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Acidic preparations of platelet concentrates release bone morphogenetic protein-2

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Abstract

Background: Growth factors released from platelets have potent effects on the mechanisms regulating fracture and wound healing. The acidic tide of wound healing, i.e., the pH within wounds and fractures, change from acidic to neutral and alkaline pH as the healing process progress. This study investigated the influence of pH on lysed platelet concentrates with respect to the release of growth factors.

Material and methods: Platelet concentrates, free of leukocyte components, were lysed and incubated in buffers between pH 4.3 – 8.6. Bone morphogenetic protein-2 (BMP-2), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF), were measured by quantitative enzyme-linked immunosorbent assays.

Results: PDGF, TGF-β, and VEGF were present in all platelet preparations but the levels were significantly influenced in a pH-dependant fashion. BMP-2 was only detected in the most acidic preparation at pH 4.3, which is interesting since it has been reported that BMP-2 is an endogenous mediator necessary for fracture repair and responsible for the initiation of fracture healing.

Interpretation: These results indicate that platelets release only significant amounts of BMP-2 in acidic milieu, i.e., the milieu associated with the critical initial stage of fracture healing.

Keywords (n = 5):
Bone morphogenetic proteins, Fracture healing, Growth factors, Growth stimulation, Platelets
**Introduction**

Platelets are abundant at sites of wound and fracture healing and thus exposed to the varying pH levels as the healing process progress from the initial inflammatory stage to the later reparative stages. The local environment in the initial fracture hematoma is acidic, which later becomes more neutral as the healing progress and, ultimately, alkaline which helps to support differentiation-related events in the healing process, e.g., expression of alkaline phosphatase and osteocalcin (Hollinger and Wong 1996). Platelet rich plasma and platelet derivates contain several potent growth factors and have, therefore, been used for growth stimulation of a variety of mesenchymal cells (Slater et al. 1995, Gruber et al. 2004, Dallari et al. 2006, Vogel et al. 2006). Platelet preparations have also been used clinically to stimulate bone formation (Marx et al. 1998) and wound healing (Steed et al. 1992). The stimulatory effect of platelets on proliferation of mesenchymal cells including osteoblasts and mesenchymal stem cells is well documented but the efficacy on differentiation and bone formation is not well understood. Some studies have not been able to demonstrate a positive effect of platelet preparations on bone formation and osteoblast differentiation (Roldan et al. 2004, Carreon et al. 2005, Gruber et al. 2006). These findings have been explained by the absence of bone morphogenetic proteins (BMPs) in the applied platelet preparations (Marx 2004). Interestingly, it has recently been reported that BMPs (BMP-2, -4, and -6) have been localized within megakaryocytes and platelets (Sipe et al. 2004). BMP-2 is of particular interest since it is required for successful fracture healing (Tsuji et al. 2006).

We have recently demonstrated that the release of growth factors from lysed platelet concentrates is pH dependant and that acidic preparations increase proliferation and alkaline phosphatase activity in human osteoblast-like cells as well as the levels of platelet-derived growth factor (PDGF) (Liu et al. 2002, Wahlström et al. 2007). This study was designed to investigate the release of BMP-2, PDGF, transforming growth factor-β (TGF-β), and vascular...
endothelial growth factor (VEGF) in platelet concentrates lysed and incubated in buffers between pH 4.3 – 8.6.

Material and methods

Platelet concentrates (1.2 – 2 × 10^{12} cells/L), free of leukocyte components, were prepared from one healthy blood donor from the Blood Center at Linköping University Hospital, Sweden, by standardized and certified procedures, and stored at –70°C prior to use. Ten platelet preparations were made for each pH from this platelet concentrate. Platelets were rapidly thawed in a water bath at 37°C and pH was adjusted by dilution 1:1 with different buffers. The pH was adjusted to pH 4.3 and 5.3 with 0.2 M sodium acetate, pH 4.0 and 5.0; to pH 6.8 and 7.2 with phosphate buffered saline, pH 6.0 and 7.4; and to pH 7.9 and 8.6 with 0.05 M Tris, pH 8.0 and 9.0, respectively. Lysed platelets buffers (LPBs) were incubated overnight (16 hours) in a water bath at 37°C. Each LPB preparation was centrifuged at 2000 × g for 5 min and the content of BMP-2, PDGF (PDGF-AB), TGF-β (TGF-β1), and VEGF were quantified in the supernatant by quantitative enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN). The minimum detection limit for BMP-2, PDGF, TGF-β, and VEGF was 11, 2, 7, and 9 ng/L, respectively.

Results

Our results demonstrate that lysed platelet preparations release growth factors in a pH-dependant fashion (Figure 1). PDGF, TGF-β, and VEGF were present in all the investigated LPB preparations but; interestingly, BMP-2 (median 57 ng/L) was only detected in the most acidic preparation at pH 4.3 and not detected at all at pH 5.3 – 8.6. The highest concentration of PDGF (median 37 μg/L) was also observed at pH 4.3. Both TGF-β and VEGF were
significantly lower at pH 4.3. The highest levels of TGF-β (median 85 μg/L) and VEGF (median 343 ng/L) were released in the more alkaline LPBs at pH 8.6.

Discussion

In this study, we found that BMP-2 was only detectable in the supernatant from LPBs in the most acidic buffer at pH 4.3. Numerous factors, such as cytokines, hormones and growth factors, are of fundamental importance for the fracture healing process. The particular importance of BMP-2 in fracture healing and bone formation is well documented (Govender et al. 2002, ) and; furthermore, BMP-2 is of specific interest since it appears to be a crucial
endogenous mediator necessary for fracture repair and responsible for the initiation of fracture healing (Tsuji et al. 2006).

It has been reported that solutions with a low pH have suppressing effects on cell proliferation and survival (Jäger et al. 2006) and, moreover, that it is not possible to activate platelets at a low pH (<5.0) (Liu et al. 2003). Lysed platelet preparations were used in this study where the release of potent growth factors was affected differently by different buffers. The mechanisms whereby platelets influence osteoblast differentiation and skeletal growth are not fully understood. It has been reported that supernatants from thrombin-activated platelets suppress the osteogenic effects of BMPs in cell cultures and, thus, a blood clot with activated platelets could therefore suppress the action of BMPs (Gruber et al. 2006). The low pH in a wound/fracture hematoma could, hypothetically, influence the release of growth factors from platelets but would still suppress cell proliferation. However, later when the microcirculation improves and pH increases at the wound site, factors suitable for wound/fracture healing have already been released from the platelet granulae. We would like to emphasize that the pH of the used buffers during the processing of the platelets is important for the activity of the platelet derivate.

The pH of the supernatant could, theoretically, affect the antibody binding in the applied ELISA and thus give erroneous results. According to the manufacturer, the estimation of all the measured growth factors in a test solution is not sensitive to its pH. So why is BMP-2 only released to the supernatant in the acidic preparation? It is, perhaps, not suitable for platelets to release BMP-2 at a neutral pH. According to Tsuji et al. (Tsuji et al. 2006), almost all BMPs contribute to bone formation, but only BMP-2 is critical for the initiation of fracture healing. Release of BMP-2 from platelets at neutral pH would be devastating since it might trigger pathological heterotopic bone formation.
The timing, i.e., when platelets are provided in a fracture/wound site, appears also to be critical and the form of the applied platelet derivative is important. It has been reported that gel versus liquid may give different results on bone growth on implants with rough titanium or calcium/phosphate coatings (Nikolidakis et al. 2006). We have not specifically studied the influence of the added ions acetate and phosphate in the different buffers; however, to the very best of our knowledge, these ions do not influence the release of growth factors from platelets, which is in contrast to calcium ions with have a well-known effect on platelets.

The results from previous clinical and experimental studies on bone formation and osteoblast differentiation with platelet rich plasma have not been as successful as the studies on soft tissue healing. Until recently the general opinion was that platelet rich plasma is not osteoinductive as a consequence of the assumed absence of BMPs in platelets (Marx 2004). Sipe et al. (Sipe et al. 2004) demonstrated that platelets contain BMPs and our results show that LPBs indeed can release BMP-2 if the platelet lysate is incubated in an acidic buffer (pH 4.3) before use. Hence, from an experimental and clinical point of view, it would be appealing to use platelet lysates in acidic buffers to obtain sufficient, or even ample, amounts of BMP-2. However, the pH of the growth factor containing supernatant from LPBs should be re-adjusted, close to physiological pH or within the buffering capacity of the cell culture medium used, before being added to the investigated cells or to the site of fracture.

These results raise a number of questions and further studies are indeed required to conclusively clarify whether acidic platelet preparations can influence osteoblast proliferation and the initial phase of fracture healing in comparison with neutral platelet preparations.
Contributions of authors

All authors were involved in the design and planning of the study. CL performed the experimental analysis. OW and AK wrote the draft manuscript and PM revised the manuscript. All authors read and approved the final manuscript.

We, the authors, have no conflicts of interest.
References


Vogel J P, Szalay K, Geiger F, Kramer M, Richter W, Kasten P. Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation