Abstract

Platelet-released supernatant (PRS) induces MSC osteoblastic differentiation via BMP-2

Introduction

Platelets play a pivotal role during wound healing. Upon being activated, they adhere to the exposed subendothelium and form a clot by binding to circulating fibrinogen molecules that cover the injured site and allow the healing process to begin. This clot is stabilized by a thick fibrin-mesh, which forms under the control of liver-produced thrombin. In addition, platelet activation and degranulation results in the release of a high number of biological factors (including Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Insulin-like Growth Factor (IGF), Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) and Transforming Growth Factor (TGF)), which attract more platelets to the site of injury, but also other growth factors and cytokines that can target further cell types (Kiuru et al., 1991; Lowery et al., 1999). Many of those factors are known to play a direct role in normal bone turnover and in the events during early bone healing (Anitua et al., 2004). This led to the idea to use platelets for bone repair in the form of platelet-rich plasma (PRP) or similar highly concentrated platelet preparations (Whitman et al., 1997; Marx et al., 1998). PRP is autologous plasma that has a platelet concentration above baseline (~1Mio platelets/μL vs. ~0.2Mio platelets/μL), but a native fibrinogen concentration. While the gel-forming ability of fibrinogen-containing platelet preparations like PRP is highly beneficial in in vivo situations, these gel-forming abilities are hindering in vitro, because the addition of PRP to cell culture medium can result in gelling of the culture medium. This is why for in vitro applications platelet-rich preparations are often prepared in buffered systems like phosphate buffered saline (PBS), instead of autologous plasma, in order to avoid gel formation in the culture dishes. Such preparations are referred to as platelet-released supernatants (PRS) (Gruber et al., 2002a; Gruber et al., 2002b).

Today, platelet preparations are used routinely in oral and maxillofacial surgery in some centers in combination with bone grafts and sometimes with autologous mesenchymal stem cells. However, the many studies performed using platelet preparations in fracture repair resulted in very divergent outcomes. Many studies showed increased bone formation (Marx et al., 1998; Kitoh et al., 2004), some showed increased implant resorption (Yamada et al., 2004a; Yamada et al., 2004b) and others...
have reported improved osseointegration (Lucarelli et al., 2005; Brodke et al., 2006) or increased vascularization (Yamada et al., 2004a; Yamada et al., 2004b; Kilian et al., 2005). Nevertheless, several studies have shown no beneficial influence of platelet preparations on bone healing (Roldan et al., 2004; Mooren et al., 2007). This divergence has to be considered carefully, since there is a great variance between the experimental setups used in these studies. There is a high variability in used species, carrier materials, cells, platelet concentration and preparation method, time points when measurements were taken, etc. But there are a few things that seem to be clear. The platelet concentration is essential and too low as well as too high concentrations are not beneficial (Graziani et al., 2006; Uggeri et al., 2007), and can even be disadvantageous (Choi et al., 2005). While it is undisputed that different carrier materials perform differently in combination with platelets (Lin et al., 2006), the presence of osteoblastic (precur sor) cells seems to be highly beneficial on the effect of platelet preparations on bone healing (Kitoh et al., 2004; Yamada et al., 2004a; Yamada et al., 2004b). Despite the many efforts taken to use platelet preparations in in vivo bone repair, very little is known about their effect at the cellular level, and the underlying mechanisms. While it is generally accepted that highly concentrated platelet preparations (i.e. PRS) have a highly beneficial effect on bone cell proliferation (Gruber et al., 2002a; Gruber et al., 2002b; Gruber et al., 2003; Lucarelli et al., 2003; Arpornmaeklong et al., 2004; Choi et al., 2005; Graziani et al., 2006; Uggeri et al., 2007), its effect on bone cell differentiation is still controversial. Some studies have shown an increase in certain osteoblastic markers in bone cells upon exposure to platelet preparations (Lin et al., 2006; Uggeri et al., 2007), while others have reported no or even a negative influence of platelet preparations on osteoblastic differentiation (Arpornmaeklong et al., 2004).

In this study, we report an increase in osteoblastic differentiation of human mesenchymal stem cells (MSC) in long-term in vitro experiments upon exposure to platelet-released supernatant (PRS). Furthermore, we propose that this effect may at least in part, be due to increased BMP-2 levels in MSC, when exposed to PRS.

Materials and Methods

Origin of bone marrow and blood aspirates
Bone marrow (60mL) and blood (100mL) aspirates in CPDA (Citrate Phosphate Dextrose Adenine)-containing S-monovettes (Sarstedt, Nürnberg, Germany) were received from patients (28 to 79 years old, with the average age being 49 years: 12 males and 4 females) undergoing routine orthopedic surgery involving iliac crest exposure, after informed consent (KEK Bern 126/03). Blood aspirates were stored at room temperature (RT) under gentle agitation and bone marrow aspirates were stored at 4°C under gentle agitation until processed within 24 hours after harvesting. The platelets were counted using a Digitana Sysmex FS-3000 (Davos Hospital, Switzerland).

MISC isolation and culture
MSC were isolated from bone marrow aspirates as previously reported (Martin et al., 1997; Bianchi et al., 2003). In brief, after homogenization, bone marrow aspirates were diluted 1:4 with IMDM (Gibco, Paisley, UK) containing 5% (v/v) FBS (Gibco). After centrifugation (5 min 200g) and removal of the fatty top layer, samples were pipetted on the top of Ficoll (1 mL sample / 2.6 mL Ficoll) (Histopaque-1077, Sigma, St. Louis, MO, USA), then centrifuged at 800 g for 20 min at RT. The mononucleated cells interphases were collected and washed twice in 5 mL of IMDM/5%FBS followed by 15 min centrifugation at 400 g. Cells were seeded at densities of 8-10x10^6 mononucleated cells per 150 cm^2 T-flask in IMDM containing 10% FBS, nonessential amino acids (Gibco) and PenStrep (100 U/mL, Gibco). After 5 days, fresh medium containing 5 ng/mL basic-FGF (R&D Systems) was added (Martin et al., 1997; Bianchi et al., 2003). Medium was changed every 3 days and cells were subcultured 1:3. After the first passage, cells were termed mesenchymal stem cells (MSC). Cells between passages 2-4 were subsequently used.

PRS preparation
The blood aspirates were transferred from the CPDA-containing monovettes into 15 mL Falcon tubes, and were centrifuged at 200 g for 30 min at RT. The resulting plasma supernatants were pooled (donor specific), transferred into a new 15 mL Falcon tube, and centrifuged 5 min at 2,000 g (RT). The resulting supernatant was discarded, and the platelet pellet was resuspended in sterile phosphate-buffered saline (PBS) at 1/10^6 of the initial blood volume. PRS was activated by freeze-thaw cycles according to the procedure described by Weibrich et al. (2002). The platelet activation efficiency was determined by measuring the release of PDGF-AB, -BB, and VEGF in the activated PRS using the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA) according the manufacturer instructions (Data not shown).

MSC culture supplementation with PRS
MSC were treated with 0.05% Trypsin-EDTA, washed, counted and 30,000 cells/cm^2 were seeded in 24-well (Falcon BD, Franklin Lakes, NJ, USA) or 12-well plates (Falcon BD) in presence of IMDM/10%FCS. Culture medium during the experiments consisted of IMDM, 10% FBS, nonessential amino acids, 0.1 mM ascorbic acid-2-phosphate (Sigma) and 10 mM β-glycerophosphate (Sigma). In the experimental cultures, MSC were supplemented with 10% PRS (Lucarelli et al., 2003) or with 10 nM Dexamethasone (Dexa) (Sigma). Media were changed twice a week. Recombinant Noggin (R&D Systems) was added at concentrations of 100 ng/mL or 500 ng/mL (Abe et al., 2000).

4Ca incorporation assay
1.25 μCi/mL of 4Ca isotope (Amersham CES3, Amersham, UK) were added to each well and the plates were incubated at 37°C o/n (Alini et al., 1994). After
medium removal and 3 washes in IMDM, 0.5 mL of 70% formic acid were added to each well and incubated at 65°C for 1 h. The formic acid solution was transferred to a scintillation tube containing 3.5 mL of scintillation liquid (OptiPhase HiSafe®, Perkin Elmer, Waltham, MA, USA) and the radioactivity was measured at day 21 of culture using a Wallac 1414 WinSpectral Liquid scintillation counter (Perkin Elmer).

**Table 1. Primers and Probes for Real-Time RT-PCR**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collagen Iα1</strong></td>
<td></td>
</tr>
<tr>
<td>Forw</td>
<td>CCC TGG AAA GAA TGG AGA TGA T</td>
</tr>
<tr>
<td>Rev</td>
<td>ACT GAA ACC TCT GTG TCC CTT CA</td>
</tr>
<tr>
<td>Probe</td>
<td>CGG GCA ATC CTC GAG CAC CCT</td>
</tr>
<tr>
<td><strong>Osteonectin</strong></td>
<td></td>
</tr>
<tr>
<td>Forw</td>
<td>ATC TTT CCT GTA CAC TGG CAG TTC</td>
</tr>
<tr>
<td>Rev</td>
<td>CTC GGT GTG GGA GAG GTA CC</td>
</tr>
<tr>
<td>Probe</td>
<td>CAG CTG GAC CAG CAC CCC ATT GAC</td>
</tr>
<tr>
<td><strong>MMP-13</strong></td>
<td></td>
</tr>
<tr>
<td>Forw</td>
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</tr>
<tr>
<td>Rev</td>
<td>TTT TGC CGG TGT AGG TGT AGA TAG</td>
</tr>
<tr>
<td>Probe</td>
<td>CTC CAA GGA CCC TGG AGC ACT CAT GT</td>
</tr>
<tr>
<td><strong>BMP-2</strong></td>
<td></td>
</tr>
<tr>
<td>Forw</td>
<td>AAC ACT GTG CGC AGC TTC C</td>
</tr>
<tr>
<td>Rev</td>
<td>CTC CGG GTT GTT TTC CCA C</td>
</tr>
<tr>
<td>Probe</td>
<td>CCA TGA AGA ATC TTT GGA AGA ACT ACC AGA AAC TG</td>
</tr>
<tr>
<td><strong>Osteopontin</strong></td>
<td></td>
</tr>
<tr>
<td>Forw</td>
<td>CTC AGG CCA GTT GCA GCC</td>
</tr>
<tr>
<td>Rev</td>
<td>CAA AAG CAA ATC ACT GCA ATT CTC</td>
</tr>
<tr>
<td>Probe</td>
<td>AAA CGC AGA CCA AGG AAA ACT CAC TAC C</td>
</tr>
<tr>
<td><strong>Runx2</strong></td>
<td></td>
</tr>
<tr>
<td>Forw</td>
<td>AGC AAG GTT CAA CGA TCT GAG AT</td>
</tr>
<tr>
<td>Rev</td>
<td>TTT GTG AAG ACG GTT ATG GTC AA</td>
</tr>
<tr>
<td>Probe</td>
<td>TGA AAC TCT TGC CTC GTC CAC TCC G</td>
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<tr>
<td><strong>BSP II</strong></td>
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</tr>
<tr>
<td>Forw</td>
<td>TGC CTT GAG CCT GCT TCC</td>
</tr>
<tr>
<td>Rev</td>
<td>GCA AAA TTA AAG CAG TCT TCA TTT TG</td>
</tr>
<tr>
<td>Probe</td>
<td>CTC CAG GAC TGC CAG AGG AAG CAA TCA</td>
</tr>
<tr>
<td><strong>Taqman Gene Expression Assays (Applied Biosystems):</strong></td>
<td></td>
</tr>
<tr>
<td>Osx :</td>
<td>Hs00541729_m1</td>
</tr>
<tr>
<td>Wnt 7b :</td>
<td>Hs00536497_m1</td>
</tr>
<tr>
<td>ALP :</td>
<td>Hs00758162_m1</td>
</tr>
</tbody>
</table>

Probes were modified at the 5’ end with the FAM fluorescent dye (6-carboxyfluorescein) and at the 3’ end with the TAMRA fluorescent dye (6-carboxy-N,N,N’,N’-tetramethylrhodamine).

**Von Kossa staining**

MSC monolayers were rinsed with Tyrode’s Balanced Salt Solution (TBSS) after 14 days in culture. Fresh silver nitrate solution (5%) was added and the cells were exposed to strong light for 20 min. After rinsing 3 times with distilled water (dH₂O), the cells were incubated in fresh 5% sodium thiosulfate for 10 min, before being rinsed 3 times with dH₂O. Then the cells were incubated in 0.1% nuclear fast red solution for 10 min, before being rinsed with dH₂O.
**ALP activity assay**

Samples for ALP activity measurement were harvested each 3rd day over a period of 18 days. After medium removal and a washing step with PBS, 500 μL of 0.1% Triton-X in 10 mM Tris-HCl (pH 7.4) were added to the MSC monolayers and incubated for 3 h at 4°C on a gyratory shaker (Alini et al., 1994). ALP activity was assessed by measuring the p-nitrophenol production during 15 min incubation at 37°C with p-nitrophenyl phosphate as substrate (Sigma Kit No.104) on a Perkin Elmer Bio Assay Reader HTS 7000.

**Gene expression analysis**

Total RNA was extracted from cells monolayers at different time points as specified in the figure legends using TRI-reagent (Molecular Research Center, Cincinnati, OH, USA) according to the protocol from the manufacturer instructions with a modified precipitation method using High Salt Precipitation solution (Molecular Research Center). Reverse transcription was performed on 1 μg of sample’s total RNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) and random hexamers to initiate transcription. Polymerase Chain Reaction (PCR) was performed on a 7500 real-time PCR system (Applied Biosystems).

Except for osterix, Wnt7b and 18S endogenous control which were from Applied Biosystems, oligonucleotide primers and TaqMan probes (Table 1) were all from Microsynth (Balghach, Switzerland) and were designed using the Primer Express Oligo Design software (Version 1.5, Applied Biosystems). The nucleotide sequences were obtained from the GenBank database and the probes were designed to overlap an exon-exon junction in order to avoid amplification of genomic DNA. The PCR was performed under thermal conditions with TaqMan Universal PCR master mix (Applied Biosystems). Relative quantification of mRNA targets was performed according to the comparative C_{T} method with 18S ribosomal RNA as endogenous control (ABI PRISM 7700 Sequence Detector User Bulletin [2], PE Applied Biosystems 1997).

**BMP-2 ELISA**

MSCs were seeded in 12 well plates at the density of 10,000 cells/cm² and grown in unsupplemented culture medium (IMDM, 10% FCS, 10 mM beta-glycerophosphate, 0.1 mM ascorbic acid), in culture medium containing 10 nM Dexa, or in culture medium containing 10% PRS for 23 days. Culture supernatants were collected twice a week during media changes, were transferred to microcentrifuge tubes and stored at -20°C until the end of the experiment. After centrifugation at 18,000 g for 2 min to remove cellular debris, the concentration of BMP-2 in the collected supernatants was determined using a Quantikine BMP-2 ELISA Kit (R&D Systems). Recombinant human BMP-2 (R&D Systems) was used as a standard.

**Statistics**

All experiments were carried out using different donors (indicated by the n values) from which, the analyses were all done in triplicates. All results are shown as mean±SEM. Statistical analysis was performed by 1-way ANOVA or by t-test using Graphpad’s Prism 4 (Graphpad, La Jolla, CA, USA). p<0.05 was considered to be statistically significant.

**Results**

The bone marrow and blood samples used in this study were obtained from healthy donors undergoing hip surgery. Isolated MSCs and PRS were used in an autogenous setting only, and samples from different donors were not pooled. There were no obvious differences in the results obtained between different donor ages or gender. However, due to the high variability in the osteogenic potential of BMSC between donors, some data (Figs. 5 and 6) are shown from one representative experiment, although, the same trends were observed in all experiments, which are represented in Tables 2 and 3.

Platelet activation was estimated by measuring the release of PDGF-AB, PDGF-BB and VEGF proteins from platelets into the supernatant upon their activation by freeze-thaw cycles (data now shown).

**PRS effect on MSC differentiation**

**mRNA levels by real-time RT-PCR:** PRS increased the mRNA levels of collagen Iα1 (p<0.05) and bone sialoprotein II (p<0.05) in MSC supplemented with PRS

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>PRS</th>
<th>PRS+nog 100</th>
<th>PRS+nog 500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene expression rel to PRS</td>
<td>Donor 1*</td>
<td>Donor 2</td>
<td>Donor 3</td>
</tr>
<tr>
<td>Coll1</td>
<td>1</td>
<td>0.77</td>
<td>0.58</td>
</tr>
<tr>
<td>OP</td>
<td>1</td>
<td>0.56</td>
<td>0.58</td>
</tr>
<tr>
<td>MMP13</td>
<td>1</td>
<td>0.50</td>
<td>0.69</td>
</tr>
<tr>
<td>BMP2</td>
<td>1</td>
<td>0.73</td>
<td>0.25</td>
</tr>
<tr>
<td>Runx2</td>
<td>1</td>
<td>0.85</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Table 2. Results all 3 donors. * = values of Donor 1 shown in Figure 5. Cells were treated with either of PRS, PRS+noggin 100 ng/mL or PRS+noggin 500 ng/mL. Results are expressed relative to PRS treatment.
Table 3. Results of all donors. *= Donor shown in Figure 6. Wnt7b gene expression was measured in MSC and in MSC + PRS. Results are presented relative to the corresponding 18S (2^-dCt) for different time points.

<table>
<thead>
<tr>
<th>Time points</th>
<th>Donor 1 (2^-dCt)</th>
<th>Donor 2 (2^-dCt)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MSC</td>
<td>MSC + PRS</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.496</td>
<td>1.867</td>
</tr>
<tr>
<td>Day 7</td>
<td>1.564</td>
<td>3.426</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.252</td>
<td>2.423</td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>0.240</td>
<td>2.426</td>
</tr>
</tbody>
</table>

Fig. 1: Gene expression of typical osteoblastic marker genes by MSC at day 28 (n = 3).
A: MSC + PRS relative to MSC only Type I collagen (Col Iα1), bone sialoprotein II (BSPII), BMP-2 and MMP-13 were significantly up-regulated. Alkaline phosphatase (ALP), osteonectin (ON), osteopontin (OP) and Runx2 were also slightly up-regulated while Osx was down-regulated. Data are expressed as mRNA levels in MSC+PRS relative to un-supplemented MSC.
B: MSC + PRS relative to MSC + Dexa. BMP-2, MMP-13 and BSP II were significantly up-regulated, while Runx2, OSX and ALP were down regulated in MSC cultures in presence of PRS when compared to Dexa treatment. (*p<0.05, **p<0.01)

Fig. 2: ALP activity of MSC cultures (n=3): Supplementation of MSC with PRS resulted in a almost two-fold increase in alkaline phosphatase activity after 15 days of culture, when compared to un-supplemented MSC. However, stimulation of MSC with dexamethasone (Dexa) resulted in more than four-fold increase of ALP activity (*p<0.01, by 1 way ANOVA).

at 28 days after confluence when compared with non treated MSC (Fig. 1A). In addition, BMP-2 (p<0.05) and MMP-13 (p<0.01) were highly up-regulated by PRS. Other osteoblastic markers including ALP, osteonectin (ON), osteopontin (OP) and Runx2 showed a trend to up-regulation without reaching significance. The transcription factor Osterix (Osx) showed a tendency to be down-regulated in the presence of PRS (not significant). When compared to cell treated with Dexa (Fig. 1B), the same trends were observed, with high up-regulation of BMP-2, down regulation of ALP, and non significant changes of Runx2 and Osx.

ALP Activity: Alkaline phosphatase activity in MSC supplemented with PRS was by day 15 found higher when compared to MSC cultured without supplementation. This trend was observed up to 18 days post-confluence. We also measured ALP activity in MSC stimulated with Dexa, which resulted, as expected, in a highly significant (p<0.01) stimulation of ALP activity compared to PRS (Fig. 2).
Ca45 incorporation: The effect of PRS on matrix mineralization by MSC was estimated by measuring $^{45}$Ca$^{2+}$ incorporation into the extracellular matrix. MSC grown in medium supplemented with PRS showed an highly significant up-regulation of $^{45}$Ca$^{2+}$ incorporation by up to 400-fold compared to un-supplemented MSC after 21 days post-confluence (Fig. 3A). This was an approximately 10 fold higher increase than when MSC were supplemented with DEXA. This result was confirmed by Von Kossa staining of MSC monolayers at day 14: Supplementation with PRS resulted in highly increased calcium deposition by MSC (Fig. 3D) compared to unsupplemented MSC (Fig. 3B). DEXA, as expected, was also able to induce matrix mineralization in MSC (Fig. 3C).

BMP-2 gene expression and protein levels: Due to the very high up-regulation of BMP-2 mRNA levels by PRS, the time course of BMP-2 gene expression over two weeks was determined using real-time RT-PCR. While MSC control cultures showed no significant increase in BMP-2 gene expression over time, MSC cultures supplemented with PRS showed a clear increase of BMP-2 gene expression after only 24 h of culture, and an approximately 14-fold significant increase in BMP-2 after 14 days post-confluence (Fig. 4A). We also measured BMP-2 protein levels in supernatant of MSC alone and MSC supplemented with either PRS or DEXA using ELISA. While the levels of BMP-2 in unsupplemented MSC or treated with DEXA was under the ELISA detection limit (11 pg/mL) for the whole duration of the experiment, MSC supplemented with PRS showed a clear increase in BMP-2 protein levels over time (Fig. 4B). After 23 days of culture, BMP-2 levels in MSC stimulated with PRS were approximately 10 times higher than at the beginning of the culture.

Noggin: Due to the clear up-regulation of BMP-2 gene expression and protein levels by PRS, we evaluated the effect of the BMP-2 antagonist Noggin on PRS-supplemented MSC cultures at two different Noggin concentrations (100 and 500 ng/mL). After 28 days of culture, the addition of both concentrations of Noggin effectively down-regulated PRS-induced osteoblastic marker genes in MSC, including BMP-2, Col I, MMP-13, BSP II, ON, OP and Runx2 (Fig. 5 and Table 2).

Wnt7b: To explain the discrepancy between up-regulation of many osteoblastic markers – in particular BMP-2 – and the down-regulation of Osterix, we studied the involvement of wnt-ligands in the effect of PRS on MSC. Interestingly, Wnt7b was always up-regulated in MSC supplemented with PRS over the whole culture period (Fig. 6 and Table 3). We could not detect elevated levels of Wnt3a or Wnt4 (data not shown).

Discussion: Due to the suspected osteoinductive effect of growth factors released by activated platelets, several forms of...
platelet preparations have recently gained popularity in maxillofacial and dental surgery, but their beneficial effect is still under debate (Kitoh et al., 2004; Marx et al., 1998; Mooren et al., 2007; Roldan et al., 2004).

In the present study, we investigated the effect of platelet preparations on the osteoblastic differentiation of human mesenchymal stem cells (MSC) by determining typical osteoblastic markers in a direct comparison with the effect of Dexa. Dexamethasone is a glucocorticoid with well established osteogenic properties in vitro (McCulloch and Tenenbaum, 1986) and potential beneficial effect in vivo (Miller et al., 2010). To date, as it is easily available and a reliable inducer of MSC osteogenic differentiation, Dexa is the most used osteogenic factor. It has been shown to stimulate most of the osteoblastic phenotype features (but not all of them) (Chen et al., 1986; Cheng et al., 1994; Cheng et al., 1996) such as increase of ALP activity and promote in vitro mineralization (Cheng et al., 1994; Cheng et al., 1996; Jorgensen et al., 2004). However, even if glucocorticoids have been shown to increase the number and size of bone nodule formation in primary cell culture, the adverse effect of increased circulating concentration...
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Fig. 6: Wnt7b mRNA levels measured by real-time RT-PCR (triplicates of a representative experiment shown; see Table III for more results): During the whole 28-days duration of the experiment, Wnt7b mRNA levels are always higher in MSC supplemented with PRS compared to MSC alone. This suggests involvement of the Wnt signaling system.

of such factors has also been described (Cooper et al., 1999; Cooper, 2004), and this could have an adverse effect if used in vivo.

In maxillofacial and dental surgery, platelet-preparations are usually administered in the form of PRP, which basically is a high concentration of platelets suspended in autologous plasma. One of the benefits of such preparations is the high gel forming ability, which in vivo helps to keep the PRP from diffusing out of the repair site. In in vitro experiments however, this gel forming capability is a problem, since it impedes proper oxygen and nutrient transport to the cells, and waste products away from the cells. We therefore used platelets resuspended in PBS instead of plasma to avoid clot formation. We validated this approach by measuring the level of several known growth factors released by activated platelets (PDGF-AB, -BB, VEGF) and were able to show that there was no significant difference between the released levels in PRP and PRS preparations (data not shown).

Our MSC preparations were prepared from bone marrow samples harvested at the iliac crest. This is a highly established procedure, which has been in use for many decades (Bolano and Kopta, 1991). In MSC supplemented with PRS, alkaline phosphatase was slightly up-regulated (approximately 2-fold at day 15) when compared to unsupplemented MSC. The differentiation effect of PRS on human MSC however was confirmed by 45Ca2+-incorporation and Von Kossa staining. Interestingly, Dexamethasone (Dexa) was able to induce ALP activity to a clearly higher level than PRS (> 3-fold higher than PRS at day 18). Measurements at the mRNA level of MSC exposed to PRS showed a significant up-regulation of collagen Iα1, bone sialoprotein II, MMP-13 and BMP-2 when compared to MSC alone. In contrast, osteonectin, osteopontin, Runx2 and Osx were not affected by PRS. Interestingly, of all the osteoblastic markers of MSC determined by real-time RT-PCR, two stuck out by being very strongly up-regulated by PRS: MMP-13 and BMP-2, this strong up-regulation was maintained when comparison was done with Dexa treated cultures. MMP-13 (collagenase-3) is a matrix metalloproteinase that has been found to be a major player during endochondral bone formation by efficiently degrading collagen types I-IV, as well as aggrecan.

Furthermore, MMP-13 is under the control of the transcription factor Runx2 and is expressed as a late differentiation marker in osteoblasts (Jimenez et al., 1999). Factors released by platelets at a fracture site are known to act as chemoattractants for neutrophil granulocytes, followed by monocyctic phagocytes (which differentiate into macrophages at the fracture site), fibroblasts, osteoblasts, vascular endothelial cells and for various precursor cells (Anderson, 1999). By degrading bone matrix components, MMP-13 can play an active role in support of the chemoattractive role of platelet-released factors. The other highly up-regulated gene was BMP-2. BMPs have the exquisite ability to induce ectopic bone formation (Wozney and Rosen, 1998). BMPs induce the expression of typical osteoblastic markers, including type I collagen, osteocalcin, MMP-13, ALP (Yamaguchi et al., 2000), and they stimulate the formation of mineralized bone-like nodules in vitro (Chen et al., 1997). These facts led us to the hypothesis that the stimulatory effect of our PRS preparations on human MSC could act at least partially through BMP-2 up-regulation. We therefore studied BMP-2 mRNA levels at earlier time points and were able to show, that BMP-2 is already 4-fold up-regulated after 24h of MSC exposure to PRS, and this up-regulation rises over time. This up-regulation was confirmed at the protein level by ELISA. Noggin is a well-known BMP antagonist and acts by binding to BMP-2, -4 and -7 receptors and therefore inhibits the BMPs from binding to their respective receptors (Zimmerman et al., 1996). We used Noggin at two different concentrations in our system and showed that most of the genes that were up-regulated by PRS were down-regulated upon exposure to PRS+Noggin. This further suggests that PRS could act through BMP-2 autocrine loop, as we showed an up-regulation of BMP-2 at both gene and protein level under PRS stimulation. It is well known that the “classical” in vitro pathway of osteoblastic differentiation especially in the murine system involves the sequential up-regulation of Runx2, Osx and ALP leading to matrix mineralization (Komori et al., 1997; Nakashima et al., 2002). This pattern has been observed in mice and rat MSC cultured either with Dexa or BMP-2 (Lee et al., 2000; Igarashi et al., 2004). This “classical” pathway also takes place when human MSC are stimulated.
with DEXA, however it has been shown that DEXA treatment does not induce up-regulation of BMP-2 gene expression in human MSC (Meury et al., 2006; Zhou et al., 2006). In contrast, human MSC treated with BMP-2 have been shown to not follow the above “classical” pathway (Dieffenbacher et al., 2003; Osyczka et al., 2004; Osyczka and Leboy, 2005; Zhou et al., 2006). In human cells, BMP-2 can induce osteoblastic differentiation independently of the transcription factors Runx2, its downstream partner Osx and ALP (Jorgensen et al., 2004; Osyczka et al., 2004; Osyczka and Leboy, 2005). This Runx2 and Osx independent pathway has also been shown in rodent cells as an alternative to the classical pathway, and it apparently involves the Wnt signaling cascade (Rawadi et al., 2003; Mbalaviele et al., 2005). Indeed, we did measure significantly elevated levels of Wnt7b in our system and this could explain our finding, that PRS did not up-regulate Runx2 and Osx. In the murine system, this alternative Runx2- and Osx-independent pathway seems to involve an increase in ALP activity (Rawadi et al., 2003; Mbalaviele et al., 2005). In contrast, in the human system, ALP activity has been shown not to be necessarily increased by BMP-2 (Jorgensen et al., 2004; Osyczka et al., 2004; Osyczka and Leboy, 2005; Kim et al., 2008). The long-term study of Jorgensen (Jorgensen et al., 2004) clearly showed that ALP, in BMP-2 treated cultures, was not increased compared to the control for up to 6 weeks, while DEXA strongly induced ALP activity. In contrast, Kim showed a 2-3 fold increase in ALP activity, when human MSC were exposed to BMP-2 (Kim et al., 2008). The increase in ALP activity in MSC+PRS cultures in our study is very low compared to the increase in ALP activity obtained by exposure to DEXA (10-15 fold increase), so whether this relatively small increase in ALP activity as seen in our study and reported by Kim can be considered biologically relevant, is presently not possible to be concluded from our results and from the above literature.

In summary, we have shown that addition of 10% of our PRS preparation induces osteoblastic differentiation and matrix mineralization in human MSC, in a comparable way to cell treatment with DEXA but the mechanisms involved clearly differ. These findings are in correlation with recent studies that reported similar effects (Lin et al., 2006; Uggeri et al., 2007). In addition, we propose that the effect of PRS on the differentiation of human MSC could at least be partially mediated by BMP-2, although other biological factors present within the PRS could also play important roles. These findings are in correspondence with recent works showing that platelet preparations potentate BMP-dependent osteoblastic differentiation (Tomoyasu et al., 2007). Furthermore, our observations suggest an involvement of the Wnt-signaling cascade, but further studies need to be performed to fully examine and characterize this last hypothesis.

In this study we have shown, that highly concentrated platelet preparations do positively influence osteoblastic differentiation of MSC and have therefore the potential to play an important role in bone healing, if applied appropriately. Besides their osteogenic effect, PRS preparations present the major advantage of being autologous. As they can be prepared intra-operatively, they constitute an easy and in a cost-effective way to obtain high concentrations of various cytokine and growth factors. The variety of the factors present in the platelet released supernatant also present the advantage of their variety, in the way that, in an in vivo situation, they can attract, proliferate and differentiate different cell types in a synergetic or sequential manner.

References


PRS induces MSC osteoblastic differentiation via BMP2

Discussion with Reviewers

Reviewer I: Based on the data you presented in the paper, what is the significance of BMP and Wnt signaling pathways in the osteoblastic differentiation of MSCs after the treatment with PRS?

Authors: Unlike Dexamethasone treatment, PRS seems to induce human MSC differentiation and matrix mineralization through a non-classical pathway which does not involve activation of runx, oxa and ALP up-regulation. MSC differentiation under PRS treatment appears to involve BMP2 protein up-regulation. When compared to cell differentiation under recombinant BMP2 treatment, similarities could be observed. The exact involvement of the Wnt pathway is currently under investigation and will be the object of a later publication.

Reviewer I: What are the pros and cons of using recombinant BMPs, autologous PRS or dexamethasone to induce osteoblastic differentiation in clinical settings?

Authors: BMPs are well known to induce osteogenic differentiation of MSC in vitro and to induce bone formation in numerous animal models. BMP-2 and BMP-7 are approved for clinical use in open fractures of long bones, non-unions and spinal fusion. However, despite significant evidence of their potential benefit on bone repair and regeneration in animal and preclinical studies, there is, to date, a lack of convincing clinical outcomes. The short life span of recombinant proteins and the inefficient delivery to target cells is also of a problem. Recombinant factors are expensive and as high doses might be required to reach the wanted therapeutic effect. An increasing number of publications questioning the efficiency-cost ratio have been published in the past few years. As compared with dexamethasone, PRS preparations have the strong advantage of being composed of a cocktail of different factors in physiological concentrations that can act in parallel, or in a sequential manner. Moreover the possibility to prepare PRS in an autologous way also reduces the risk of immunogenic reaction or disease transmission. Using other stimulating factors, like BMP or dexamethasone, the questions concerning the


appropriate dosage for in vivo application, linked to the “un-controlled” release problem are still an issue. Furthermore, the patient inter-individual variation is still poorly understood. PRS might constitute a good alternative, as it can be prepared intra-operatively in a cost effective way and thus provides the surgeon with an autologous high concentration cocktail of growth factors and cytokines, although further research evaluating the PRS growth factors composition need to be performed, in order to avoid inter-patient variation.

**Reviewer III:** Platelet released factors are proposed as a potential alternative to classic MSC differentiation methods. Looking at the different features of MSC stimulated by PRS and dexamethasone (e.g., increased matrix mineralization vs. alkaline phosphatase activity), could it be that MSC triggered by PRS reach a different osteoblastic phenotype/lineage as compared to the one typically obtained using conventional differentiation factors? 

**Authors:** This is certainly a possibility. Alternatively, one could also speculate that due to the heterogeneous cell populations present within the MSC pool (which are at different differentiation stages and with different lineage progression potential) each different trigger (PRS, Dexam and BMPs) could act preferentially on a cellular subset of that pool.