2\%$ in diabetic patients vs $4.1 \pm 2.2\%$ in control subjects, $p=0.008$).

To explore the possible contribution of proliferation and apoptosis to difference in MFPC numbers, three additional control- and diabetes-derived MFPC cultures were analysed after 3, 7 and 17 days. After 7 days, all three diabetes-derived cultures contained higher MFPC numbers per ml blood than those from the three control subjects ($9.7 \pm 4.1 \times 10^4$ vs $4.2 \pm 0.9 \times 10^4$; Fig. 2b). These observations were within the range of the initial experiment (significance level of accumulated data, $p=0.003$). The number of adherent cells in patients with diabetes tended to be higher already at day 3 of culture ($37.2 \pm 14.7 \times 10^4$ vs $20.7 \pm 6.4 \times 10^4$, $p=0.16$), although this was not significant with this low number of observations ($n=3$ per group). Cumulative proliferation of MFPCs was significantly higher in patients with diabetes after 7 days (3.5 ± 0.3 vs $0.5 \pm 0.3\%$, $p=0.01$) and 17 days (6.7 ± 0.2 vs $4.0 \pm 1.0\%$, $p=0.04$; Fig. 2c), while apoptotic activity was significantly lower in diabetes-derived MFPCs after 3 days of culture (31.0 ± 1.5 vs $43.2 \pm 2.7\%$, $p=0.03$; Fig. 2d).

BMP6 expression is reduced in diabetes-derived MFPCs

Of the genes present on the 19 K oligochip, those involved in TGF- β /BMP signalling pathways, including receptors, intra- and extracellular modulators, downstream mediators, transcription factors and known targets of TGF- β and BMP

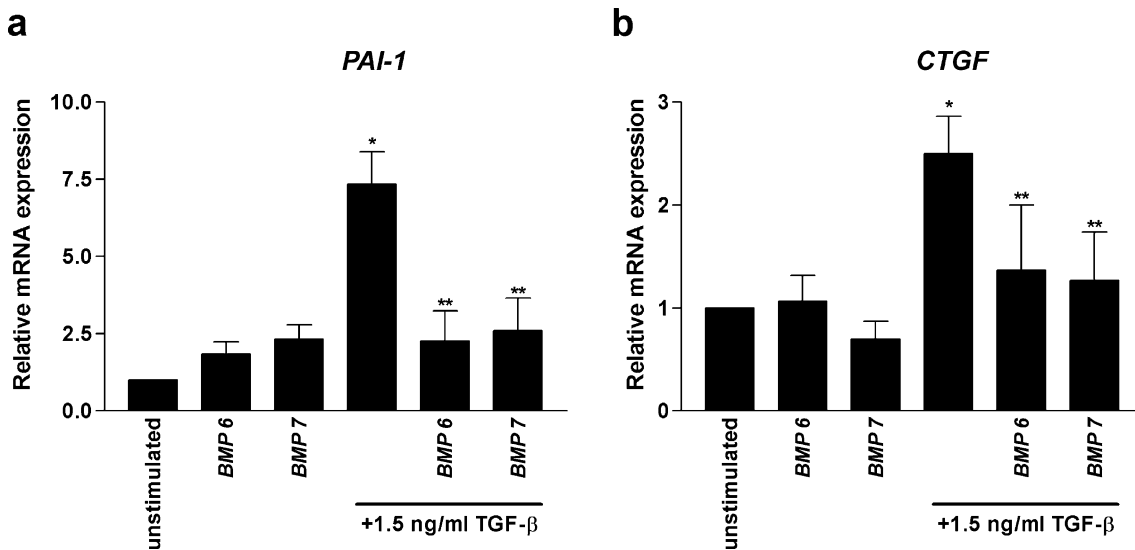


Fig. 4 Effects of BMP6 and BMP7 on the TGF- β -induced expression of *PAI-1* (now known as *SERPINE1*) and *CTGF* mRNA in renal fibroblasts. TK173 cells were cultured in serum-free medium, and stimulated with and without TGF- β 1 (1.5 ng/ml) in the presence and absence of BMP6 (50 ng/ml) and BMP7 (200 ng/ml). After 4 h of stimulation, total RNA was isolated and reverse-transcribed into cDNA for measurement of *PAI-1* and *CTGF*

levels by quantitative PCR. Treatment with TGF- β resulted in a 7.3-fold increase in *PAI-1* mRNA expression (a) and a 2.5-fold increase in *CTGF* mRNA expression (b). Addition of BMP6 or BMP7 equally reduced *PAI-1* (by 70%) and *CTGF* mRNA levels (by 50%). Results are expressed as means \pm SD of triplicate experiments. * $p < 0.05$ vs unstimulated condition; ** $p < 0.05$ vs stimulation with 1.5 ng/ml TGF- β

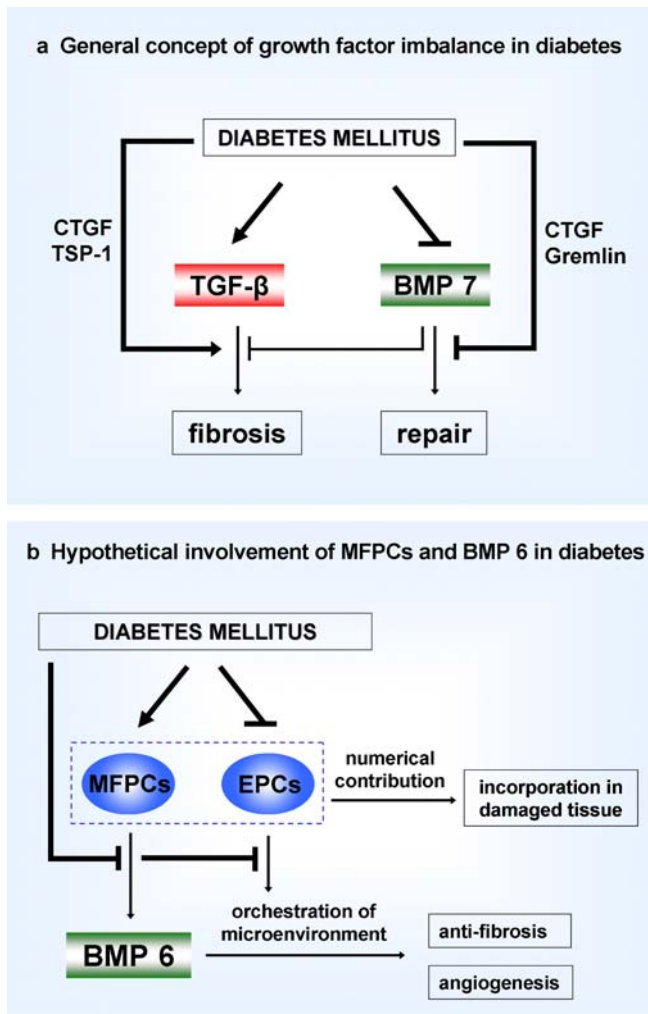


Fig. 5 Concept of growth factor imbalance affecting the contribution of MFPCs in diabetes mellitus. **a** Increased TGF- β signalling in diabetes, which is amplified by factors such as CTGF and thrombospondin-1 (*TSP-1*), results in fibrosis, while repair is hindered by the downregulation of BMP7 signalling, as well as the diabetes-induced BMP antagonists CTGF and gremlin. **b** The number of EPCs is decreased in diabetes, whereas the number of MFPCs is increased, along with the downregulation of *BMP6* expression. In view of the antifibrotic and pro-angiogenic effects of BMP6, we propose that the contribution of BMP6-deficient MFPCs in diabetes might involve inappropriate orchestration of resident cells in their microenvironment, in addition to fibroplasia due to increased numbers of infiltrating MFPCs per se

signalling, were identified by searching PubMed (<http://www.ncbi.nlm.nih.gov>) and the Biomolecular Interaction Network Database (BIND; <http://www.bind.ca>) [29–31]. Of all 426 genes thus identified, only *BMP6* and *GDF9* proved to be significantly regulated in both experiments. In MFPCs derived from patients with diabetes compared with MFPCs derived from control subjects, both *BMP6* and *GDF9* showed a mean downregulation of 1.7-fold.

To validate the microarray data, *BMP6* and *GDF9* mRNA levels were assessed by quantitative PCR. In pooled, amplified RNA samples, quantitative PCR showed 2.7-fold downregulation of *BMP6* and 2.6-fold down-

regulation of *GDF9* in MFPCs from patients with diabetes compared with control subjects. However, in samples from individual patients and control subjects, only *BMP6* mRNA expression proved to be significantly downregulated in diabetes-derived MFPCs (3.9-fold reduction, $p=0.007$; Fig. 3a), while mRNA expression of *GDF9* was particularly variable in both groups and not significantly different ($p=0.24$; data not shown). Separate analysis of data from only diabetes-derived MFPCs revealed a significant inverse correlation between *BMP6* mRNA level and the number of outgrowing MFPCs ($r=-0.85$, $p=0.03$; Fig. 3b). When these parameters were analysed in diabetic patients and control subjects together, a similar trend was observed, but this did not reach statistical significance ($r=-0.48$, $p=0.11$).

Measurement of BMP6 protein in culture supernatants by ELISA revealed that diabetes-derived MFPC conditioned media contained approximately 45% less BMP6 protein per 10^6 cells than supernatants of control-derived MFPCs ($p=0.04$; Fig. 3c).

BMP7 was below the detection limit of microarray, quantitative PCR and ELISA.

BMP6 inhibits TGF- β -induced CTGF and PAI-1 mRNA expression in human renal fibroblasts

Treatment of TK173 cells with 1.5 ng/ml TGF- β resulted in a 7.3-fold increase in *PAI-1* (now known as *SERPINE1*) mRNA expression and a 2.5-fold increase in *CTGF* mRNA expression. Addition of BMP6 (50 ng/ml) or BMP7 (200 ng/ml) equally reduced *PAI-1* and *CTGF* mRNA levels by approximately 70 and 50%, respectively (Fig. 4).

Discussion

The two main findings of the present study are the increased number of outgrowing MFPCs obtained from peripheral blood of patients with diabetes and the decrease in BMP6 expression in diabetes-derived MFPCs. Taking into account that diabetes- and control-derived MFPCs were cultured under identical conditions in the presence of 20% fetal bovine serum, these are remarkable findings, which suggest robust alternative programming of cPCs in these diabetic patients. It would be interesting to know whether an increased number and altered gene expression of MFPCs in diabetes is already evident in freshly drawn blood. However, in contrast to EPCs, which express CD34, CD133 and kinase insert domain receptor (KDR) in peripheral blood and could therefore be isolated directly from the circulation [32], no such specific markers are known to exist for circulating MFPCs. Thus far, the only way to obtain MFPCs is by ex vivo cell culture. Hence, it is not possible to analyse the number of MFPCs in freshly drawn blood.

The increased number of MFPCs in cultures derived from patients with diabetes might be related, at least in part, to the lower apoptotic activity and increased proliferation

observed in diabetes-derived MFPC cultures. Interestingly, a reduced proliferation rate and augmented apoptotic activity has been observed in cultures of control-derived EPCs under hyperglycaemic conditions [33], which could explain the remarkable observation of increased MFPCs in contrast to decreased EPCs in diabetes. The precise origin of MFPCs is still unclear. We observed that MFPCs were negative for acLDL uptake and *Ulex* lectin staining. Interestingly, in vitro EPCs, which are acLDL⁺-*Ulex* lectin⁺ cells, can be derived from monocytes/macrophages [34]. Whether MFPCs can also be derived from monocytes/macrophages remains to be established.

The differential gene expression in diabetes- vs control-derived MFPCs suggests robust programming in patients with diabetes, since it appeared to be resistant to the expected equalising effect of prolonged culture under identical conditions in 20% fetal bovine serum. Similarly, prolonged maintenance of aberrant function/gene expression in patients with diabetes has been observed in EPC cultures [12] and in cultured skin fibroblasts [35]. Furthermore, others have shown that bone marrow-derived mesangial cell progenitors are able to transmit a disease phenotype to normal glomeruli [36] and also play a crucial role in the development of diabetic nephropathy [37].

The origin and nature of aberrant programming of gene expression in diabetes is not known. One possibility is that this relates to growth factor imbalance in vivo, which is also involved in the misdirection of the tissue response to injury in the diabetic condition. In particular, TGF- β activity is increased in diabetes, whereas the BMP7 pathway is downregulated (Fig. 5a). The increased TGF- β production and signalling, which is further amplified by, for example, CTGF and thrombospondin-1, results in fibrosis [13, 15, 22, 38], while the maintenance and repair of functional tissue is hindered by the downregulation of BMP7 signalling, which is due to reduction of ligand and receptor expression by the diabetic condition, as well as to the diabetes-induced BMP antagonists CTGF and gremlin [24, 39].

To examine whether gene expression in diabetes-derived MFPCs is indicative of TGF- β /BMP signalling imbalance, we performed microarray experiments followed by quantitative PCR validation. Data analysis was focused on genes related to TGF- β /BMP superfamily members, i.e. inducers, ligands, receptors, modulators, mediators and targets. Of 426 genes identified, according to our analyses, only *BMP6* was significantly (down)regulated in diabetes-derived MFPCs. The observed downregulation of *BMP6*, which was evident also at the protein level, is of particular interest because of the pivotal role of BMP6 in angiogenesis [40]. This is an interesting analogy with the release by EPCs of potent pro-angiogenic growth factors [34], which supports the hypothesis that the in vivo angiogenic activity of EPCs may include a role in the orchestration of their microenvironment. The expression of the angiogenic factor BMP6 by MFPCs (which is reduced in diabetes) might suggest similar involvement of these cells in the tissue response to injury.

Although BMP7 has received much attention as a renoprotective factor in diabetes and other conditions, no reports are available concerning a possible role of BMP6 regulation in diabetes. However, BMP6 and BMP7 are structurally very similar and both act as specific ligands for the BMP-receptor activin receptor-like kinase-2 (ALK-2), unlike other BMPs [29]. Furthermore, crosstalk between BMP6 and TGF- β has been observed [41], and BMP6 has the capacity to substitute for BMP7 in kidney development [42]. We demonstrate here for the first time that, like BMP7, BMP6 antagonises profibrotic TGF- β activity by decreasing *PAI-1* and *CTGF* expression in renal interstitial fibroblasts. We observed comparable antagonistic effects on TGF- β activity in mesangial cells and tubular epithelial cells (data not shown).

BMP7 expression was below the detection limit of microarray, quantitative PCR and ELISA in our MFPC cultures. These data suggest that, as far as MFPCs are concerned, BMP6 rather than BMP7 expression is aberrantly regulated in diabetes. Interestingly, decreased BMP6 expression has also been observed in (myo-) fibroblasts obtained from fibrotic Dupuytren's lesions compared with fibroblasts harvested from normal palmar fascia [43]. BMP7 expression in (myo-)fibroblasts was below the detection limit in that study, as was the case for MFPCs in the present study. Since BMP6 levels were still very low in culture supernatant of MFPCs at day 3 (which had not yet obtained the myofibroblast phenotype), we propose that BMP6 expression occurred mainly during or after the acquisition by the cells of a mesenchymal phenotype. In agreement with this notion, BMP6 expression in the developing kidney is mainly present in loose mesenchymal stromal cells, in contrast to BMP7, which is expressed in epithelial cells [44].

Together with the known angiogenic effect of BMP6 on endothelial cells, our novel observations suggest that the contribution of MFPCs in diabetes might involve the inappropriate orchestration of resident cells in their microenvironment, in addition to fibroplasia due to increased numbers of infiltrating MFPCs per se (Fig. 5b). According to this concept, aberrant programming of cPCs would act as a double-edged sword in the adverse remodelling of damaged tissue in diabetes.

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