Eur J Med Res (2004) 9: 337-344

Effects of Platelet Growth Factors on Human Mesenchymal Stem Cells and Human Endothelial Cells in vitro

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Abstract: The aim of the present in vitro study has been to investigate the effects of a enriched platelet derived growth factors on proliferation and migration of human endothelial and mesenchymal stem cells and on osteogenic differentiation of stem cells. Platelet rich plasma has been produced, yielding a four time higher number of thrombocytes than normal plasma. Degranulation of platelets has been performed by means of calcium and thrombin. Plasma has served as a control, whereas plasma in combination with calcium and thrombin was used to distinguish the difference between calcium and/or thrombin mediated effects and growth factor induced effects on the cells. The observed enhanced proliferation and migration of endothelial cells towards the platelet derived growth factors was driven by the plasma component of these preparations. However PDGF solely stimulated the migration and proliferation of mesenchymal stem cells. The increased osteogenic differentiation of growth factor treated mesenchymal stem cells was mostly driven by the high level of calcium used for the platelets degranulation. In summery, the different components of platelet derived growth factors work together to influence human endothelial and mesenchymal stem cells. This is of special clinically interest regarding the stimulation of bone healing in orthopaedic and traumatic surgery.

Key words: Platelet growth factor, PDGF, TGF-beta, endothelial cell, mesenchymal cells

Introduction

The reconstruction of bone defects continues to be an evolving discipline in orthopaedic, trauma and maxillo-facial surgery. Growth factors by single application such as recombinant bone morphogenetic proteins, accelerate and enhance newly bone formation. The major source of autologous growth factors, including platelet derived growth factor (PDGF), transforming growth factor-beta (TGF-beta), insulin-like growth factor (IGF) and epidermal growth factor (EGF) are localized in platelets. Growth factors are released upon activation of thrombin and calcium and degranulate at fracture site in response to injury and inflammation. Platelet-rich plasma, PRP, is obtained by sequestration and concentration of platelets by gradient density centrifugation, which produces a concentration of about 400% of normal platelet counts in blood [37] . PRP has been proven to be an effective valuable accessory in wound healing and new bone formation in maxillofacial surgery [2, 8, 11, 12, 18, 23, 37, 44]. Applying thrombin and Ca^{2+} ions to platelet rich plasma induces a significant release of growth factors resulting in a concentration of platelet factor (PLF). Growth factors are involved in cellular proliferation, differentiation and morphogenesis of tissues and organs during embryogenesis and postnatal growth. PDGF in human serum is known to be the major mitogen for mesenchymal cells and endothelial cells [41, 43]. The major target of transforming growth factor-beta are fibroblasts, marrow stem cells and preosteoblast, in which TGF-beta act as chemoattractant and mitogen. TGF-beta has been observed to both inhibit and stimulate osteoblastic cell proliferation in vivo, depending on its concentration, cell density, species and the stage of osteoblast differentiation [7]. IGF stimulates osteoblastic cell proliferation and differentiation [33], and recent studies have shown that IGF infusion increased bone formation and bone volume in normal rats [45]. EGF is a strong mitogen for mesodermal and endothelial cells, and acts as a differentiation factor for different cell lines. The mitogenic activity of EGF for endothelial cells can be potentiated by thrombin [20]. EGF is also a chemoattractant for fibroblasts and endothelial cells and, either alone or in combination with other cytokines is a mediator for wound healing processes. Furthermore, it is able to down-regulate the expression of TGF-beta receptor, so that it can be assumed that some activities are indirect [36]. Concentrated platelet factors preparation comprises not only the effect of one growth factor, but affects every special growth factor in the presence of other growth factors, resulting in a cascade of signals, leading to induction or enhancement of proliferation, differentiation and migration. The purpose of this study was to investigate the effects of the cytokines in PLF on two cell population, endothelial cells and mesenchymal stem cells, which play a major part in the process of bone healing. Therefore, proliferation and migration of human mesenchymal and endothelial cells and the effect of PLF on differentiation of mesenchymal cells into osteoblasts were analyzed.

Material and Methods

Preparation of Plasma and Platelets Factors (PLF)

500 ml of peripheral blood were centrifuged at 100 x g for 10 min at room temperature. Platelet rich plasma was withdrawn and again centrifuged at 300 x g for 10 min. Plasma was then withdrawn and centrifuged at 1000 x g for 30 min. The plasma fraction was withdrawn and filtered through a filter with the pore size of 0.45 µm (Millipore, Eschborn, Germany) to remove resident platelets. The platelet-rich plasma supernatant was carefully squeezed into a pre-connected bag for further preparation of the growth factors. 1000 IE thrombin (Gentrac Inc., Middleton, USA) and 10 ml 8.4 % calcium-gluconate (Braun, Melsungen, Germany) were added for aggregation and degranulation of the platelets. After degranulation the liquid supernatant was filled into tubes and stored at –20 °C. Plasma was heat-inactivated at 65 °C for 30 min and again centrifuged at 1000 x g for 30 min. The liquid supernatant was then filled into tubes and stored at – 20 ° C.

Measurement of Cytokine Concentration

Commercially available ELISA kits for PDGF, TGF-beta, VEGF, bFGF, IGF and EGF were used (Quantikine™, R & D Systems, Minneapolis, MN, USA) for the measurement of cytokine concentration. The beta-thromboglobulin concentration was measured by asserachrom beta-TG-ELISA (Diagnostica Stago, Asnières-sur-Seine, France).

Migration Assay

Tissue culture inserts (NUNC) in 24-well size with 8 µm-sized pores were used for assessment of cell migration. Polycarbonate membranes were coated with bovine collagen type 1 (0.1mg/ml; Biochrom) in 0.2 N acetic acid according to the following procedure. Inserts were soaked overnight in 0.5 M acetic acid. The membranes were rinsed with PBS. Collagen type I was diluted in 0.2 N acetic acid to a final concentration of 0.1 mg/ml. The inserts were soaked in collagen solution for 30 min and air dried. Low passage number HUVEC (passage 3-5) were cultured in EBM-2 (Biowhitaker) until migration assay. Mesenchymal stem cells were cultured in an Alpha medium (PAA) including 20% FBS (Invitrogen) until migration assay. Cells were suspended in migration assay medium, M199 (Sigma) plus 1% FBS, to a cell density of 4 x 10^5 cells/ml and added on top of the membranes. Migration assay medium plus indicated volumes of platelet factors were filled in the bottom chamber. Assembled plates were incubated for 4 hours at 37 °C in 5% CO_2 without any further disturbance. Afterwards, the filters were removed and stained with hematoxylin solution (Sigma). Non-migrated cells on the top of the membranes were removed by wiping with tissue. Density of stained filters were quantified using the Quantity One® Quantitation Software from BIO-RAD.

Calcium Assay

Cell layers were washed twice with Tyrodes salt solution (Sigma) and fixed with aceton-3% glutaraldehyd (Sigma) for 30 min at room temperature. Afterwards, cell layers were washed twice with deionized water and air dried. The calcium was extracted from cell layers by shaking overnight at room temperature with 1 ml of 0.69 N HCl solution. The resulting supernatant was used for quantitative calcium determination according to the manufacturer (Sigma). Absorbance of samples was read at 575 nm. Total calcium was calculated from standard solutions prepared in parallel, and expressed as µg per dish.

Von Kossa Staining

For the measurement of the mineralization potential wells were washed twice with Tyrodes salt solution and then fixed with aceton-3% glutaraldehyd for 15 min at room temperature. Subsequently, wells were washed three times with deionized water and 1ml of 2% silver nitrate solution was added. Cell layers were incubated for 10 min in the dark. Plates were rinsed three times with deionized water and exposed to UV light under continuous application of deionized water to avoid drying for 15 min. After that wells were washed for three times with deionized water and 1 ml of 100% ethanol was added. Plates were kept at 4 °C.

Proliferation Assay

6.2 x 10^3 mesenchymal stem cells were plated in 24 well culture dishes and incubated for 7 days with indicated concentrations of platelet factors. Subsequently, culture medium was removed and cells were incubated for 3 h with a 1:20 dilution of Alamar blue‘ (Biosource) in MSCBM-medium (Biowhitaker). Fluorescence was measured at 585 nm after excitation with 485 nm.

1.0 x 104 endothelial cells were plated in 24 well culture dishes with EBM-2MV medium and incubated overnight at 37 °C, 5% CO_2. The next day, the medium was exchanged to M199/1% FCS with determined concentrations of platelet factors, plasma or a mixture of plasma, calcium and thrombin and cultured for another 3 days. Cells were then incubated for 3 h with 100 µl Alamar blue™ (Biosource) and added to the cultures. Fluorescence was measured at 585 nm after excitation with 485 nm.

Osteogenic Differentiation

6.2 x 10^3 mesenchymal stem cells were plated in 24 well culture dishes in normal growth medium. 24 h later, culture medium was removed and cells were cultured in 600 µl osteogenic growth medium for a period of four to five weeks at 5% CO_2 and 37 °C. The osteogenic growth medium consisted of DMEM low glucose plus 10 % FBS, 0.1µM dexamethasone, 0.05 mM ascorbic acid-2-phosphate and 10mM β-glycerophosphate (all chemicals from Sigma). Medium was exchanged every three to four days.

Preparation of Mesenchymal Stem Cells

Mesenchymal stem cells were either prepared from frozen bone marrow mononuclear cells (Biowhitaker) or purchased from Biowhitaker. Bone marrow mononuclear cells were thawed and plated in T75 flasks in MSCGM-media (Biowhitaker). Medium was exchanged the next day. When the cultures reached 90% of confluence, cells were recovered by the addition of a solution containing 0.25% trypsin-EDTA (Biowhitaker). All cultures were positive for CD44, CD73, CD105, CD166 and CD29 and could differentiate into adipocytes, chondrocytes and osteocytes.

Statistical Analysis

Statistical analysis was performed with the independent t-test. Confidence levels of <95% ($p>0.05$) were considered not insignificant.

Results

Platelet Count and Growth Factors

The concentration of platelets after enrichment was four times higher than in blood (Table 1). Degranulation with calcium and thrombin led to the release of TGF-beta 1, PDGF AA, PDGF BB, PDGF AB, EGF, IGF I and II. Predominant growth factors in the PLF-preparations are TGF-beta (69087 pg/ml) and PDGF AB (162164 pg/ml). Neither bFGF and VEGF could be detected in the preparations.

Table 1. Platelet count (gpt/ml), growth factor concentration (pg/ml)and beta-thromboglobulin (IU/ml) in platelet rich plasma (platelet count in blood: 267300 +/- 33100 gpt/ml), n.d. no detectable

	platelet rich plasma	**plasma** [lit.]
platelet count	1021900 ± 345956	
TGF beta1	69087 ± 20418	410 -4930 [24, 29]
PDGF AA	639 ± 423	
PDGF BB	578 ± 486	
PDGF AB	162164 ± 68466	165.9 ± 119.1 [15]
VEGF	n.d.	0.39 ± 0.26 [42]
EGF	311.8 ± 221.6	0.75 ± 0.10 [1]
bFGF	n.d.	1.89 ± 1.30 [31]
IGF-I	57.42 ± 19.6	
IGF-II	284	
thromboglobulin	28814 ± 8406	

Influence of Platelet Factors on Proliferation and Migration of Endothelial Cells

Addition of platelet factors enhanced the proliferation of endothelial cells in a dose dependent manner. Platelet factors are prepared by the induced degranulation of plasma suspended platelets through addition of thrombin and calcium. In order to determine the effect solely driven by the action of the released platelet factors, we tested the influence of plasma and the combination of plasma, thrombin and calcium on the proliferation of endothelial cells. Both additives stimulated the proliferation of the endothelial cells as well as the platelet factors in a similar manner (Fig. 1). Enhanced migration of human endothelial cells towards control medium was also observed with platelet factors, plasma and also with plasma/calcium/thrombin (Fig. 2A+B). Observed migration here was stronger than migration induced by the addition of 10 ng/ml VEGF (data not shown). There was no significant difference between platelet factors, plasma or the combination of plasma, calcium and thrombin.

Influence of Platelet Factors on Proliferation and Migration of Mesenchymal Stem Cells

Since differentiation of mesenchymal stem cells to osteocytes occurs during bone healing we kept these stem cells during a period of seven days under conditions that induce osteocyte differentiation. The addition of plasma did not alter stem cell proliferation in comparison to control cultures (Fig. 3). Addition of

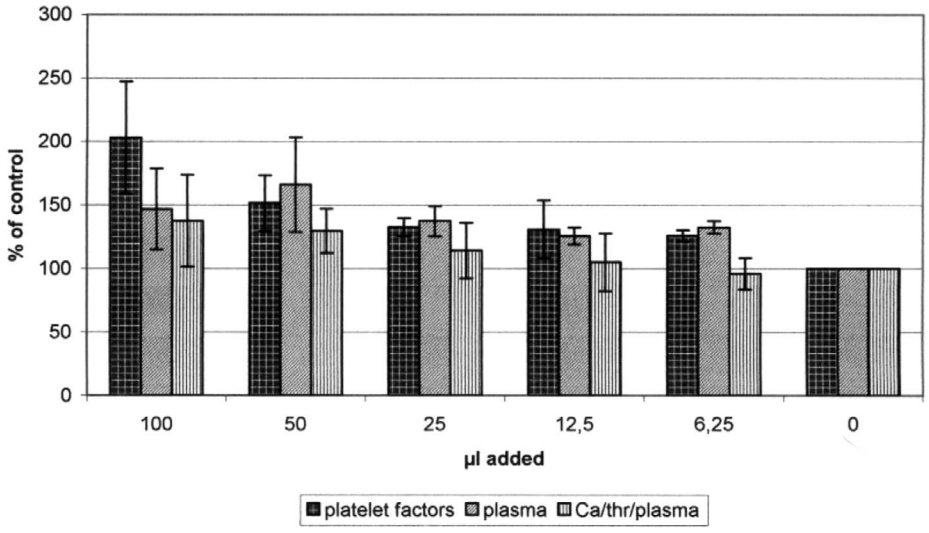
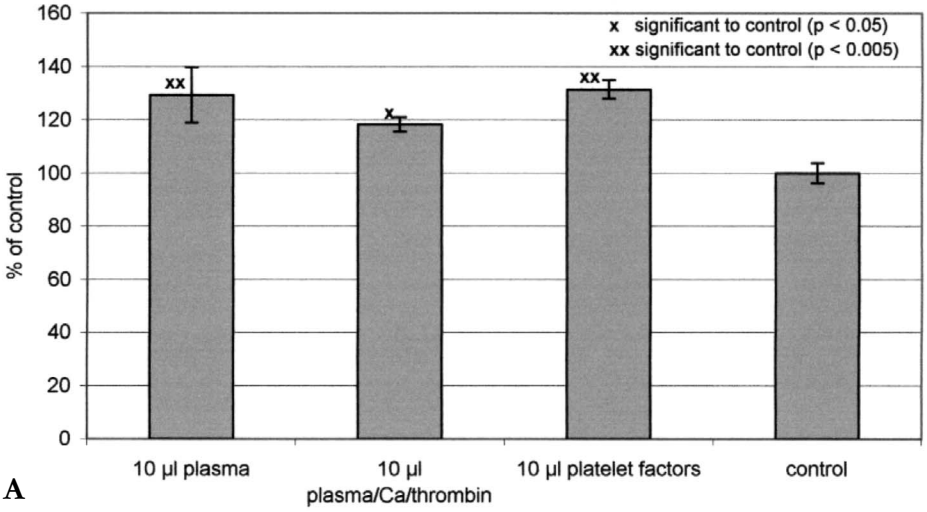


Fig. 1. Proliferation of endothelial cells in the presence of platelet factors. Endothelial cells were cultured for 4 days in the presence of indicated concentrations of platelet factors, plasma with or without calcium and thrombin in medium 199 with 1% FBS. The figure shows the mean ± S.D. of three independent experiments expressed as percent of the unstimulated control.



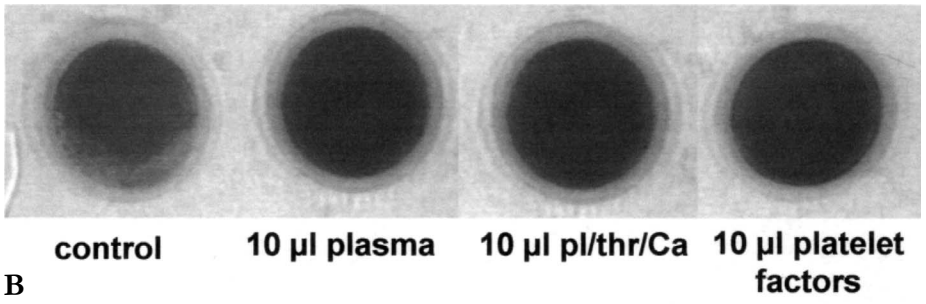


Fig. 2. Migration of endothelial cells in the presence of platelet factors. A: Hemotoxylin stained membranes after migration of endothelial cells towards 10 µl of plasma, 10 µl of the mixture of plasma, calcium and thrombin and 10 µl platelet factors. B: Density of stained membranes expressed as percent of control (migration assay medium without any additives). The figure shows the mean ± S.D. of three independent experiments.

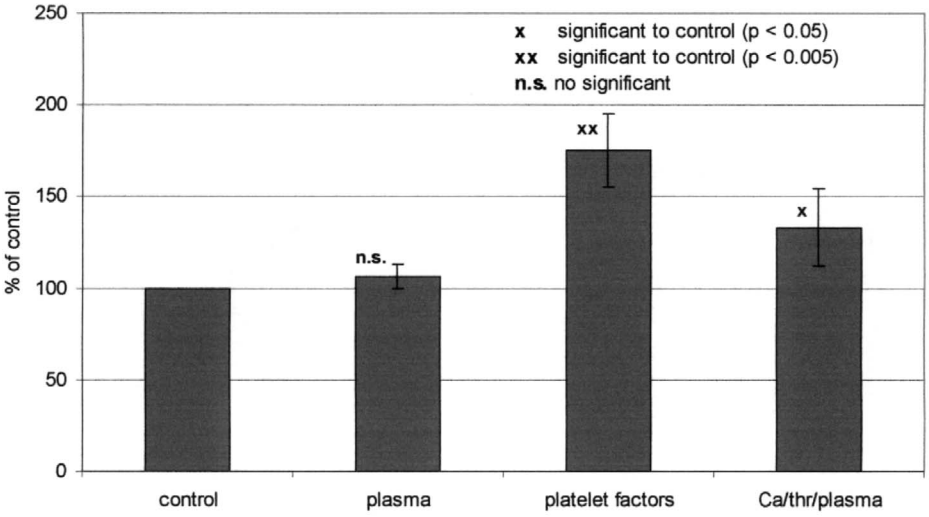


Fig. 3. Proliferation of mesenchymal stem cells in the presence of platelet factors. Mesenchymal stem cells were cultured for 7 days in the presence of 20 µl of platelet factors, plasma with or without calcium and thrombin in 600 µl osteocyte induction medium. The figure shows the mean ± S.D. of four independent experiments expressed as percent of the unstimulated control.

the combination of plasma, thrombin and calcium slightly enhanced proliferation (133.0 ± 21.17% of control). However remarkable enhancement of proliferation was seen in cultures with platelet factors (175 ± 19.7 % of control).

During the process of bone healing the migration of osteocyte precursor cells to the places where bone healing should occur is required. Therefore, we analyzed the chemotactic effect of platelet factors on mesenchymal stem cells in a migration assay. Indeed the presence of platelet factors significantly attracted mesenchymal stem cells (134.2 ± 1.8% of control), whereas neither plasma nor the combination of plasma, calcium and thrombin enhanced their migration in comparison to control (Fig. 4 A+B).

Influence of Platelet Factors on Differentiation of Mesenchymal Stem Cells

Differentiation capacity of mesenchymal stem cells into osteocytes is tested by cultivation of these cells in osteocyte induction media which provide optimal growth conditions for differentiation of these cells. We confirmed the hypothesis that platelet factors could further improve the osteocyte differentiation of mesenchymal stem cells. Therefore, osteocyte differentiation of mesenchymal stem cells was performed in the presence of platelet factors, plasma or the combination of plasma, calcium and thrombin. Starting of mineralization could be already detected after 10 days in cultures with platelet factors and with the mixture of

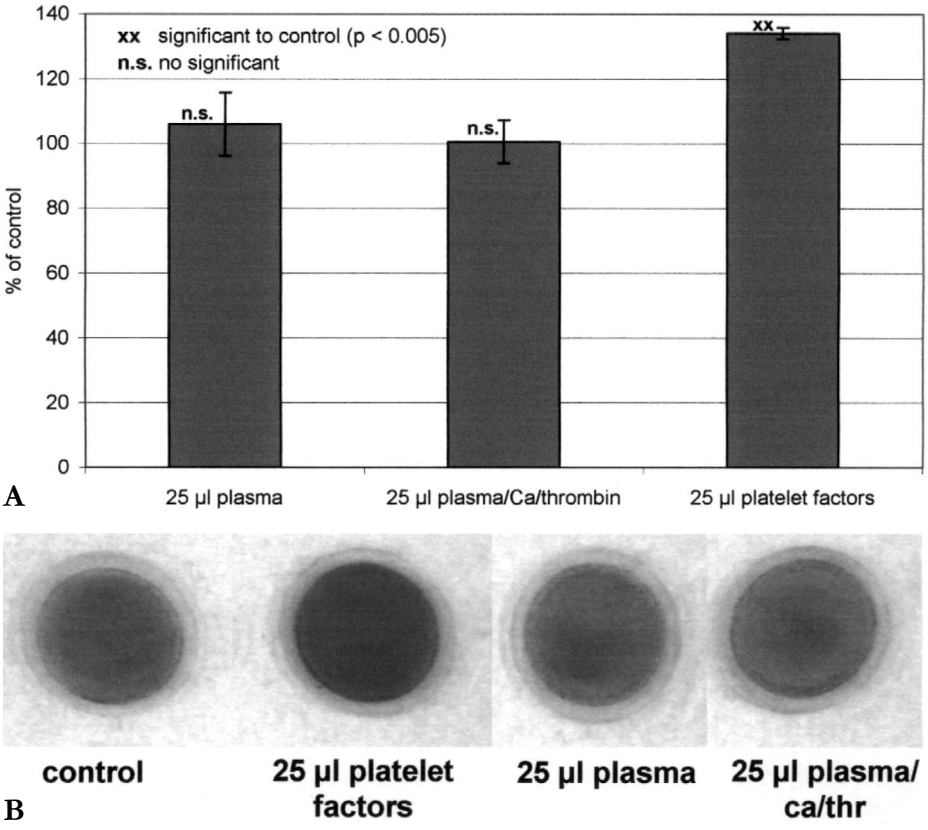


Fig. 4. Mesenchymal stem cells migrate towards platelet factors. A: Hemotoxylin stained membranes after migration of mesenchymal stem cells towards 25 µl of plasma, 25 µl of the mixture of plasma, calcium and thrombin and 25 µl platelet factors. B: Density of stained membranes expressed as percent of control (migration assay medium without any additives). The figure shows the mean ± S.D. of three independent experiments.

calcium, plasma and thrombin, whereas no signs of mineralization could be detected at this time in control cultures and in plasma incubated cultures (Fig. 5A). Cultures were continued for 3-4 weeks and analyzed for calcium content and stained with von Kossa staining. Extracted calcium levels were highest in cultures that were treated with the combination of plasma, calcium and thrombin followed by the cultures treated with platelet factors. Whereas in the presence of plasma there was no detectable increase in the calcium content in compare to control cultures. Von Kossa staining of these cultures confirmed the obtained results (Fig. 5B).

Discussion

In the present in-vitro study the effects of platelet factors concentrate (PLF) on proliferation and migration capacity of human mesenchymal stem cells and human endothelial cells has been investigated. Moreover, the influence of platelet factor concentrate on the differentiation from mesenchymal stem cells into osteoblasts was examined.

Immediately after preparation, the number of thrombocytes was four times higher in platelet-rich plasma than in blood, as shown in Table 1. Degranulation of platelets by thrombin and calcium significantly increases the concentration of the growth factors PDGF, and remarkably the isoform PGDF-AB, TGF-beta1 and EGF compared with the levels of these cytokines found in normal plasma [9, 37, 47]. However, neither VEGF nor bFGF could be detected in measurable concentration in the PLF. This might be due to the fact, that VEGF and bFGF are synthesized, especially in hypoxia in case of injury or during development, but not in normal homeostasis [4].

The proliferation assay of endothelial cells (Fig. 1) revealed no significant differences after incubation of the cells with PLF, the combination of plasma/calcium/thrombin or plasma. Possibly the mitogenic activity of different PLF factors on endothelial cells are more or less neutralized by the inhibiting effect of the high amount of TGF-beta1 (69 ng/ml) in the PLF. TGF-beta inhibits proliferation of endothelial cells when applied in high doses [27, 28, 39]. In contrast, TGF-beta1 concentration of 0.5ng/ml stimulates EC-proliferation in cell culture environment. The plasma concentrations of TGF-beta are up to approximately 4 ng/ml which might explain the stimulatory capacity of plasma in our experiments. Moreover, interactions of PDGF-AB, which is highly concentrated in PLF, with the PDGF receptor _ might explain the lacking additional effect of PLF on the proliferation of endothelial cells. The PDGF receptors alpha and beta induce mitogenic signal to a different extent. PDGF receptor beta, which is known to be the major transducer for proliferation [5, 21, 30], binds the PDGF isoform BB and partially PDGF isoform AB, which presents the largest isoform in platelet factor concentrate, whereas PDGF receptor alpha, whose effects on proliferation are much lower, mainly binds PDGF-AA [16, 38]. Additionally, De Marchis [17] reported an inhibitory effect of PDGF-BB induced by interaction with the PDGF receptor alpha on bovine aorta endothelial cells.

Migration of endothelial cells was significantly enhanced by the addition of platelets factors, the combi-

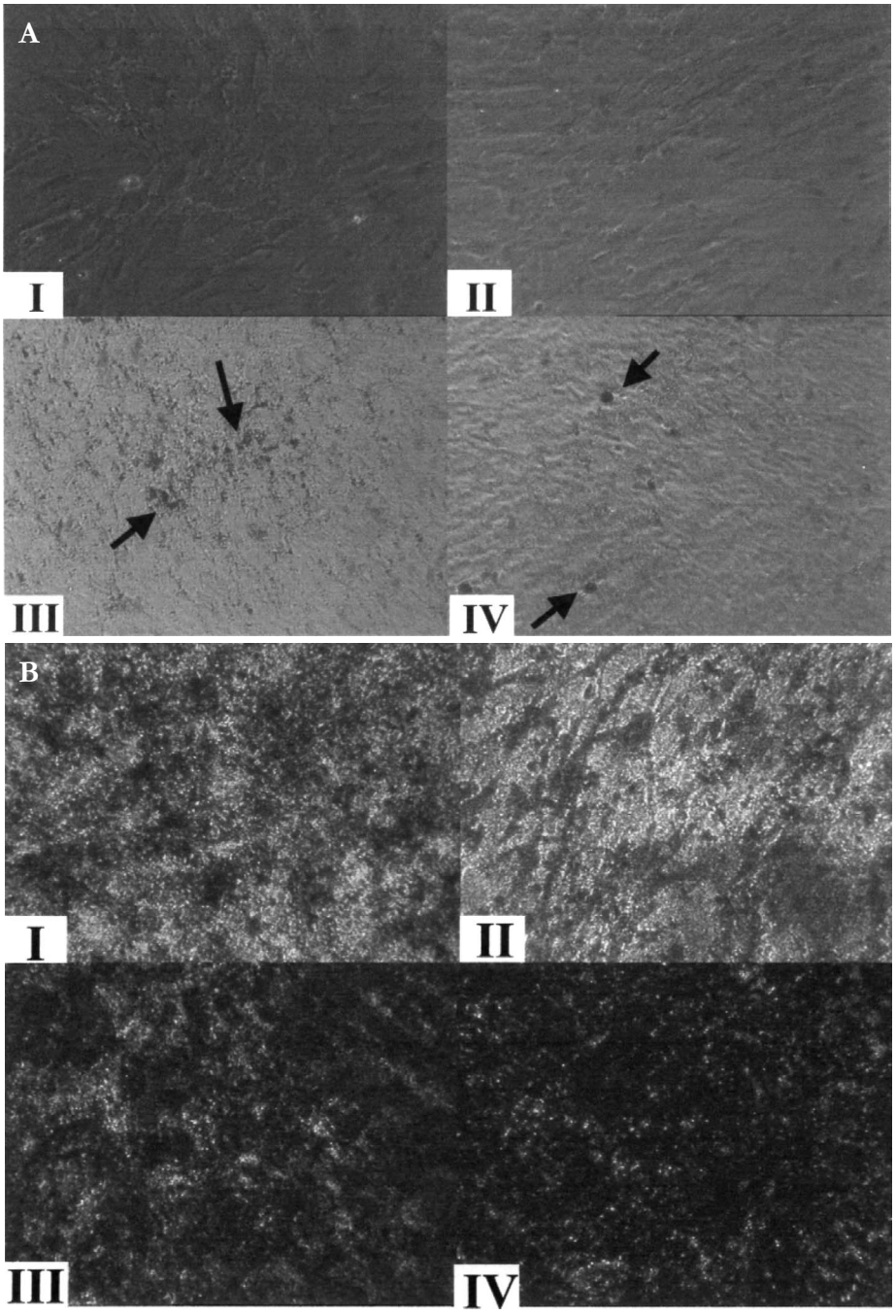


Fig. 5. Mineralization of mesenchymal stem cell cultures in the presence of platelet factors. A: Morphology of mesenchymal stem cell cultures after 10 days in I) osteocyte induction medium, II) osteocyte induction medium + 20 µl of plasma, III) osteocyte induction medium + 20 µl of platelet factors, IV) osteocyte induction medium + 20 µl of plasma, calcium and thrombin. The arrows indicate the beginning of mineralization. B: Von Kossa staining of mesenchymal stem cell cultures after 30 days in I) osteocyte induction medium, II) osteocyte induction medium + 20 µl of plasma, III) osteocyte induction medium + 20 µl of platelet factors, IV) osteocyte induction medium + 20 µl of plasma, calcium and thrombin. Magnification 200x.

nation of plasma/calcium/thrombin and plasma in comparison to control, but the presence of PLF did not alter the migratory activity in comparison to plasma or the mixture of thrombin, calcium and plasma (Fig. 2). There are several reasons which might explain this lacking effect of PLF. 1) The concentration of the most potent chemotactic factor for endothelial cells in PLF which is VEGF is not higher as in normal plasma. 2) The concentration of bFGF in PLF which was also described to have chemotactic activity for endothelial cells [17] is also not higher in the PLF preparations than in plasma. 3) The most predominant factor in PLF is PDGF-AB, however probably due to its binding to the PDGF receptor alpha it inhibits the migratory activity of the endothelial cells.

Proliferation activity of mesenchymal stem cells increased significantly after incubation of the cells with platelet factors and plasma/calcium/thrombin (Fig. 3). However the highest stimulation of mesenchymal cell proliferation was obtained by application of PLF. These data are in agreement with the publication of Gruber et al [26] and Lucarelli et al [35].

The existence of the growth factors TGF-beta and PDGF in the PLF preparations may explain this potent stimulation of proliferation. Accordingly, Cassiede et al. [13] found in vitro proliferative effects of TGF-beta1 and of PDGF-BB on mesenchymal stem cells at concentrations of 5 ng/ml. In the current study, approximately 0.5 ng/ml PDGF-BB and approximately 16 ng/ml PDGF-AB were added to the cultures via the application of PLF. Investigations by Lennon et al.

[32] also showed a stimulating effect of PDGF-BB on the proliferation of mesenchymal stem cells. The impact of PDGF-AB on the proliferation of mesenchymal stem cells is unknown. A synergic effect to PDGF-BB through coupling to PDGF receptor-beta seems to be most likely [19].

Thrombin [46] and calcium might be responsible for the increase of proliferation rates after addition of plasma/thrombin/calcium compared to plasma.

Migration of mesenchymal stem cells was significantly stimulated after treatment on the cells with platelet factors (Fig. 4). The high amounts of PDGF-AB found within the PLF might be responsible for the chemotactic activity of the PLF on mesenchymal stem cells. TGF-beta and bFGF had no influence. PDGF-BB is a crucial factor for in vitro migration of mesenchymal stem cells. Fiedler et al. [22] reported an effect of PDGF-BB on chemotaxis in concentrations of 0.001 -1.0 ng/ml. This effect is approximately eight fold stronger than chemotactic effects of bone morphogenetic protein 2 and 4. However the main factor in PLF is PDGF-AB. Coupling is done via PDGF receptor-beta to which PDGF-AB also binds partially [16, 38].

Pluripotent mesenchymal stem cells exist in bone marrow and differentiate osteogenic in culture medium by application of beta-glycerolphosphate, ascorbic acid and cortison [40]. Differentiation of human mesenchymal stem cell to osteoblast takes place in stages of osteogenic progenitor cell and preosteoblast [10, 25].

Regarding the temporal pattern of mineralization, the first signs became evident within the osteocyte differentiation medium (control) after 4 weeks. Interestingly, due to the treatment with platelet factors and with plasma/thrombin/calcium fraction, mineralization could already be detected after 10 days, as shown by the typical morphology of osteocytes and clusters of mineralization. After 30 to 35 days, a significant increase of calcium deposits was seen in response to platelet factor. However, as significant enhancement of mineralization which was higher than induced by the treatment with platelet factors was seen after the incubation of the cells with the plasma/calcium/thrombin preparation. These observations indicate that the osteogenic differentiation is partially inhibited by the presence of PLF. This is in accordance to Gruber et al. [26], who described a decrease of osteogenic differentiation of human mesenchymal stem cells by application of a PLF. prepared with thrombin used for cytokine release. In addition, Arpornmaeklong et al. [3] reported a dose-dependent inhibition of osteogenic differentiation by the addition of PLF in comparison to a combination of plasma, calcium and thrombin. However observed mineralization in the presence of PLF was higher than in control cultures. We assume that calcium plays a predominant role in the process of differentiation from mesenchymal stem cells into osteoblasts.

Accordingly, Chang et al. [14] has demonstrated that calcium and phosphate supplementation induce mineralization in rat osteoblastic-like cell cultures, when calcium ions interact with calpain receptors of preosteoblasts.

The reduction of calcium deposition in the PLF preparations in compare to the combination of plasma, calcium and thrombin might be explained by the action of TGF-beta1 which is highly enriched in our PLF preparation. Lu et al. [34] reported that TGF-beta1 concentration of 5-10 ng/ml inhibits ALP-activity of bone marrow derived mesenchymal stem cells whereas low concentrations of TGF-beta, 1ng/ml, which can be found in plasma increases this activity

All in all, our results have demonstrated that different factors within the platelet factors (PLF) in platelet preparations work together to promote migration and proliferation of human endothelial cells and mesenchymal stem cells and on osteogenic differentiation of mesenchymal stem cells. The addition of calcium to concentrated platelets in plasma seems to be critical for the osteogenic differentiation of mesenchymal stem cells to overcome inhibitory effects of high level TGF-beta1 within these preparations. PDGF must be regarded as the main mediator of migration, whereas mesenchymal stem cell proliferation is predominantly induced by PDGF and TGF-beta1. These results indicate that the use of calcium for the release of PLF is favourable to solely thrombin released preparations for the potentation of angio- and osteogenesis during fracture repair processes. Actually we investigate the effect of PLF in the bone defect healing on a minipig model .

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Received: March 29, 2004 / Accepted: July 12, 2004

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