Composition of platelet-rich plasma gel: A Western blot analysis

Abstract

Objective

Platelet-rich plasma (PRP) gel is an autogenous blood-derived material that may be used as a regenerative agent of oral structures. The regenerative capacity of PRP is largely attributed to its composition, including many different growth factors. Thus far, no study has identified the molecular content of this gel. Therefore, it was the purpose of this study to assess the presence of different growth factors in PRP gel, using the Western blot technique.

Materials and methods

Blood samples were collected from 20 healthy donors and then processed to obtain PRP gel samples. The Western blot technique was used to determine the presence of the following growth factors: vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β1), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF).

Results

Western blot analysis showed positive electrophoretic bands corresponding to molecular weights of the examined platelet growth factors. These bands were observed in every sample of PRP, demonstrating their presence as constituents of PRP gel.

Conclusion

Data from our study showed that PRP gel contains varying amounts of VEGF, TGF-β1, bFGF, PDGF-A, IGF-1 and EGF.

Keywords

Platelet-rich plasma, growth factors, tissue engineering, wound healing, Western blotting.
Introduction

Tissue integrity and blood vessel repair are essential after destructive and reconstructive events, such as surgery, trauma and regenerative procedures. The seeking for and identification of reliable and safe techniques or therapeutic methods that would predictably enhance the regenerative capacity in damaged tissue has become a major focus of current research. A large number of cells are involved in wound healing, including platelets, which play a crucial role in controlling coagulation and releasing growth factors and cytokines related to tissue regeneration. Platelet-derived products isolated from the patient’s own blood have been extensively studied and tested because platelets are considered a source of cytokines and growth factors, which amplify wound healing and tissue repair.

Platelet-rich plasma (PRP) offers much potential owing to its autogenous nature and a supposed molecular content. PRP was originally defined as a product with a high concentration of platelets, obtained from autologous blood, that contains different growth factors that may potentially influence cells involved in wound healing and bone regeneration. Besides its adhesive and hemostatic properties, from a biological standpoint, the rationale for the use of PRP is rooted in the idea that regenerative advantages are obtained after the application of this product, given the modulating activity that is supposed to be exerted by molecules released from the α-granules, which are stored in the cytoplasm of platelets. A wide variety of molecules with different biological roles are known to be contained in the different platelet granules, such as serotonin, coagulation factors, proteoglycans, membrane-associated proteins and different types of proteases. However, some researchers believe that the activity of specific mitogenic/growth factors, concretely stored in the α-granules, is of major importance in regenerative events. Growth factors, including those that have been classically associated with PRP, are included in a family of polypeptides of low molecular weight with a very short life span. Growth factors can modulate cell behavior, alter gene expression of target cells and ultimately lead to promotion of cell migration, proliferation, differentiation and eventually maturation. The growth factors that have been reported to be in PRP include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β1), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF).

When PRP was introduced in dentistry, most of the knowledge that justified its attributed regenerative potential came from experimental animal models and several human case reports. Nowadays, clinical trials conducted to assess the clinical validity of PRP have shown controversial results, many of them advocating for no significant effect when PRP is used alone. However, it has been reported that the addition of PRP to bone substitutes promotes enhancement of osseointegration of dental implants, optimizing the expansion of new bone cells, and improvement of the aggregation and cohesiveness of particulate-based bone substitutes. In order to understand how PRP works, it is important to know its structure and molecular composition. It is important to know whether the described factors are present in an amount that can be easily detectable and in what proportion of the population. However, only limited evidence is currently available. Hence, it was the aim of this study to conduct a qualitative analysis to screen the molecular composition of PRP.

Materials and methods

Preparation of PRP

Venous blood was obtained from 20 healthy volunteers (16 males and four females; mean age of 27.4), who gave their informed consent and met the following inclusion criteria after a complete medical history recording and examination: no medication taken in the last two weeks, no dental or intra-oral surgical treatment within the last month, and no vaccine received and no infection history within the last three months. Briefly, 20 cm³ of blood was drawn per patient and 5 cm³ placed into each of four Vacutainer tubes (Becton Dickinson, Oxford, U.K.), which contained 0.1 cm³ of 3.8% (w/v) sodium citrate. In order to minimize platelet activation during blood collection, a 19-gauge butterfly needle with a light tourniquet was used and the first 2 mL of blood was discarded. For the preparation of the PRP, a modification of the original procedure proposed by Anitua in 1999 was followed. Immediately after collection, the tubes were placed in a centrifuge machine to spin at 1,500 rpm for 7 min
In order to separate the blood fractions. In order to produce PRP, 500 mL from the volume of plasma situated just over the top of the red fraction was collected from every sample. To each 500 mL of plasma, 250 mL of calcium chloride was added. Finally, the tubes were placed in a 37°C warm water bath for 20 min to accelerate the formation of a PRP gel. The remaining contents of the tubes was discarded.

Monoclonal and polyclonal antibodies

The Western blot technique was performed using monoclonal antibodies for the selected platelet growth factors: anti-VEGF (C-1: sc-7269; Santa Cruz Biotechnology, Santa Cruz, Calif., U.S.), anti-PDGF-A (E-10; sc-9974; Santa Cruz Biotechnology), anti-human TGF-β1 (Clone 9016.2; Sigma-Aldrich, St. Louis, Mo., U.S.), anti-human IGF-1 Goat (Sigma-Aldrich), anti-human bFGF (Clone FB-8; Sigma-Aldrich) and anti-human EGF (Clone EGF-10; Sigma-Aldrich). Fluorochrome-marked polyclonal immunoglobulin-G antibodies able to bind the previously mentioned antibodies were purchased from Santa Cruz Biotechnology to be used as secondary antibodies.

Western blot technique

Gels for electrophoresis (12% SDS-PAGE) were initially prepared for each experiment. Final samples consisting of a volume of 15 mL of PRP clot, previously diluted in a sample buffer (at a proportion of 1 mg/mL), were properly identified and placed on each well of the electrophoresis gels. The proteins were electrophoretically run at a rate of 60 V and 45 mA for 2 h, using the PowerPac HC electrophoresis kit (Bio-Rad, Laboratories, Hercules, Calif., U.S.). Transference of the proteins located in the running gel was performed using a Trans-Blot SD device (Bio-Rad), at 25 V/60 mA for 50 min. Once the transference to nitrocellulose membranes, designed for protein transference, had been completed, specimens were submerged in 20 mL of blocking suspension (5 mg of fat-free powder milk diluted in 100 mL of 1 × TBS) for 60 min, at room temperature. After that, the membranes were incubated with the mentioned specific monoclonal antibodies for different growth factors in agitation, for 24 h at 4°C. Three 5 min washes using a washing solution (0.5 mL of 0.1% TWEEN 20 in 500 mL of 1 × TBS) were performed prior to the incubation of the membranes with the secondary antibodies.
Table 1

<table>
<thead>
<tr>
<th>Donor</th>
<th>VEGF 45 kDa</th>
<th>TGF-β1 25 kDa</th>
<th>bFGF 16–18 kDa</th>
<th>PDGF-A 15 kDa</th>
<th>IGF-1 7.6 kDa</th>
<th>EGF 6.4 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>JF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Discussion

PRP has been suggested to be a novel agent that could promote not only hard-tissue regeneration but also soft-tissue healing. Although controversy exists regarding its ability to promote regeneration, especially in hard tissue, many clinicians remain loyal to its clinical usage. In order to enhance understanding of PRP, our research group has focused on analyzing the structure and composition of PRP gel using flow cytometry and the scanning electron microscope. Our results have shown PRP’s microstructural composition, essentially constituted by fibrin in relation to the different cellular elements in the clot, identified as largely platelets in different stages of activation.

Following the line traced by those previous findings, our group has designed a further in vitro study to determine the presence of different growth factors in the PRP gel samples obtained from donors, after Western blotting.
growth factors known to be present in the α-granules of the platelets in PRP gel samples. Results obtained from the present study indicate that PRP gel contains the six tested growth factors: VEGF, PDGF, TGF-β1, FGF, IGF-1 and EGF. These growth factors have been known for their influence at different stages of the healing processes.\(^8\),\(^19\) Basically, growth factors are low-weighted molecules produced and released by many different cell types under variable stimuli and play a determinant role in the development and maturation of different tissues in mammals. For instance, VEGF is an important regulator of angiogenic processes, increasing vessel permeability and contributing essentially to neangiogenesis. Its presence in the clot of PRP can partially justify the positive outcomes obtained in some studies regarding the beneficial effect of PRP on capillary growth in soft-tissue wound healing.\(^27\) Several in vitro and animal studies have illustrated the modulating effect that growth factors exert on different cell types. PDGF is primarily responsible for tissue healing and has been shown to induce proliferation of gingival fibroblasts and osteoblasts\(^28\) and adherence of periodontal ligament cells to root surfaces.\(^29\) TGF-β1 is essential for normal tissue remodeling and wound healing; it is chemotactic for human fibroblasts,\(^30\) enhances the proliferation and differentiation of osteoblasts,\(^31\)\(^–\)\(^33\) and intervenes in angiogenesis and immunomodulation.\(^34\) BFGF induces stimulation of periodontal ligament cell proliferation, osteoblastic cell proliferation and growth and fibroblasts, and plays a role in angiogenesis.\(^35\)\(^–\)\(^37\) IGF-1, also known as somatomedin, is a mediator in the activity of growth hormone\(^38\) and a positive regulator of cell proliferation and differentiation for most cell types.\(^39\) EGF enhances the proliferation of keratinocytes\(^40\) and is implicated in epithelialization, wound contraction and remodeling.\(^41\)

Thus far, the majority of studies reporting detection of one or some of the previously mentioned growth factors in PRP samples have been conducted on nonclotted samples, without any platelet activator added.\(^3\),\(^42\)\(^–\)\(^45\) In our opinion, direct analysis of the presence of growth factors in PRP gel would provide a more accurate idea regarding which are the bioactive constituents of PRP gel.

El-Sharkawy et al. performed a similar study based on a different technique, enzyme-linked immunosorbent assay (ELISA), and the same growth factor content of PRP was found.\(^9\) They quantified platelet and growth factor levels. PDGF, TGF-β1, IGF-1, EGF, VEGF and bFGF were also identified in their PRP samples. They attributed the biological properties of these growth factors, such as proliferation of fibroblasts and periodontal ligament cells and extracellular matrix formation, to PRP. Lu et al. also used the same technique, ELISA, and they identified and quantified PRP growth factors released (PDGF, TGF-β1 and IGF-1), obtaining similar results, although no search for EGF, VEGF or bFGF was conducted.\(^2\)

Despite the contribution of our findings to the understanding of PRP biology, they also raise more questions that need to be addressed. The identification of these growth factors in PRP gel only suggests their presence in the clot, without providing evidence supporting its biological benefit after its clinical usage, especially for regenerative approaches. Growth factors are proteins that, once released from the producer cell, exert a very localized action at specific ratios,\(^46\) owing to their short life span. They are labile molecules highly susceptible to denaturalization mediated by proteases present in the wound site and to phagocytosis and might even become solubilized in the carrier.\(^47\) These undesirable events usually lead to the inactivation or annulment of the biological properties of these mediators. For instance, PDGF cannot be detected in circulating blood in normal conditions, and when it is intravenously injected, its life span is around 2 min.\(^48\) This lack of long-term activity associated with the short half-life of this platelet growth factor may require repeated applications over time to maintain their therapeutic effect.

Both the vehicle and concentration (dose) of biological mediators, such as platelet growth factors, may be critical factors to consider when seeking to achieve controlled modulation of cellular events in the desired time interval.\(^49\) However, it has to be taken into consideration that there is no currently available information about the optimal dosages of PRP needed to achieve the highest effectiveness.\(^1\)

It is also important to note that the technique used in this study for the detection of growth factors, Western blotting, allows detection of the presence of a determined protein in a sample by highly specific binding of antibodies to epitopes of that polypeptide. However, it is not possible to ultimately determine whether the target protein is biologically active and therefore able to exert its effect when applied to a wound. This fact, along with the arguments discussed before,
moves us to reason that the beneficial effects obtained after application of autologous PRP gel may not be mediated only by the action of growth factors.

Considering this data, it is logical to outline several questions and doubts regarding the variable amount of growth factors, presenting a true ability to interact with the environment, that may be released from the PRP gel sample that is clinically applied. Are those growth factors the exclusive or major mediators responsible for a change in the biological conditions that lead to an improvement in tissue regeneration? Does the fibrillar component of the clot have a role in modulating the biological response that permits a better clinical response? Or, is it a synergistic effect between the fibrillar scaffold and different molecular components of blood plasma, among which growth factors are present, that induces those beneficial effects?

Understanding the role of each component of PRP gel in wound-healing events and the optimal concentration of molecules with the capacity of modulating these processes remains one of the major challenges for researchers in this field of tissue engineering. In order to address those questions, further in vitro and in vivo studies seeking to determine the physical interaction of PRP elements with cell types present in the craniofacial surgical field and its impact on cell proliferation and differentiation are needed.

Competing interests

The authors do not have any financial interests, either directly or indirectly, in the products listed in the study.

Acknowledgments

This article was supported partially by Research Group #CTS-583 (Junta de Andalucía, Spain).

References

Composition of PRP


28. Zhang L, Leeman E, Barnes DC, Graves DT. Human osteoblasts synthesize and respond to platelet-derived growth factor.  


31. Centrella M, McCarthy TL, Canalis E. Transforming growth factor β is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone.  


34. Ghosh D, Li L, McGurr D, Matyunina LV, McDonald JF, Dawson MR. Integral role of platelet-derived growth factor in mediating transforming growth factor-β1-dependent mesenchymal stem cell stiffening.  

35. Sennez AB, Castelnuovo J. Applications of basic fibroblastic growth factor (FGF-2, bFGF) in dentistry.  

36. Terranova VP, Odziemiec C, Tweedt KS, Spadone DP. Repopulation of dentin surfaces by periodontal ligament cells and endothelial cells: effect of basic fibroblast growth factor.  


38. Laron Z. Clinical use of somatomedin-1: yes or no?  


43. Giannobile WV. Periodontal tissue engineering by growth factors.  

44. Nimni ME. Polypeptide growth factors: targeted delivery systems.  