Acidic preparations of platelet concentrates release bone morphogenetic protein-2

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Background and purpose **Growth factors released from platelets have potent effects on fracture and wound healing. The acidic tide of wound healing, i.e. the pH within wounds and fractures, changes from acidic pH to neutral and alkaline pH as the healing process progresses. We investigated the influence of pH on lysed platelet concentrates regarding the release of growth factors.**

Material and methods **Platelet concentrates free of leukocyte components were lysed and incubated in buffers with pH between 4.3 and 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 varied in a pH-dependent fashion. BMP-2 was only detected in the most acidic preparation (pH 4.3), which is interesting since BMP-2 has been reported to be an endogenous mediator of fracture repair and to be responsible for the initiation of fracture healing.**

Interpretation **Our findings indicate that platelets release substantial amounts of BMP-2 only under conditions of low pH, the milieu associated with the critical initial stage of fracture healing.**

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There is an abundance of platelets at sites of wound and fracture healing. These are thus exposed to the varying levels of pH as the healing process progresses from the initial inflammatory stage to the later reparative stages. The local environment in the initial fracture hematoma is acidic, which later becomes neutral as the healing progresses 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 derivatives contain several potent growth factors and have therefore been used to stimulate growth 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 effect on differentiation and bone formation is not well understood. Some authors have been unable 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, Nikolidakis et al. 2006). These findings have been explained by the absence of bone morphogenetic proteins (BMPs) in the platelet preparations used (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).

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We have recently shown that the release of growth factors from lysed platelet concentrates is pH-dependent, and that acidic preparations encourage proliferation and alkaline phosphatase activity in human osteoblast-like cells (Liu et al. 2002, Wahlström et al. 2007). In this study we wanted to investigate the release of BMP-2, platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) in platelet concentrates lysed and incubated in buffers of pH between 4.3 and 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 the pH was adjusted by 1:1 dilution with different buffers. The pH was adjusted to 4.3 or 5.3 with 0.2 M sodium acetate, pH 4.0 and 5.0, respectively; to pH 6.8 or 7.2 with phosphate buffered saline, pH 6.0 and 7.4; and to pH 7.9 or 8.6 with 0.05 M Tris, pH 8.0 and 9.0, respectively. Lysed platelet buffers (LPBs) were incubated overnight (16 h) in a water bath at 37°C. Each LPB preparation was centrifuged at 2,000 × g for 5 min and the levels 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 limits for BMP-2, PDGF, TGF-β, and VEGF were 11, 2, 7, and 9 ng/L, respectively.

Results

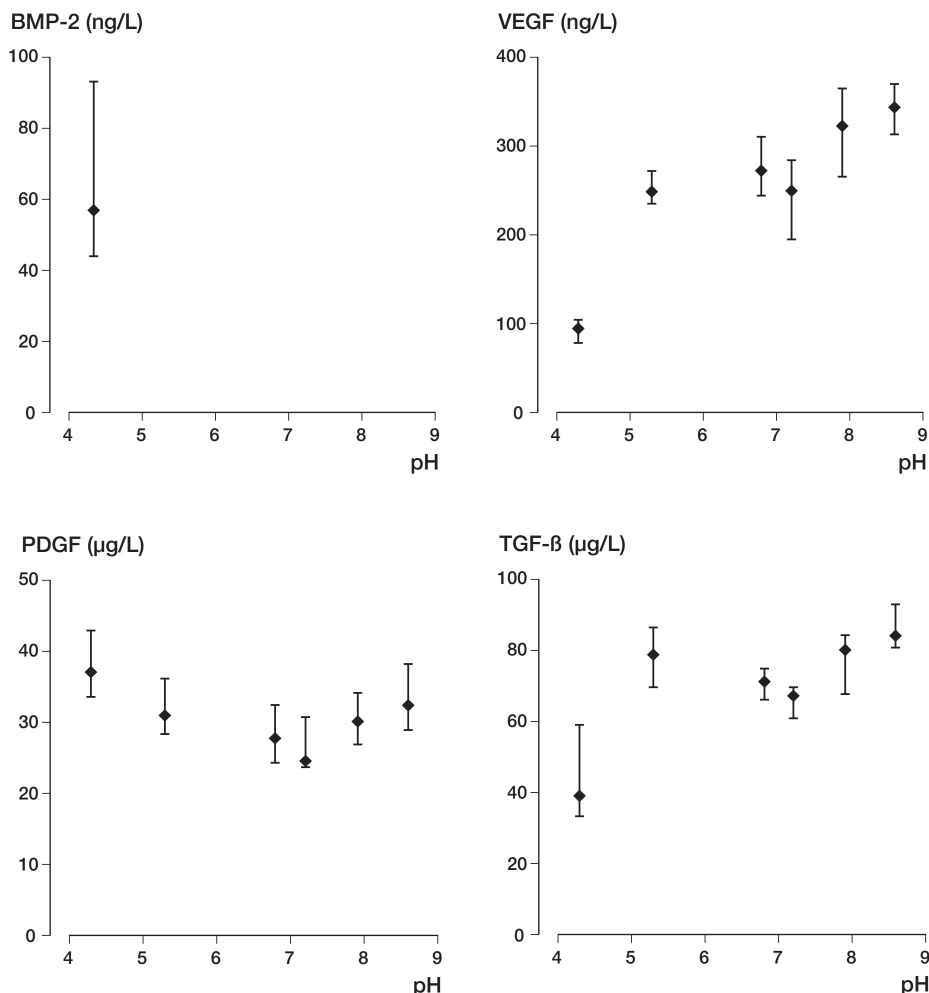
Lysed platelet preparations released growth factors in a pH-dependent fashion (Figure). PDGF, TGF-β, and VEGF were present in all the LPB preparations investigated; interestingly, however, BMP-2 was only detected in the most acidic preparation at pH 4.3 (with a median level of 57 ng/L) and was not detected at all at pH 5.3–8.6. The highest median concentration of PDGF (37 µg/L) was also found at pH 4.3. Levels of both TGF-β and VEGF were lower at pH 4.3 than at higher pH. The highest median levels of TGF-β (85 µg/L) and VEGF (343 ng/L) were found in LPBs at pH 8.6.

Discussion

We found that BMP-2 was only detectable in the supernatant from lysed platelets 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 BMP-2 is also of specific interest since it appears to be a crucial endogenous mediator of fracture repair and to be responsible for the initiation of fracture healing (Tsuji et al. 2006).

It has been reported that low-pH solutions have suppressive effects on cell proliferation and survival (Jäger et al. 2006) and, in addition, that it is not possible to activate platelets at low pH (< 5.0) (Liu et al. 2003). In this study using lysed platelet preparations, the release of potent growth factors was affected differently by different buffers. The mechanisms by which 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 culture; thus, a blood clot with activated platelets could suppress the action of BMPs (Gruber et al. 2006). The low pH in a wound or fracture hematoma could, hypothetically, influence the release of growth factors from platelets but might still suppress cell proliferation. However, later when the microcirculation improves and the pH increases at the wound site, factors suitable for wound or fracture healing have already been released from the platelet granulae. We want to emphasize that the pH of the buffers used 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 ELISA used and thus give erroneous results. According to the manufacturer, measurement of several growth fac-



Levels of BMP-2, VEGF, PDGF, and TGF-β determined in LPB preparations at pH 4.3, 5.3, 6.8, 7.2, 7.9, and 8.6. BMP-2 was only detected in the most acidic LPB preparation (pH 4.3), which also contained significantly higher amounts of PDGF in comparison with the other LPB preparations (ANOVA, $p < 0.001$). VEGF and TGF-β levels were significantly lower in the LPB preparation at pH 4.3 ($p < 0.001$). Values are presented as median ± SD; n = 10.

tors in a test solution was not found to be sensitive to pH. So why would BMP-2 only be released into the supernatant in the acidic preparation? It is perhaps not suitable for platelets to release BMP-2 at neutral pH. According to 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 might be devastating since it would trigger pathological heterotopic bone formation.

The timing of when platelets are provided in a fracture/wound site also appears to be critical, and the form of the platelet derivate supplied is important. It has been reported that gel and liquid may give different results regarding bone growth on implants with rough titanium or calcium/phosphate coatings (Nikolidakis et al. 2006). We have not specifically studied the influence of the acetate and phosphate ions present in the different buffers. To our knowledge, however, these ions do not influence the release of growth factors from platelets; this contrasts with calcium ions, however, which have a well-known effect on platelets.

The results of previous clinical and experimental studies on bone formation and osteoblast differentiation with platelet-rich plasma have not been as

successful as studies on soft tissue healing (Slater et al. 1995, Gruber et al. 2004, Dallari et al. 2006, Vogel et al. 2006). Until recently, the general consensus was that platelet-rich plasma is not osteoinductive as a consequence of the assumed absence of BMPs in platelets (Marx 2004). Sipe et al. (2004) demonstrated that platelets contain BMPs and our results show that LPBs can indeed release BMP-2 if the platelet lysate is incubated in an acidic buffer (pH 4.3) before use. Thus, 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 supernatant from LPBs (containing growth factors) should be re-adjusted to be close to physiological pH or should be within the buffering capacity of the cell culture medium used, before being added to the cells under investigation or to the site of fracture.

Our findings raise a number of questions, and further studies are certainly required to determine conclusively whether acidic platelet preparations (as opposed to neutral ones) can influence osteoblast proliferation and the initial phase of fracture healing.

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 it. All authors read and approved the final manuscript.

No competing interests declared.

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