Reinhard Gruber
Franz Varga
Michael B. Fischer
Georg Watzek

Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes

Authors' affiliations:
Reinhard Gruber[†], *Georg Watzek*, School of Dentistry, Department of Oral Surgery, University of Vienna, and Ludwig Boltzmann Institute of Implantology, Vienna, Austria
Franz Varga, Ludwig Boltzmann Institute of Osteology, Hanusch Hospital, Vienna, Austria
Michael B. Fischer, Department of Transfusion Medicine University of Vienna, Vienna, Austria

Correspondence to:
Reinhard Gruber, Ph.D.,
School of Dentistry
Department of Oral Surgery
University of Vienna
Waehringerstraße 25a
A-1090 Vienna
Austria
Tel: +43 4277 67152
Fax: +43-1-4277-67159
e-mail: reinhard.gruber@akh-wien.ac.at

Date:
Accepted 28 January 2002

To cite this article:
Gruber R, Varga F, Fischer MB, Watzek G. Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes
Clin. Oral Impl. Res, **13**, 2002; 529–535

ISSN 0905-7161

Key words: membranes; microparticles; osteoblast; platelet-derived growth factor; platelet-rich plasma.

Abstract: Platelets have been implicated in accelerated bone regeneration in grafting applications. The beneficial effects of platelets may involve their ability to stimulate the proliferation of osteoblasts. We therefore determined the mitogenic response of human trabecular bone-derived cells to human platelets and supernatants of thrombin-activated platelets. We can show a ~ 50-fold increase in DNA-synthesis of bone cells (BC) cultured in the presence of platelets as determined by [^{3}H]-thymidine incorporation. Preventing cell-to-cell contact by a membrane filter did not abrogate the stimulatory effect, indicating the release of soluble factor(s) that are mitogenic for BC. The lipid fraction of the platelets had no effect on [^{3}H]-thymidine uptake into the DNA of BC. Platelet-released supernatant (PRS) increased the rate of [^{3}H]-thymidine incorporation to ~ 20-fold and retained 56% of their activity after incubation at 56 °C, and 27% at 100 °C, respectively. Neutralizing antibodies raised against platelet-derived growth factor (PDGF) partially suppressed the mitogenic potential of PRS. Gel exclusion chromatography analysis showed that molecules ranging from 25 kDa to more than 70 kDa within the PRS can stimulate BC proliferation. The highest amount of PDGF was detected in fractions corresponding to a molecular weight of 28–37 kDa as determined by immunoassay. The mitogenic activity was not restricted to soluble growth factors because microparticles in the PRS and platelet membranes also increased BC proliferation. Our data indicate that native platelets, the respective PRS, microparticles, and platelet membranes can stimulate the mitogenic activity of BC, thereby contributing to the regeneration of mineralized tissue.

Platelets are characteristically activated at sites of injury where they create a physical barrier to limit blood loss and accelerate the generation of thrombin to intensify the coagulation process.[8] Platelets can also release local mediators with phylogistic potential and interact with leukocytes and endothelial cells modulating the inflammatory reaction (Bazzoni et al. 1991; Klinger 1997). In addition to haemostasis and inflammation, platelets are involved in wound healing and repair of mineralized tissue (Gentry 1992; Barnes et al. 1999). As bone is a highly vascularized tissue, the biologic activities of platelets occur as immediate responses to fractures where the degranulation of platelets takes place during formation of a heamatoma. Vascular disruption also occurs in microfractures without any clinical sign of injury (Zuo & Zhu 1991). Platelet-released growth factors are chemoattractant for mesenchymal cells of the external soft tissue and the bone marrow (Barnes et al. 1999). Growth factors that are highly abundant in platelets can stimulate the proliferation and the dif-

ferentiation of periosteal cells, and attract granulocytes and macrophages to the fracture haematoma (Joyce et al. 1990; Barnes et al. 1999). Little is known regarding the interaction of platelets with cells of the osteogenic lineage.

Native platelets can be mitogenic for osteoblastic cells derived from fetal bone (Slater et al. 1995). Two clinical studies have investigated the effects of platelets to reconstruct mandibular and vertebral defects (Marx et al. 1998; Lowery et al. 1999); the application of platelet-rich plasma results in a higher bone density within autologous bone grafts after 6 months suggesting that endosteal osteoblasts that line the cancellous bone surface survive transplantation and are responsive to the platelet-released growth factors (Marx et al. 1998). Among these growth factors, platelet-derived growth factor (PDGF) has been shown to stimulate bone cell replication (Hock & Canalis 1994; Yu et al. 1997). PDGF increases bone matrix apposition and *in vivo* studies on tibial osteotomies in rabbits showed that exogenous PDGF has a stimulatory effect on fracture healing (Pfeilschifter et al. 1990; Nash et al. 1994). Despite these results, it remains unclear whether PDGF is the only factor that mediates the interaction of platelets with BC.

Activation of platelets by physiologic agonists such as thrombin also results in the release of small (1 μM) membrane vesicles from their surface, termed microparticles (MP) (Barry & FitzGerald 1999; Hughes et al. 2000). Similar to growth factors, MP act in the local environment of their formation. MP have pro- as well as anticoagulant properties and their number is increased in the circulation of patients with syndromes of platelet activation (Barry & FitzGerald 1999). Moreover, MP modulate the function of platelets, monocytes, and vascular endothelial cells, and stimulate the proliferation of coronary artery smooth muscle cells, independent of PDGF (Barry et al. 1998; Weber et al. 2000). Given that the release of MP accompanies the vascular disruption at the fracture sites, we suggest that MP can have an effect on endosteal osteoblasts.

Here, we addressed the question whether native platelets and PRS can stimulate the proliferative activity of human trabecular BC. Our results show that platelets were genuinely highly mitogenic to BC, mediated, at least in part, by PDGF and MP, and also that platelet membranes are involved in this process. These findings suggest a mechanism where platelets can contribute to the healing of skeletal defects and the regeneration of grafted bone matrix.

Material and methods

Human bone cells (BC)

Trabecular bone specimens were obtained from patients (62–88 years old) during routine hip replacements from the Department of Traumatology (Dr Mayer, Hospital Baden, Baden, Austria). Human trabecular BC were prepared as described by Evans and coworkers with minor modifications (Evans et al. 1990). In brief, bone chips were extensively washed with PBS and placed in T-75 tissue-culture flasks (Costar, Cambridge, MA, USA) in the presence of DMEM/F12 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Life Technologies), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies). This medium is termed serum-containing medium (SC-medium). Cells that grew out from the bone chips were cultured in a humidified atmosphere at 37 °C in 5% CO_2 for at least 2 weeks. Medium was changed 3 times a week. Cells of the second and the third passage were used for experiments.

Isolation of platelet concentrates

Aphaeresis platelet concentrates from volunteer donors were prepared by standard aphaeresis procedures using an MCS$^+$ device (Haemonetics, Braintree, MA, USA) and acid citrate dextrose (ACD-A; 22 g/l sodium citrate, 24.5 g/l glucose monohydrate and 8 g/l citric acid monohydrate; Haemonetics) as anticoagulant at a ratio of 1 : 10. The platelet concentrates were automatically leucodepleted by a negatively charged pall filter (LRF^{TM}, XL), and fewer than 3×10^3 white blood cells/ml remained in the platelet concentrates. The platelet concentrates were gently agitated at 21 °C on a platelet agitator (Helmer Laboratories, Nobesville, IN, USA) and aliquots were aseptically withdrawn after 3–4 h.

Preparation of washed platelets, platelet supernatants, platelet membranes, and MP

Platelets (3×10^9) from platelet concentrates were washed in Tyrode's buffer, pH 6.4, and centrifuged at 1400*g* for 10 min Pellets were resuspended in 3 ml serum-free medium (SF-medium), which is a DMEM/F12 medium supplemented with 2.5 μg/ml insulin-transferrin-selenium (Boehringer Mannheim, Germany) and antibiotics/antimycotics. The platelet suspension was diluted at a 1 : 5 ratio, resulting in platelet numbers of 2×10^8/ml (I); 4×10^7/ml (II); 8×10^6/ml (III); 1.6×10^6/ml (IV); and 3.2×10^5/ml (V).

Release of platelet products into the supernatant was induced by adding 1 U/ml of human thrombin (Sigma, St. Louis, MO, USA) to 1×10^9 platelets/ml SF-medium for 20 min at room temperature. After centrifugation at 1400*g* for 10 min, the PRS was diluted in the same way as described for experiments with washed platelets. PRS were also incubated for 5, 15, and 30 min at 56 °C and 100 °C before dilution. Platelet membranes were prepared from resuspended pellets of thrombin-activated platelets after washing with PBS.

MP were prepared as described by Weber et al. (2000) with minor modifications. In brief, 1 ml of the platelet supernatant was further centrifuged at 100000*g* for 1 h at 4 °C. The particle-free supernatant was collected and the MP-pellet was resuspended in 1 ml SF-medium. Both the MP and the corresponding particle-free PRS were diluted at the 1 : 5 ratio in SF-medium for analysis.

Mitogenic assay

The proliferation of human BC was measured by incorporation of radiolabeled [^{3}H]-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, England) into the replicating strands of DNA produced during BC mitosis as recommended by the supplier of the gamma-counter with minor modifications (Topcount®, Packard, Meriden, CT, USA; http://www.packardbioscience.com/reference_matl/531.asp). In brief, BC were plated at 10000 cells/cm^2 in 96-well plates (Packard) and grown for 2–3 days in SC-medium. The SC-medium was changed to SF-medium with and without the indicated concentrations (I–V, see above) of washed platelets, platelet membranes and supernatants for 24 h. Cells were pulse-labeled with [^{3}H]-thymidine (0.5 μCi/well) for the last 6 h of culture, washed extensively with PBS, and the [^{3}H]-thymidine incorporation was measured directly in the culture plate with a liquid scintillation counter (Packard).

To determine the involvement of cell-to-cell contact for the proliferative effect, human BC were grown in 24-well culture dishes (Costar) in the presence of various concentrations (I–V) of washed platelets for 24 h, but separated by a membrane filter (Transwell, 6.5-mm diameter, 3.0-μm pore size, Costar). BC were pulsed with [^{3}H]-thymidine (3 μCi/well) for the last 6 h of culture. After removing unincorporated radioactivity, osteoblasts were released from the culture plate with 0.2% trypsin (Life Technologies) and harvested onto Unifilter-plates (Packard). Relative [^{3}H]-thymidine incorporation was determined by liquid scintillation counting.

Methanol/chloroform extraction of cellular lipids

Washed platelets (1×10^9/ml SF-medium) were lysed by sonification (Branson Sonifire 250; Schwäbisch Gmünd, Germany) for 2 min at room temperature. Lipids were extracted according to a modified version of Bligh & Dyer (1959). In brief, 1 ml of disrupted platelets were incubated with 3.75 ml chloroform/methanol (1 : 1, Merck, Darmstadt, Germany) for 10 min with continuous shaking. Thereafter, 1.25 ml chloroform and 1.25 ml H_2O were added and after extensive vortexing, the tubes were centrifuged for 10 min at 1400 *g*. The lower phase containing the lipid fraction was evaporated and dissolved in 100 μl ethanol. For analysis, 900 μl SF-medium was added to the lipid extract that was then diluted at the 1 : 5 ratio. The cell remnants within the interface were removed, washed two times with SF-medium, resuspended in 1 ml of the same medium and diluted as described above.

Blocking of PDGF with an neutralizing antibody

Human BC were cultured with PRS corresponding to 8×10^7 platelets/ml (Dilution II) in the presence of neutralizing antibodies raised against human PDGF (Clone AB-20-NA, R&D systems, MN, USA) for 24 h and assayed for mitogenic activity. According to the information provided by the supplier, the half-maximal inhibition is 3–5 μg antibody/ml for 10 ng/ml natural human PDGF; hence, 1 : 100 dilutions can block 30 ng PDGF/ml.

Gel exclusion chromatography of platelet supernatants and determination of PDGF

Chromatographic analysis was performed based on the handbook 'Gel Filtration, Principles and Methods' provided by the supplier of the equipment (Amersham Pharmacia). 800 μl of PRS, corresponding to 1×10^9 platelets/ml, was applied to a Sephadex G-75 column (1.6 × 40 cm; Amersham Pharmacia Biotech) equilibrated with SF-medium and eluted at a flow rate of 50 ml/h at room temperature. The column was calibrated using bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (12.7 kDa). Fractions (1 ml) were collected and assayed for their mitogenic activity and for their concentration of PDGF. PDGF was determined by a commercially available immunoassay (R&D systems). The PDGF-immunoassay detects the $PDGF_{AB}$ isoform with 10% cross-reactivity with $PDGF_{AA}$ and 2% cross-reactivity with $PDGF_{BB}$.

Statistical analysis

Statistical analysis was performed by Student's *t*-test and by multiple range tests, with significance assigned at the $P < 0.05$ level.

Results

Isolated platelets stimulate the proliferation of human BC

The effects of native platelets on BC proliferation were examined in a coculture model. BC cultured for 24 h with SF-medium alone showed little mitogenic activity, whereas BC incubated with platelet suspensions resulted in a concentration-dependent increase of [^{3}H]-thymidine incorporation (Fig. 1). Summarizing data from four experiments, 2×10^8 platelets/ml (Dilution I) increased the mitogenic activity of BC 46 ± 15-fold when compared to SF-medium controls.

Cell-to-cell contact is not necessary for the effects of platelets on bone cell proliferation

We performed experiments where platelets were separated from BC by a 3.0-μm pore size filter to determine whether the biologic activity of platelets depends on the cell-to-cell contact. Similar to the cocul-

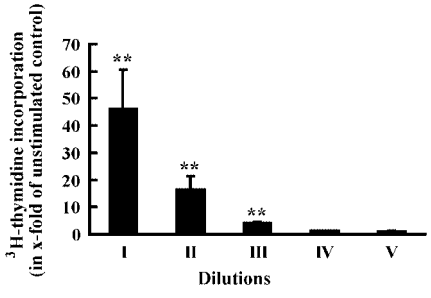

Fig. 1. Platelets increase the proliferation of human BC. Subconfluent human BC were cultured in 96-well plates, and incubated for 24 h with and without the addition of washed platelets. The platelet suspension was diluted, resulting in platelet numbers of 2×10^8/ml (I); 4×10^7/ml (II); 8×10^6/ml (III); 1.6×10^6/ml (IV); and 3.2×10^5/ml (V). Values are given as x-fold stimulation over untreated controls. Data are means ± SD of results from 4 experiments resulting from 2 different BC and 2 platelet preparations. $^{**}P < 0.01$ vs. control.

Table 1. **Platelets stimulate mitogenic activity of BC, independent of cell-to-cell contact**

	I	II	III	IV	V
Cell proliferation	67 ± 15**	29 ± 20**	4 ± 1**	1 ± 0.5	1 ± 0.6

Human BC grown in 24-well culture plates were incubated with washed platelets that were separated by a 3.0-μm nylon mesh. Platelets were diluted to reach concentrations of 2×10^8/ml (I); 4×10^7/ml (II); 8×10^6/ml (III); 1.6×10^6/ml (IV); and 3.2×10^5/ml (V). Cells were pulsed with [^{3}H]-thymidine and values are given as x-fold stimulation over untreated controls. Data are means ± SD of results from experiments with 2 different BC- and 4 platelet preparations.** $P < 0.01$ vs control.

Table 2. **Platelet-derived lipid extracts are not mitogenic to human BC**

	I	II	III	IV	V
BC 1/Lipid extracts	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.3
BC 2/Lipid extracts	1.4 ± 0.3	1.4 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.1 ± 0.2
BC 1/Platelet remnants	2.1 ± 0.1*	1.6 ± 0.1*	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
BC 2/Platelet remnants	5.5 ± 0.6**	3.3 ± 0.4**	2.7 ± 0.7*	2.3 ± 0.3*	1.8 ± 0.4

Lipid extracts and the respective platelet remnants were prepared as described in Material and methods. Preparations corresponding to platelet numbers of 2×10^8/ml (I); 4×10^7/ml (II); 8×10^6/ml (III); 1.6×10^6/ml (IV); and 3.2×10^5/ml (V) were assayed for their mitogenic activity. Values are given as x-fold stimulation over untreated controls. Data are means ± SD of results from quadruplicates. * $P < 0.05$ vs. control; ** $P < 0.01$ vs. control.

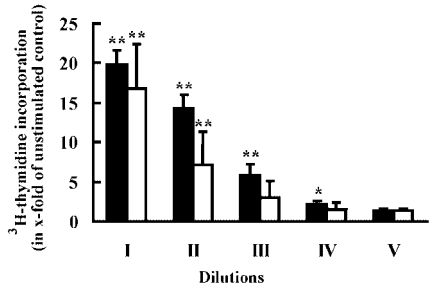

Fig. 2. PRS increase the proliferation of human BC. Subconfluent human BC were cultured in 96-well plates, and incubated for 24 h with and without the addition of PRS (black bars) and platelet membranes (white bars). The stock solutions were diluted corresponding to platelet numbers of 2×10^8/ml (I); 4×10^7/ml (II); 8×10^6/ml (III); 1.6×10^6/ml (IV); and 3.2×10^5/ml (V). Values are given as x-fold stimulation over untreated controls. Data are means ± SD of results from experiments resulting from 2 different BC and 2 platelet preparations. $^*P<0.05$ vs. control; $^{**}P<0.01$ vs. control.

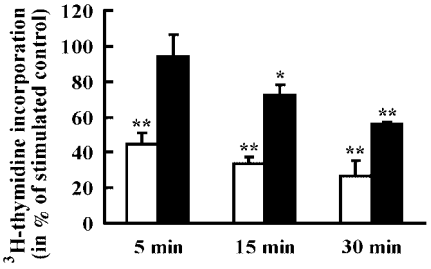

Fig. 3. Preincubation of PRS at 56 °C and 100 °C partially reduced their mitogenic potential. Subconfluent human BC were cultured in 96-well plates, and incubated for 24 h with and without the addition of PRS. The supernatants were preincubated at 56 °C (black bars) and 100 °C (white bars) for the indicated time periods and diluted corresponding to platelet numbers of 2×10^8/ml (Dilution I). Values are given as % of stimulated control. Data are means ± SD of results from quadruplicates. $^*P<0.05$ vs. control; $^{**}P<0.01$ vs. control.

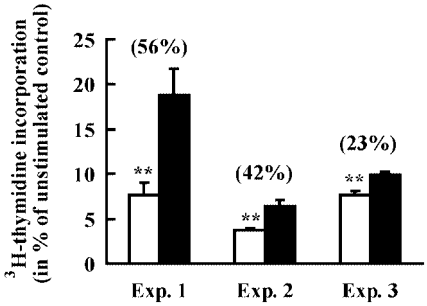

Fig. 4. Antibodies against PDGF decreased the mitogenic activity of PRS. Human BC were incubated with PRS with or without the addition of antibodies specific for PDGF (10 μg/ml) for 24 h in SF-medium. The PRS are representative of platelet numbers of 8×10^7/ml (Dilution II). Values are given as percent of unstimulated controls. Percent difference between white bars (anti-PDGF antibody) and black bars (without antibody) are indicated in parentheses. Data are means ± SD of results from quadruplicates. $^{**}P<0.01$ vs. without antibody.

ture experiments, the mitogenic activity of BC in response to platelets was increased in a dose-dependent mode of action up to 67 ± 15-fold, as indicated in Table 1. The proliferative response observed in the co-culture was not significantly different from experiments where platelets were separated by a membrane filter.

The lipid fraction of platelets does not stimulate the mitogenic activity of BC

The lipid fraction of the platelets was not mitogenic for BC, whereas the lipid-extracted cell remnants retained a mitogenic activity, shown by the dose-dependent increase in BC proliferation (Table 2).

PRS enhance the proliferation of BC

Based on the observation that platelets can mediate their effects independently of cell-to-cell contact, we investigated the proliferation of BC in response to PRS obtained from thrombin-stimulated cells. In these experiments, the addition of the PRS resulted in a dose-dependent stimulation of [^{3}H]-thymidine uptake into the DNA of BC when compared to unstimulated controls (Fig. 2). Summarizing four experiments, PRS corresponding to 2×10^8 platelets/ml (Dilution I) increased the mitogenic activity of BC 20 ± 2-fold when compared to SF-medium controls. Supernatants from platelets that were either stimulated with ADP (2.5 μM), serotonine (1 μM), and collagen type I (0.1 μg/ml) or disrupted by repeated freezing/thawing cycles also enhanced the mitogenic activity of BC (data not shown). Platelet membranes that remain in the pellet after centrifugation were also mitogenic for BC, resulting in an 17 ± 7-fold increase in [^{3}H]-thymidine incorporation (Fig. 2).

The mitogenic activity of PRS decreased at higher temperatures

In order to characterize the heat sensitivity of the mitogenic components, PRS were preincubated at 56 °C and 100 °C before being added to BC. As shown in Fig. 3, the incubation of the PRS at 100 °C reduced the mitogenic activity to 27 ± 9% of unheated controls. The incubation of the PRS at 56 °C resulted in a time-dependent decrease of the rate of [^{3}H]-thymidine uptake into the DNA of BC, to 56 ± 1% after 30 min. Additional experiments showed that the storage of the PRS at −70 °C did not abrogate their mitogenic potential (data not shown).

Neutralization of PDGF decreased bone cell proliferation

Blocking experiments with a neutralizing antibody raised against human PDGF were performed to determine whether the mitogenic effects of the PRS were mediated by PDGF. In these experiments, the addition of the PDGF neutralizing antibody (at 1 : 100 dilution) blocked the stimulatory effect of supernatants corresponding to 4×10^7 platelets/ml (Dilution II) between 23% and 56% (Fig. 4). Purified platelet-derived PDGF, which contains all the isoforms that are naturally released from activated platelets (R&D systems), also increased the mitogenic activity of BC, although to a much lesser extent than PRS (data not shown).

Gel exclusion chromatography of PRS resulted in one major peak

We used G-75 gel filtration chromatography to correlate the molecular weight of molecules within the PRS with their mitogenic activity. The data show that platelet-released molecules with a molecular weight higher than 25 kDa are mitogenic for BC, as shown in Fig. 5. PDGF could be detected in fractions corresponding to a molecular weight around 30 kDa (Table 3). The addition of PDGF-neutralizing antibodies did not completely block the mitogenic activity of the 30 kDa fraction (data not shown). Fractions corresponding to molecular weights higher than 68 kDa (Fractions 16–18) were also mitogenic for BC.

Platelet-derived MP stimulate the proliferation of BC

To examine whether platelet-derived MP, released upon stimulation with thrombin, are part of the mitogenic potential of PRS, BC were incubated with MP suspended in SF-medium, and with MP-free supernatants. Both MP and their respective supernatants enhanced the incorporation of [^{3}H]-thymidine by BC when compared to SF-medium controls. Preparations of MP

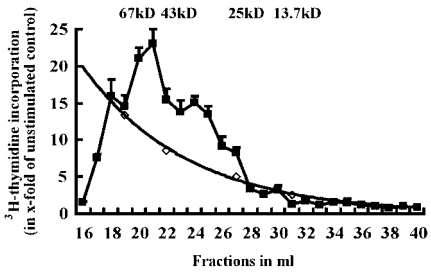

Fig. 5. Sephadex G-75 gel chromatography of platelet supernatants showed a wide range of mitogenic activity. PRS corresponding to 1×10^9 platelets/ml SF-medium, was applied to a Sephadex G-75 column (1×40 cm) and eluted at a flow rate of 50 ml/h at room temperature. Fractions (1 ml) were collected and assayed for their mitogenic activity. The calibration by bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa) is indicated in the figure. Values are given as x-fold stimulation over untreated controls. Data are means ± SD of results from quadruplicates.

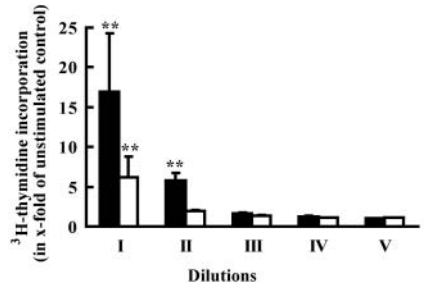

Fig. 6. MP and the corresponding particle-free supernatants stimulate the proliferation of BC. Human BC were incubated with platelet-released MP (white bars) and their respective particle-free supernatants (black bars) for 24 h in SF-medium. MP and the particle-free supernatants were obtained from PRS and diluted corresponding to platelet numbers of 2×10^8/ml (I); 4×10^7/ml (II); 8×10^6/ml (III); 1.6×10^6/ml (IV); and 3.2×10^5/ml (V). Values are given as x-fold stimulation over untreated controls. Data are means ± SD of results from 2 experiments. $^{**}P < 0.01$ vs. control.

were less stimulatory than the MP-free supernatants. Summarizing four experiments, MP-suspensions corresponding to 2×10^8 platelets/ml (Dilution I) increased the mitogenic activity of BC 6 ± 2.6 –fold, and MP-free supernatants 17 ± 7-fold, respectively (Fig. 6) (Table 3).

Table 3. **PDGF-concentration in fractionated PRS**

Fractions	16–18	19–21	22–24	25–27	28–30
Molecular size	69 kDa	68–44 kDa	37–28 kDa	25–20 kDa	18–15 kDa
PDGF	26 ± 15	80 ± 35	700 ± 230	80 ± 27	33 ± 19

Supernatant of thrombin activated platelets. corresponding to 1×10^9 platelets/ml SF-medium. was applied to a Sephadex G-75 column (1.6×40 cm). Fractions (1 ml) were collected and assayed for their PDGF-concentration. Values are given in pg PDGF/ml. Data are means ± SEM.

Discussion

Bone is a highly vascularized tissue and activation of platelets occurs immediately after a fracture, during formation of the haematoma. It is suggested that platelet-released growth factors act locally at the site of injury, where they mediate the early events of fracture repair (Barnes et al. 1999). Clinical studies have shown that the autologous application of platelets to bone grafts result in a higher bone density of the reconstructed mandibular defect (Marx et al. 1998). Platelets are mitogenic for BC released from embryonic tissue, but no data exist with regard to cells of mature bone (Slater et al. 1995).

Here we report that platelets strongly increased the proliferation of BC from adult individuals, supporting our hypothesis that platelets can act as local regulators of fracture repair and bone regeneration. A membrane filter that prevented cell-to-cell contact did not decrease the mitogenic effects, indicating the involvement of small platelet-released products. Based on observations by Ammon and coworkers that lipid extracts of platelets can stimulate the differentiation of monocytic cells, we investigated whether lipids may also account for the mitogenic activities on BC (Ammon et al. 1998). In contrast to monocytic cells, lipid fractions of platelets did not stimulate [^{3}H]-thymidine uptake into BC.

These findings prompted us to analyze the supernatants of thrombin-activated platelets. PRS increased BC proliferation within a range corresponding to $2 \times 10^8 - 8 \times 10^6$ platelets/ml, concentrations that reflect the physiologic number of platelets in the circulation. Purified thrombin can stimulate BC proliferation, although to a much lesser extent when compared to PRS (Frost et al. 1999). The mitogenic activity of the PRS does not depend on thrombin-activation because similar results were obtained when platelets were treated either with ADP, serotonin, and collagen type I, or disrupted by repeated freezing/thawing (data not shown). It seems likely that thrombin can only partially contribute to the PRS-mediated [^{3}H]-thymidine uptake.

Native platelets were more potent stimulators for BC-proliferation than the respective PRS; these findings prompted us to determine whether platelet membranes that remain in the pellet after thrombin-activation can account for the different mitogenic activity. Resuspended platelet membranes increased BC proliferation, suggesting that the differences may be due to the mitogenic activity of platelet membranes and/or to growth factors that remained in the pellet after centrifugation.

We next investigated the heat sensitivity of PRS to get information about the thermostability of the molecules that cause the proliferative response. The data showed that PRS retained their mitogenic activity after incubation at 56°C to 56%, vs. 27% at 100°C, indicating that the increased [^{3}H]-thymidine incorporation into BC is mediated, at least in part, by heat stable growth factors.

PDGF is a heat stable molecule, highly abundant in platelets, that has been shown to increase the proliferation of osteoblastic cells, suggesting that it may account for the 'heat-insensitive' mitogenic effects of the supernatant (Strain et al. 1982; Hock & Canalis 1994). Our data showed that the mitogenic effect of the PRS does not depend exclusively on the presence of PDGF because neither could the antibody against PDGF completely abolish the increase in DNA synthesis, nor could the PDGF-protein, purified from human platelets, reach the same mitogenic activity when compared to PRS. These observations indicate that PDGF is one of a larger number of platelet-released molecules with a mitogenic potential for BC (Kasperk et al. 1995). The data are consistent with reports based on other *in vitro* models, showing that the effects of platelets are mediated by PDGF, but not exclusively.

In order to identify other components that may account for the observed mitogenic activity, PRS were size-fractionated by gel exclusion chromatography. The highest PDGF levels were detected in frac-

tions corresponding to a molecular weight of ~30kDa. Blocking experiments showed that even in this particular fraction the mitogenic effect on BC could not be abolished by a PDGF-neutralizing antibody, indicating the presence of additional mitogenic growth factors with a similar molecular weight (Heldin et al. 1979; Heldin & Westermark 1999). Moreover, fractions with a molecular weight higher than ~70kDa also increased BC proliferation. The findings obtained by gel exclusion chromatography further suggest that PDGF is part of a larger number of mitogenic molecules that are released from activated platelets.

Published data and our own observations have led us to examine the effects of larger platelet-released products, the MP (Barry & FitzGerald 1999). Indeed, MP increased [^{3}H]-thymidine incorporation into BC in a dose-dependent manner, though the MP-free supernatant was more potent in this regard. Based on the fact that MP are released from platelets in response to physiologic stimuli and enhance BC proliferation, it follows that, in addition to growth factors, MP may also contribute to the regeneration of mineralized tissue.

In summary, we have demonstrated that platelets stimulated the mitogenic activity of BC independent of cell-to–cell interaction, and that the proliferation rate of BC was increased in response to PRS, MP, and platelet membranes. These findings suggest that soluble factors and MP being released upon platelet activation, as well as platelet membranes can contribute to the regeneration of skeletal defects.

Acknowledgements: We thank Ms M.-T. Krauth for reading the manuscript. The authors are grateful to the surgeons and the OP-staff at the Department of Traumatology, Hospital Baden, Austria. The work was supported by the Austrian Nationalbank (grant no. 9269) and by the 'Fonds des Bürgermeisters der Bundeshauptstadt Wien' (grant no. 1811).

Résumé

Les plaquettes ont rendu la regeneration osseuse plus rapide dans les systémes de greffe. L'effet bénéfique des plaquettes pourrait comprendre leur halbilité à stimuler la proliferation des ostéoblastes. La response mitogénique des cellules derives de l'os trabéculaire humain sur les plaquettes humaines et les surnageants de plaquettes actives par la thrombine a été déterminée. Une augmentation de 50 fois de la synthès d'AND des cellules osseuses (BC) en culture en presence de plaquettes a été mise en evidence par l'incorporation de Thymidine ^{3}H d'une vingtaine de fois et retenait 56% de leur activité après incubation à 56 degrés et 27% à 100 degrés. Neutraliser les anticorps contre les facteurs de croissance derives des plaquettes (PDGF) supprimait partiellement le potentiel mitogénique de PRS. L'analyse par chromotographie d'exclusion a montré que les molecules de 25 à plus de 70 kD à l'intérieur du PRS. L'analyse par chromotographie d'exclusion a montré que les molecules de 25 à plus de 70 kD à l'intérieur du PRS pouvaient stimuler la proliferation des BC. La quantité la plus importante de PDGF a été détectée dans les fractions correspondant à un poids moléculaire de 28 à 37 kD comme cela a été determiné par essai immunitaire. L'activité mitogénique n'était pas réduite au facteur de croissance soluble parce que des microparticules dans le PRS et les membranes des plaquettes augmentaitent également la prolifération des BC. Les plaquettes, les PRS, les microparticules et les membranes des plaquettes peuvent donc stimuler l'activité mitogénique des BC, contribuant ainsi à la regeneration du tissue mineralise.

Zusammenfassung

Plättchen werden zur Beschleunigung der Knochenregeneration bei Transplantaten verwendet. Die günstigen Effekte der Plättchen rühren daher, das sie die Fähigkeit besitzen, die Proliferation von Osteoblasten zu stimulieren. Wir bestimmten daher die mitogene Antwort von menschlichen Zellen aus trabekulärem Knochenmark auf menschliche Plättchen und thrombin-aktivierte Plättchen. Wir können eine ~50-fache Steigerung der DNA-Synthese der Knochenzellen (BC) zeigen, wenn Plättchen vorhanden sind. Die DNA-Synthese wurde mittels Inkorporation von [3H]-Thymidin bestimmt. Wenn der Zellkontakt mit einem Membranfilter verunmöglicht wurde, so hatte das keinen Einfluss auf den stimulierenden Effekt. Das deutet auf die Freisetzung von löslichen Faktoren hin, welche mitogen für BC sind. Die Fraktionierung der Lipide der Plättchen hatte keinen Effekt auf die [3H]-Thymidin-Inkorporation in die DNA der BC. Die von Pättchen freigesetzten Ueberbleibsel (PRS) steigerten die Rate der [3H]-Thymidin-Inkorporation auf ~20-fach und behielten 56% ihrer Aktivität nach Inkubation bei 56°C bzw. 27% bei 100°C. Neutralisierende Antikörper gegen Wachstumsfaktoren von Plättchen (PDGF) unterdrückten teilweise das mitogene Potential von PRS. Die gel-ausschlusschromatographische Analyse zeigte, dass Moleküle von 25kD bis zu mehr als 70kD innerhalb der PRS die BC-Proliferation stimulieren können. Die grösste Menge an PDGF wurde in Fraktionen, welche einem Molekulargewicht zwischen 28–37kD entsprachen, entdeckt. Die Bestimmung erfolgte mittels Immunoassay. Die mitogene Aktivität war nicht auf die löslichen Faktoren beschränkt, da Mikropartikel in PRS und Plättchenmembranen ebenfalls die BC-Proliferation steigerten. Unsere Daten zeigen, dass native Plättchen, die entsprechenden PRS, Mikropartikel und Plättchenmembranen die mitogene Aktivität von BC stimulieren können. Dadurch tragen sie zur Regeneration von mineralisiertem Gewebe bei.

Resumen

Se ha implicado a las plaquetas en la regeneración ósea acelerada en aplicaciones de injerto. Los efectos beneficiosos de las plaquetas pueden incluir su habilidad para estimular la proliferación de osteoblastos. Por lo tanto hemos determinado la respuesta mitogénica de las células derivadas del hueso trabecular humano a las plaquetas humanas y a los supernatants de las plaquetas activadas por trombina. Podemos mostrar un incremento de 50 veces en la síntesis de DNA de las células óseas (BC) cultivadas en presencia de plaquetas tal como se determina por la incorporación de [3H]-Timidina. El hecho de prevenir el contacto célula a célula por una membrana no abolió el efecto estimulador, indicando la liberación de factor(es) solubles que con mitogénicos para (BC). La fracción lipídica de las plaquetas no tuvo efecto sobre la toma de [^{3}H]-Timidina en el DNA de las BC. Los supernatants liberados por plaquetas (PRS) incrementó el índice de incorporación de [^{3}H]-Timidina en 20 veces y retuvo el 56% de su actividad tras incubación a 56°C, y el 27% a 100°C, respectivamente. Los anticuerpos neutralizantes aumentaron contra el factor de crecimiento derivado de las plaquetas (PDGF) parcialmente suprimidos por el potencial mitogénico de PRS. El análisis de cromatografía de gel de exclusión mostró que las moléculas que varían desde 25 kD a más de 70 kD dentro de PRS puede estimular la proliferación de BC. La mayor cantidad de PDGF se detectó en fracciones correspondiente a un peso molecular entre 28 a 37 kD determinado por inmunoensayo. La actividad mitogénica no se restringió a los factores de crecimiento solubles porque micropartículas en el PRS y en las membranas de las plaquetas también incrementó la proliferación de BC. Nuestros datos indican que las plaquetas nativas, los PRS respectivos, las micropartículas y las membranas plaquetarias pueden estimular la actividad mitogénica de BC, por tanto contribuyendo a la regeneración de tejido mineralizado.

要旨

移植において血小板は骨再生を促進すると考えられているが、その効果には血小板の骨芽細胞増殖刺激能が関与している可能性がある。従って我々はヒト血小板及びトロンビンで活性化された血小板の上清に対する、ヒト骨梁骨由来細胞の有糸分裂誘起反応を調べた。血小板の存在下で培養した骨細胞（ＢＣ）のＤＮＡ合成は約５０倍増加することが、チミジン取り込み反応の測定によって示された。メンブレン・フィルターによって細胞と細胞の接触を防いでも刺激効果は排除されなかった事から、ＢＣの有糸分裂を促進する可溶性因子の放出が示唆される。血小板の脂質分画はＢＣのＤＮＡへのチミジン取り込みに影響を及ぼさなかった。血小板を放出した上清（ＰＲＳ）のチミジン取り込み率は約２０倍増加し、その活性は５６℃で培養した場合は５６％、１００℃では２７％維持された。血小板由来の成長因子（ＰＤＧＦ）に対する中和抗体は、ＰＲＳの有糸分裂促進能を部分的に抑制した。ゲル排除クロマトグラフィーの分析は、ＰＲＳ内の２５ｋＤから７０ｋＤ以上までの分子がＢＣの増殖を刺激できることを示した。イムノアッセイの測定によると２８から３７ｋＤの間の分子量に呼応する分画においてＰＤＧＦの最高量が検出された。ＰＲＳ及び血小板膜中の微粒子もまたＢＣ増殖を増加させたので、有糸分裂誘起活性は可溶性成長因子に限定されてはいなかった。我々のデータは、生来の血小板、ＰＲＳ、微粒子、血小板膜はＢＣの有糸分裂誘起活性を刺激し、石灰化組織の再生に寄与することを示唆している。

References

Ammon C., Kreutz M., Rehli M., Krause S.W. & Andreesen R. (1998) Platelets induce monocyte differentiation in serum-free coculture. *Journal of Leukocyte Biology* **63**: 469–476.

Barnes G.L., Kostenuik P.J., Gerstenfeld L.C. & Einhorn T.A. (1999) Growth factor regulation of fracture repair. *Journal of Bone Mineral Research* **14**: 1805–1815.

Barry O.P. & FitzGerald G.A. (1999) Mechanisms of cellular activation by platelet microparticles. *Thrombosis Haemostasis* **82**: 794–800.

Barry O.P., Pratico D., Savani R.C. & FitzGerald G.A. (1998) Modulation of monocyte–endothelial cell interactions by platelet microparticles. *Journal of Clinical Investigations* **102**: 136–144.

Bazzoni G., Dejana E. & Del Maschio A. (1991) Platelet–neutrophil interactions. Possible relevance in the pathogenesis of thrombosis and inflammation. *Haematologica* **76**: 491–499.

Bligh E.G. & Dyer W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**: 911–917.

Evans C.E., Galasko C.S. & Ward C. (1990) Effect of donor age on the growth *in vitro* of cells obtained from human trabecular bone. *Journal of Orthopaedic Research* **8**: 234–237.

Frost A., Jonsson K.B., Ridefelt P., Nilsson O., Ljunghall S. & Ljunggren O. (1999) Thrombin, but not bradykinin, stimulates proliferation in isolated human osteoblasts, via a mechanism not dependent on endogenous prostaglandin formation. *Acta Orthopaedica Scandinavica* **70**: 497–503.

Gentry P.A. (1992) The mammalian blood platelet: its role in haemostasis: inflammation and tissue repair. *Journal of Comparative Pathology* **107**: 243–270.

Heldin C.H. & Westermark B. (1999) Mechanism of action and *in vivo* role of platelet-derived growth factor. *Physiological Review* **79**: 1283–1316.

Heldin C.H., Westermark B. & Wasteson A. (1979) Platelet-derived growth factor: purification and partial characterization. *Proceedings of the National Academy of Sciences of the USA* **76**: 3722–3726.

Hock J.M. & Canalis E. (1994) Platelet-derived growth factor enhances bone cell replication. but not differentiated function of osteoblasts. *Endocrinology* **134**: 1423–1428.

Yu X., Hsieh S.C., Bao W. & Graves D.T. (1997) Temporal expression of PDGF receptors and PDGF regulatory effects on osteoblastic cells in mineralizing cultures. *American Journal of Physiology* **272**: C1709–C1716.

Hughes M., Hayward C.P., Warkentin T.E., Horsewood P., Chorneyko K.A. & Kelton J.G. (2000) Morphological analysis of microparticle generation in heparin-induced thrombocytopenia. *Blood* **96**: 188–194.

Joyce M.E., Roberts A.B., Sporn M.B. & Bolander M.E. (1990) Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. *Journal of Cell Biology* **110**: 2195–2207.

Kasperk C., Wergedal J., Strong D., Farley J., Wangerin K., Gropp H., Ziegler R. & Baylink D.J. (1995) Human bone cell phenotypes differ depending on their skeletal site of origin. *Journal of Clinical Endocrinology and Metabolism* **80**: 2511–2517.

Klinger M.H. (1997) Platelets and inflammation. *Anatomical Embryologicy (Berlin)* **196**: 1–11.

Lowery G.L., Kulkarni S. & Pennisi A.E. (1999) Use of autologous growth factors in lumbar spinal fusion. *Bone* **25**: 47S–50S.

Marx R.E., Carlson E.R., Eichstaedt R.M., Schimmele S.R., Strauss J.E. & Georgeff K.R. (1998) Platelet-rich plasma: Growth factor enhancement for bone grafts. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **85**: 638–646.

Nash T.J., Howlett C.R., Martin C., Steele J., Johnson K.A. & Hicklin D.J. (1994) Effect of platelet-derived growth factor on tibial osteotomies in rabbits. *Bone* **15**: 203–208.

Pfeilschifter J., Oechsner M., Naumann A., Gronwald R.G., Minne H.W. & Ziegler R. (1990) Stimulation of bone matrix apposition *in vitro* by local growth factors: a comparison between insulin-like growth factor I, platelet-derived growth factor and transforming growth factor beta. *Endocrinology* **127**: 69–75.

Slater M., Patava J., Kingham K. & Mason R.S. (1995) Involvement of platelets in stimulating osteogenic activity. *Journal of Orthopaedic Research* **13**: 655–663.

Strain A.J., McGowan J.A. & Bucher N.L. (1982) Stimulation of DNA synthesis in primary cultures of adult rat hepatocytes by rat platelet-associated substance (s). *In Vitro* **18**: 108–116.

Weber A., Koppen H.O. & Schror K. (2000) Platelet-derived microparticles stimulate coronary artery smooth muscle cell mitogenesis by a PDGF-independent mechanism. *Thrombosis Research* **98**: 461–466.

Zuo Z.J. & Zhu J.Z. (1991) Study on the microstructures of skull fracture. *Forensic Science International* **50**: 1–14.