Autologous platelets as a source of proteins for healing and tissue regeneration

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Summary

Platelets are known for their role in haemostasis where they help prevent blood loss at sites of vascular injury. To do this, they adhere, aggregate and form a procoagulant surface leading to thrombin generation and fibrin formation. Platelets also release substances that promote tissue repair and influence the reactivity of vascular and other blood cells in angiogenesis and inflammation. They contain storage pools of growth factors including PDGF, TGF-β and VEGF as well as cytokines including proteins such as PF4 and CD40L. Chemokines and newly synthesised active metabolites are also released. The fact that platelets secrete growth factors and active metabolites means that their applied use can have a positive influence in clinical situations requiring rapid healing and tissue regeneration. Their administration in fibrin clot or fibrin glue provides an adhesive support that can confine secretion to a chosen site. Additionally, the presentation of growth factors attached to platelets and/or fibrin may result in enhanced activity over recombinant proteins. Dental implant surgery with guided bone regeneration is one situation where an autologous platelet-rich clot clearly accelerates ossification after tooth extraction and/or around titanium implants. The end result is both marked reductions in the time required for implant stabilisation and an improved success rate. Orthopaedic surgery, muscle and/or tendon repair, reversal of skin ulcers, hole repair in eye surgery and cosmetic surgery are other situations where autologous platelets accelerate healing. Our aim is to review these advances and discuss the ways in which platelets may provide such unexpected beneficial therapeutic effects.

Keywords

Platelets, growth factors, cytokines, healing, dental implant surgery, tissue repair

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Introduction

Much is known about how platelets fulfil their function in haemostasis. Under the influence of shear, the GPIb-IX-V complex assures transient adhesion to exposed subendothelium, an interaction stabilised through the involvement of the collagen receptors α2β1 and GPVI (1, 2). The αIIbβ3 integrin is the principal mediator of platelet aggregation through its ability to bind multivalent adhesive protein ligands on the activated platelet (3). Fibrinogen and von Willebrand factor (VWF) are the major ligands forming bridges that crosslink platelets together. Soluble substances such as ADP released from injured vascular cells, red blood cells or adhered platelets, and newly generated thrombin, react with receptors of the seven transmembrane domain family ($P2Y_1$ and $P2Y_{12}$ for ADP; PAR-1 and PAR-4 for thrombin) and act in synergy with newly synthesised metabolites such as thromboxane A_2 (TXA_2) to promote platelet plug formation (4). Newly released or exposed proteins and other substances stimulate tissue repair and vascular remodelling. Specific cells are targeted, and leukocyte accumulation may be promoted. Components of the extracellular matrix (collagens, glycosaminoglycans, adhesive proteins) bind growth factors establishing chemotactic gradients for cell recruitment as well as a storage pool that can be secondarily released by metallo-

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proteases active in the matrix. Platelet-derived factors can influence cellular growth, morphogenesis and differentiation. The ability of platelets to release within a growing clot makes the latter a natural source of growth factors and cytokines that can be used therapeutically to accelerate natural healing processes.

How platelets can provide a benefit

We first briefly detail the proteins and other substances that are provided by platelets and which can participate in healing. Many are stored in distinct populations of granules easily distinguishable by electron microscopy and shown in cartoon form in Figure 1. Active metabolites are released by diffusion across the membrane, while the activated platelet provides a catalytic surface for thrombin generation as well as releasing procoagulant microparticles.

Dense granules

Substances stored in and released from dense granules are shown in Figure 1. Purinergic signalling by way of nucleotide binding to members of the P2Y and P2X receptor families can influence cell migration and proliferation and may determine vascular tone (5). ADP promotes platelet aggregation while ATP can act on $P2X_1$ and participates in the platelet response to collagen under flow. Ca^{2+} is a necessary cofactor for platelet aggregation and fibrin formation. It is also a potential central regulator in wound healing; for example Ca^{2+} can modulate keratinocyte proliferation and differentiation (6). Serotonin has receptors on vascular cells and its release leads to vasoconstriction and increased capillary permeability. Histamine can have pro- and anti-inflammatory effects. Thus already we can see how platelets profoundly influence the environment in the vicinity of their activation.

α-granules

Figure 1 groups and Table 1 details proteins stored in and secreted from α-granules (7, 8). Already substantial, this list will increase greatly as proteomics and genomics are applied to platelets. We have subdivided the proteins according to their functional properties. Abundant are the adhesive proteins: fibrinogen (Fg), fibronectin (Fn), vitronectin (Vn), and thrombospondin-1 (TSP-1). In haemostasis, a proportion become attached to platelet receptors during secretion and participate directly in thrombus growth. Even such an abundant protein as Fg may act as a mitogen, being first shown to potentialise the effect of interleukin-3 (IL-3) on human haematopoietic progenitors (9). Fn and Vn also participate in wound repair (10). Among fibrinolytic proteins, plasminogen activator inhibitor type I (PAI-1), as well as regulating fibrinolysis, can bind to vitronectin (Vn) promoting Vn multimer formation and enhancing cell/matrix interactions (11). Another protein from platelets able to form a complex with plasminogen and anchor it to collagen is osteonectin, a protein also secreted by osteoblasts (12). Recent studies have shown the release of thrombin activatable fibrinolysis inhibitor (TAFI) from platelets (13).

Among the stored mitogenic factors essential for wound repair are platelet-derived growth factor (PDGF) with the -AB and -C isoforms predominating, transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived epidermal

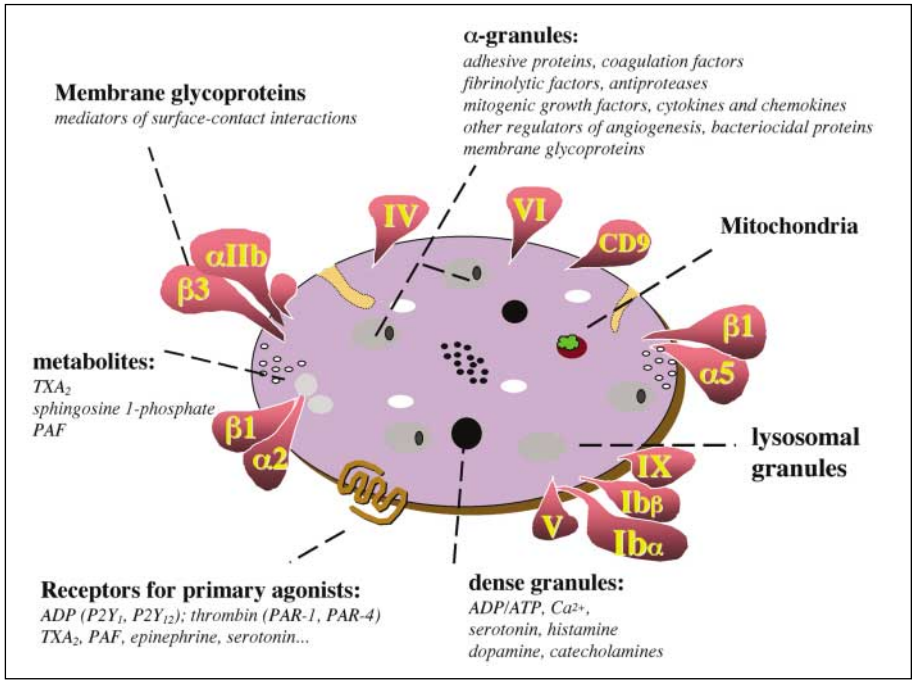

Figure 1: Cartoon showing the structure of a platelet with the major membrane glycoprotein mediators of the platelet involvement in haemostasis. A single seven transmembrane domain receptor regroups the primary receptors for ADP, thrombin, TxA_2 and others. Highlighted are intracellular organelles whose contents are secreted on activation and active metabolites synthesised during activation.

Table I: Platelet α-granule contents and their functional categories. This list of platelet α-granule proteins has been largely compiled from refs 8 and 13 although we have made additions. Proteins are classified in groups, yet many may have several potential functions. Secreted proteins with reported pro-angiogenic properties are underlined while those with anti-angiogenic potential are also identified (*). It should be noted that some of these may have both pro- and anti-angiogenic properties depending on the situation at the time of their release and/or the expression of cryptic sites. CD40L, tissue factor and P-selectin are membrane glycoproteins but may be cleaved from the platelet surface and released in soluble form or produced by alternative splicing in soluble form.

Category	Term	Biological activities
Adhesive proteins	VWF + pro-peptide, Fg, Fn*, Vn, TSP-1*, laminin-8	Cell contact interactions, clotting, extracellular matrix composition
Clotting factors and associated proteins	Factor V/Va, Factor XI, multimerin, gas6, protein S, high-molecular weight kininogen*, antithrombin*, tissue factor pathway inhibitor (TFPI)*	Thrombin production and its regulation, angiogenesis
Fibrinolytic factors and associated proteins	Plasminogen, PAI-I*, u-PA, osteonectin*, α2-antiplasmin* , histidine-rich glycoprotein, TAFI, α2-macroglobulin	Plasmin production and vascular modelling
Proteases and anti-proteases	Tissue inhibitor of metalloprotease-4 (TIMP-4)*, metalloprotease-4, platelet inhibitor of FIX, protease nexin-2*, C1 inhibitor, α1-antitrypsin*	Angiogenesis, vascular modelling, regulation of coagulation, regulation of cellular behaviour
Growth factors cytokines and chemokines	PDGF, TGF-β-1* and-2, EGF*, IGF-1, VEGF (A and C), bFGF and FGF-2, hepatocyte growth factor*, RANTES, IL-8, MIP-1α, growth-regulated oncogene-α, ENA-78, MCP-3, angiopoietin-1, IL-1β, IGF BP-3, neutrophil chemotactive protein	Chemotaxis, cell proliferation and differentiation, angiogenesis
Basic proteins and others	PF4*, β-thromboglobulin*, platelet basic protein, connective-tissue-activating peptide III, neutrophil-activating-peptide-2, endostatins*	Regulation of angiogenesis, vascular modelling, cellular interactions
Anti-microbial proteins	Thrombocidins	Bactericidal and fungicidal properties
Others	Chondroitin 4-sulfate, albumin, immunoglobulins	Diverse
Membrane glycoproteins	αIIbβ3, αvβ3, GPIb, PECAM-1, most plasma membrane constituents, receptors for primary agonists, CD40L, tissue factor, P-selectin	Platelet aggregation and adhesion, endocytosis ,of proteins, inflammation, thrombin generation, platelet-leukocyte interactions

growth factor (PDEGF) and insulin-like growth factor-1 (IGF-1) (8, 14-20). These are variously involved in stimulating chemotaxis, cell proliferation and maturation. PDGF is a powerful chemoattractant and stimulator of cell proliferation. All are potent angiogenic factors and endothelial cell mitogens. TGF-β is another 2-chain polypeptide and is abundant in platelets as well as in bone. Its effect is regulated by its activation from a latent form, and it may negatively influence angiogenesis although it promotes production of matrix proteins (14, 21). Whereas most growth factors are synthesised by megakaryocytes, IGF-1 and a modulating bone protein, IGFBP-3, resemble Fg in that they are captured by endocytosis prior to storage (22). IGF-1 is a single chain protein that binds to a specific cell surface receptor (IGF-I-R) and directly stimulates bone matrix formation and replication of osteoblasts and their precursors.

Electron microscopy reveals a dark nucleoid in α-granules, and proteoglycans such as chondroitin 4-sulphate are localised here. A family of basic proteins including platelet factor 4 (PF4) and β-thromboglobulin are packaged in close association with the proteoglycans. PF4, a CXC-chemokine, is a negative regulator of angiogenesis and a powerful inhibitor of endothelial cell proliferation (23, 24). PF4 can stimulate cells by binding to proteoglycans on their surface, although it also interacts with growth factors and IL-8. PF4 shares anti-angiogenic properties with other stored proteins including TSP-1 and the endostatins (see below), yet a majority of proteins secreted from α-granules react positively on angiogenesis (see 14 and Table I). PF4 is also a chemotactant for neutrophils and fibroblasts. Platelets store antibacterial and fungicidal proteins that could help prevent infection although this has yet to be proved. Two such proteins, termed thrombocidins, are C-terminal deletion products of CXC

chemokines being variants of neutrophil-activating peptide-2 and connective tissue-activating peptide-III (25). Anti-microbial peptide sequences are present in PF4, RANTES, platelet basic protein, and thymosin beta-4 as well as fibrinopeptides A and B released during clotting (26).

Concordant with a role in healing, platelets are a rich source of cytokines and chemokines (Table I). An example is RANTES, a chemokine deposited on inflamed endothelium by a platelet P-selectin-dependent mechanism, a deposition that creates a cell-associated signal leading to monocyte arrest (27). Proteoglycans recognise RANTES through its heparin-binding motifs. Other released chemokines of the CXC family include IL-8, MIP-1α, growth-regulated oncogene-α, ENA-8 and MCP-3 (28). These attract leukocytes and activate other platelets as well as modulating the production of inflammatory molecules by endothelial cells.

A much studied platelet cytokine is an intrinsic membrane glycoprotein known as CD40 ligand (CD40L) (29). Known for its role in the immune response, binding of CD40L to its receptor, CD40, on vascular cells leads to inflammation and integrin production, synthesis of interleukins and chemokines (30). CD40L is a substrate for a metalloprotease and released from activated platelets in a soluble form (sCD40L). Interestingly, this can rebind to αIIbβ3 through a KGD sequence suggesting that integrin clustering can present zones of CD40L with high avidity (31). Tissue factor (TF), the initiator of the extrinsic pathway of blood coagulation, is also a natural regulator of angiogenesis (32). Monocytes were originally thought to be the source of TF in circulating blood, but platelets are already labelled within a growing thrombus (33). In fact, TF is a membrane glycoprotein and it too is transported to the platelet surface from α-granules during exocytosis (34). TF can regulate plasminogen binding and activation (35) and may contribute to wound healing by, for example, inducing migration of cultured smooth muscle cells (36). Finally, the α-granule membrane mirrors the glycoprotein composition of the plasma membrane of unstimulated platelets. Present is the αIIbβ3 integrin, which recycles to and from the surface and assures the endocytosis of Fg (37). Also found are receptors for primary agonists (38). However, the most well studied of granule membrane glycoproteins is P-selectin, which on surface expression mediates platelet-leukocyte interactions (39).

Lysosomal granules

Platelets also contain lysosomal granules which can secrete acid hydrolases, cathepsins D and E, elastase and other degradative enzymes (7, 8).

Newly synthesised active metabolites

Platelets provide eicosanoids synthesised from arachidonic acid released from membrane phospholipids. TXA_2 is a powerful vasoconstrictor but is also involved in the injury-induced vascular proliferative response, a process regulated by prostacyclin (40). Also released is sphingosine 1-phosphate, a novel active metabolite able to stimulate mitogenesis. This is liberated from activated platelets during clot formation and stimulates fibronectin matrix assembly through a Rho-dependent signalling pathway (41). As well as promoting endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement, this sphingolipid can induce TF expression on endothelial cells (42, 43). Platelet-activating factor (PAF) is another platelet-derived bioactive lipid and can play a role in mediating leukocyte arrest and activation on endothelial cells or adherent platelets through P-selectin-dependent mechanisms 44).

Thrombin generation

Activation-dependent transport of phosphatidylserine (PS) to the outer surface of the platelet plasma membrane and microvesicle release result in procoagulant surfaces and thrombin generation independent of TF (45). Newly expressed PS participates in the binding of coagulation factors leading to a rapid formation of an activated factor Xa/Va complex that transforms prothrombin into thrombin in a Ca^{2+}-dependent process. Platelet α-granules store factor V associated with an abundant large protein termed multimerin (7, 8), and provide a source of subsequently activated factor V for the prothombinase complex. Thrombin is a powerful mitogen, and even factor Xa can elicit specific cellular responses (46).

Ways of administrating platelet-derived proteins and metabolites

It was in the early 1990s that fibrin glue (fibrin sealant or fibrin gel) was developed as a biomaterial with haemostatic and adhesive properties. Addition of platelets came later (47, 48). These preparations have since been variously described, and the literature contains terms such as PRP-gel, platelet gel, PRP-clot or plasma rich in growth factors (PRGF). Originally, platelet concentrates were prepared using a plasmapheresis system. Recently, machines have been tailored to achieve the optimal centrifugal separation of PRP for therapeutic use from small amounts (10-50 ml) of citrated blood (data critically reviewed in 49, 50). Basically, the aim is to rapidly prepare a PRP with a platelet count in excess of 300,000/μL. Clotting may be induced just prior to application at the therapeutic site. Although bovine thrombin has been used by some authors (47), this is to be avoided through the risk of antibody formation and induced Factor V deficiency (51). The use of recombinant human thrombin is a possible alternative. In our own studies in dentistry (see below), we take peripheral blood (minimum 10 ml) by venipuncture directly into 3.8 % (wt/vol) sodium citrate (1 vol : 9 vol). PRP is prepared by centrifugation at 460 x g for 8 min at room temperature and the 0.5 ml plasma fraction located just above the sedimented red cells but not including the buffy coat is collected. Glass tubes containing the PRP are incubated at

37°C in the presence of 22.8 mM $CaCl_2$ to start clot retraction. Following tooth extraction, we recommend that the developing clot be installed prior to retraction, a process that will result in proteins being extruded from the clot *in situ* (52). For some uses, the clot is allowed to develop *in situ*. One potential handicap is that platelet PDGF levels may vary between individuals and the need for pre-therapy dosing has been evoked (53).

Platelets and bone regeneration in dentistry and oral-maxillofacial surgery

Situations where guided secretion of autologous platelet products can promote healing and wound repair are listed in Table 2. The most widespread use of PRP-clots is in dentistry and oral-maxillofacial surgery. Platelets are activated at tooth extraction sites as a natural consequence of vascular disruption. There is substantial evidence that bone regeneration can be enhanced by positioning an additional source of autologous platelets in a fibrin clot at the extraction site and/or around the implant. Alternatively tried treatments include the application of recombinant bone morphogenetic proteins (BMPs) or growth factors (54, 55).

Bone regeneration requires the recruitment, proliferation and maturation of osteoblasts which are derived from mesenchymal stem cells (MSC) (56, 57). Although MSC are mainly localised in the bone marrow, a subpopulation is present in peripheral blood. Culture of MSC with dexamethasone or BMP-2 leads to the rapid development of the osteogenic cell lineage. *In vivo*, osteoblast differentiation involves cell-cell and cell-matrix interactions as well as multiple hormonal (parathyroid hormone, oestrogen) and local autocrine/paracrine factors. These include BMPs, themselves members of the TGF-β superfamily, whose activity is potentiated by $1,25(OH)_2D_3$ (58). Also involved are IGF-1, FGF and TGF-β (59). TGF-β may signal through Smad proteins (59). Osteoblast maturation involves transcriptional regulation (Runx2, AJ18, Dlx5…) and specific genes have been identified as being responsible for high bone mass (57). Human platelet concentrate was shown to promote the proliferative and functional activity of human fœtal osteoblast-like cells in both short term and long-term culture (60). In vitro studies suggest that platelet releasates also promote the migration of MSC (61).

Autologous platelet gel was first used by Whitman et al. (47) in reconstructive oral and maxillofacial surgery and as an adjunctive procedure related to the placement of osseointegrated titanium implants. Marx et al. (48) evaluated the effect of autologous PRP during bone graft reconstruction of mandibular continuity defects. PDGF and TGF-β from platelets were shown to have been adsorbed onto the grafts and it was concluded that addition of PRP accelerated the rate and degree of bone formation (48). In these and subsequent studies, platelets were hypothesised to provide a concentrated and directed supply of growth factors that stimulated migration and maturation of mesenchymal and epithelial cells (49). Autografts, allografts, xenografts and alveolar ridge augmentation procedures remain ways of increasing bone density in difficult cases, but even here the use of autologous platelets can positively affect the outcome (62, 63). Thus with PRP, radiographically, significant amounts of new bone were visible as early as 2 months postoperatively. PRP is also thought to accelerate soft tissue healing by promoting a more rapid revascularisation and also the re-epithelialisation of flaps caused by the surgical incision. The use of deproteinated bovine bone and PRP has been successfully tried in maxillary sinus augmentation with simultaneous insertion of endosseous implants (64).

Table 2: Autologous PRP-clots as an aid to healing

Therapeutic use of autologous PRP-clots
Maxillofacial surgery and bone grafts
Dental implant surgery
Orthopaedic surgery and bone reconstruction
Facial plastic and cosmetic surgery
Skin ulcers
Eye surgery - retinal hole repair
Sports medicine - cartilage and tendon repair

Our laboratory first studied the deposition of a PRP-clot with or without autogeneous bone in a series of 20 patients undergoing tooth extraction due to vertical fractures or severe periodontal disease (52). Subsequent implant placement was another precondition. In some patients with multiple extractions, PRP-clot was deposited into the socket on one side of the mouth while the other served as the control. Bone biopsies were taken at extraction sites between 10 and 16 weeks. In most of the patients with PRP-clot, bone regeneration was extensive and bone tissue was compact with well-organised trabeculae. In contrast, in the control group the cavity was mainly filled with connective tissue. Typical histochemical observations on biopsies from this study are shown in Figure 2. Epithelialisation was also clearly improved in the PRP group. Subsequently, we investigated the benefit of adding a platelet-rich clot around titanium implants used to anchor the dental prostheses (65, 66). Here, the implant surface is moistened with PRP and inserted into the alveolus. Then the peri-implant surface is irrigated with calcified PRP (3 vol) mixed with extruded supernatant (1 vol) from a retracted clot. This preparation clots within 15-30 seconds. Histochemical studies on biopsy sections clearly showed how implants installed with a platelet-rich clot had more dense bone with better-organised trabeculae at 2 to 3 months. Early studies with mice had shown that newly generated bone forms a tight interface with the titanium implant (67). Scanning environmental electron microscopy clearly shows how the plasma proteins react with the bioactive titanium implant surface (Fig. 3). Interestingly, the αIIbβ3 integrin mediates an initial platelet adhesion to titanium surfaces probably via adsorbed fibrinogen (68). An improved platelet interaction is seen with

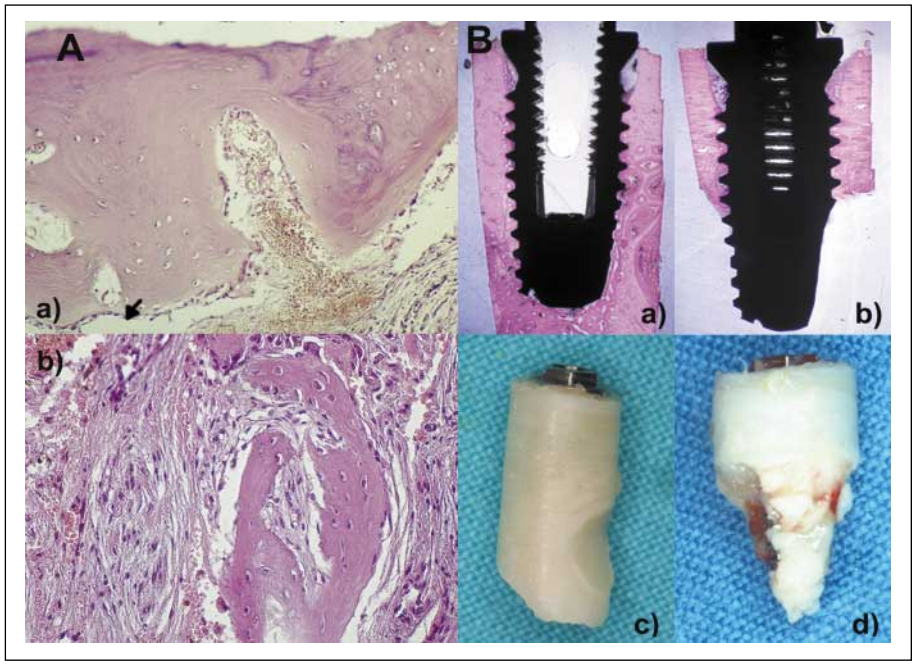

Figure 2: Illustrations of how the presence of a PRP-clot can promote bone regeneration following tooth extraction and implant surgery. Left hand side: A/Two tooth extraction sites from the same patient. Panel (a) shows a biopsy from the regenerated area of a cavity receiving a PRP-clot and which after 3 months contained hard compact bone and well-organised bone trabeculae. Panel (b) shows the site without a PRP-clot, at 3 months a large part of the cavity was filled with a dense fibrous connective tissue with few bone trabeculae. Right hand side: B/ Analysis of the effect of a PRP-clot on bone regeneration around a titanium implant in an animal model. Implants were placed in the tibia of anaesthetised goats in the presence or not of a PRP-clot. Bone structure was examined histologically after 8 weeks. In panel (a), a stained section shows the compact cortical structure of the bone surrounding the entire implant. A typical section from a control implant is shown in panel (b) where soft tissue has been lost from the apical portion during the biopsy. Panels (c) and (d) respectively show macroscopic views of similar treated and control implants.

titanium surfaces that are micro-roughened rather than smooth (69). A direct osteoblast adhesion to titanium surfaces has also been described with integrin-mediated intracellular signalling and activation (70). Osteoblasts appeared to proliferate and differentiate on the titanium surface while the role of integrins suggests that adsorbed plasma proteins such as fibronectin served as intermediates for their attachment.

Studies using animal models confirmed the results being seen with humans. Thus, Anitua and Andia Ortiz (65) showed that adding PRP-clot around roughened titanium implants at the moment of their implantation in goats improved both the extent and the quality of bone regeneration around the implant. Typical findings from this study are illustrated in Figure 2. In more recent work, Zechner et al. (71) assessed the time course of local bone formation in minipigs following the application of PRP-clot during titanium implant placement. These authors used a split mouth model where after premolar extraction, PRP-clot was added on one side with the other acting as the control. Animals were sacrificed at 3, 6 and 12 weeks. Histomorphometric analysis showed that addition of PRP-clot resulted in increased bone-to-implant contact at 3 and 6 weeks and was primarily effective during the early healing phase. In another model, Stefani et al. (72) had earlier shown that a combination of recombinant PDGF and IGF-1 promoted peri-implant bone regeneration in the early phase.

Bone remodelling involves forming a balance between the resorbing activity of osteoclasts and the matrix-generating capacity of osteoblasts (57). Osteoclasts are haematopoietic cells derived from the monocyte/macrophage lineage that differentiate in the bone microenvironment. Osteoblasts/stromal cells secrete osteoprotegerin (OPG), a dimeric member of the tumour necrosis factor receptor superfamily and an inhibitor of bone resorption (73, 74). OPG reacts with a membrane receptor known as RANKL (TRANCE and OPGL). RANKL binds to RANK receptor (receptor activator of nuclear factor κB) and modulates transcription factor expression in differentiating osteoclasts (75). Increasing evidence points to RANKL and OPG as paracrine regulators of bone metabolism and vascular function (76). Interestingly, platelet releasates stimulate formation of osteoclast-like cells through a COX-2/RANKL-dependent mechanism (77). Endogenously produced PGE_2 was proposed to increase the RANKL:OPG ratio. Osteoclastic bone resorption requires cell-matrix contact, a process that was abnormal in β3 integrin knockout mice but rescued following

full expression of the full-length β3 integrin showing an absolute requirement for αvβ3 (78). A role for β3 signalling was proposed.

PDGF, a multiple mitogen, stimulates osteoblast replication and bone collagen degradation and is a key factor in bone metabolism. Osteoblasts express PDGF receptors and PDGF induces their proliferation (79, 80). PDGF-AA was identified as an autocrine of normal human adult osteoblasts and its expression is upregulated in foetal cells by exogenous PDGF-AA, PDGF-BB, TGF-β and bFGF (80). Interestingly, there is already evidence that TGF-β may regulate the response of cells to PGDF and PDEGF (14) while it has been known for some time that TGF-β is able to initiate bone formation (81). IGF-1 is another factor coming from platelets but it is also synthesised by primary osteoblast cultures where its activity is modulated by IGF-binding proteins whose secretion is regulated by PGE_2 (82). So in some ways platelets mimic and up-regulate the osteoblasts own activation machinery.

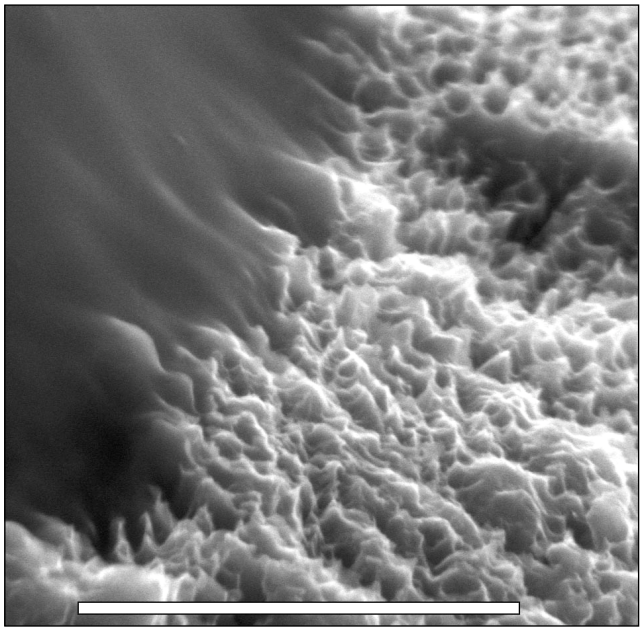

Figure 3: Visualisation of the interface between a PRP-clot and the titanium surface of the implant (BTI industry, Vitoria, Spain). A small volume (100 µl) of citrated PRP was placed on the implant surface in the presence of Ca^{2+}. The surface boundary was directly visualised using an Electroscan 2020 environmental scanning electron microscope without prior specimen preparation. Note the tight interface and close contact between the clot (dark zone) and the micro-roughened surface of the implant. Magnification × 6000

Other situations where autologous platelets have been shown to aid healing

The wound-healing process is typically divided into three phases (inflammatory, proliferative and remodelling). As discussed in the first section, platelets and their released cytokines and growth factors are pivotal elements of each of these processes.

Plastic surgery

Autologous platelets are especially useful for the soft tissue and bony reconstruction encountered in facial plastic and reconstructive surgery (83). Their use results in a decrease in operative time, necessity for drains and pressure dressings, and incidence of complications. Reduced infections and length of hospital stay in plastic surgery was the conclusion of Valbonesi et al. (84) who used autologous fibrin-platelet glue in 14 patients with skin and soft tissue losses caused by recent trauma or chronic pathology. This points to useful bactericidal properties as well as cell proliferation promoting properties; proteins capable of both are present in platelet releasates (Table I). Anti-inflammatory properties with reduced oedema and ecchymosis was associated with the use of autologous platelet gel in 8 women after deep plane rhytidectomy (85). Autologous platelet-rich gel was also shown to be effective in stopping capillary bleeding in the surgical flaps of a series of 20 patients undergoing cosmetic surgery (face lifts, breast size changes or neck lifts) (86). An increased recovery time is often observed.

Wound healing (ulcers)

As early as 1990, autologous human platelet-derived wound healing factors (HPDWHF) were proposed to regulate wound healing of recalcitrant skin ulcers by promoting the formation of granulation tissue in the early healing phase (87). This conclusion was based on studies on 23 patients with 27 skin ulcers who had shown no signs of healing after an average period of 25 weeks conventional wound care. Strikingly, 100% healing was seen an average of 10 weeks after the application of HPDWHF. Interestingly, more recent studies have shown an increased expression of αvβ3 integrin after application of HPDWHF during healing of venous leg ulcers and this was linked to a potentiation of angiogenesis at sites of newly formed granulation tissue (88). Factors potentiating angiogenesis are released from platelets (Table I). Foot ulceration is a common complication of diabetes. The wounds are often multifactorial but arise in the setting of peripheral neuropathy and/or vascular complications. Platelet releasate has been used on thousands of patients over a ten year period in the USA, and an analysis of results for these patients in an American Health Service database allowed Margolis et al. (89) to conclude that use of platelet releasate is of proven efficacy, especially for patients with more severe wounds. The success of such a treatment is illustrated by two European case reports. First, Tarroni et al. (90) successfully used a platelet gel composed of a mix of concentrated platelets and cryoprecipitate activated by batroxobin in the presence of calcium chloride for a chronic foot ulcer in a 62-year-old diabetic man. His ulcer recovered after 8 platelet applications and leg amputation was avoided. The repeated application of a lysate of autologous platelets as a source of

PDWHF was recently also shown to be successful for a β-thalassemia patient with an ankle ulcer of > 4 years duration. Complete healing was seen within a month (91).

Orthopedic surgery

Autologous growth factor concentrate (AGF) prepared by ultra-concentration of platelets is being used in patients undergoing lumbar spinal fusion. As with bone regeneration around titanium implants, the hypothesis is that platelets release multiple growth factors having a chemotactic and mitogenic effect on mesenchymal stem cells and osteoblasts and therefore accelerate bone healing. Lowery et al. (92) reported on their experience using AGF in a 39 patient study group in the USA. In particular, they concentrated on 19 patients with at least a 6 months follow-up (15 posterior and 4 anterior intradiscal fusions). AGF was used with autograft and coraline hydroxyapathite in all posterior fusions, and with autograft, coral and intradiscal spacer in intradiscal fusions. Autologous iliac crest bone graft was used in 15 cases and local autograft in 5 cases. Solid bone fusion was reported in 5 patients, although this was a study without controls. Interestingly, in an animal model evaluating mandibular construction, Fennis et al. (93) reported that PRP appeared to enhance bone healing considerable in goats after being mixed with a particulate bone graft. Clinical uses in healing complicated bone fractures and during total joint replacement are often now considered.

Eye surgery

A novel potential use of platelets is in retina repair. In a double-masked randomised trial on 110 French patients undergoing surgery for stage 3 or 4 idiopathic full-thickness macular holes, half of the patients additionally received an injection of autologous platelet concentrates (94). One month after surgery, the anatomic success rate for hole closure was significantly greater for those receiving platelet concentrates. In fact, 52 out of 53 patients showed reapposition of the edge of the hole with no complications due to the use of the platelets. In the control group, anatomic success was seen in 47 of 57 patients. Gehring et al. (95) showed anatomic closing of Stage II to IV macular holes in 18 of 19 patients treated with autologous platelet concentrates. Using a rabbit model, Cullinane et al. (96) showed that adjuvant treatment with autologous platelet concentrate resulted in an increased proliferative cellular response in the healing of retinal wounds.

Tendon and ligament repair

The strength and quality of abdominal wall hernia repair with a resorbable PGA (polyglycolic acid) mesh was improved in a rat model by the use of a sublay procedure with fibrin glue or platelet releasates (97). Among the findings were an increased amount of collagen fibres and increased numbers of fibroblasts. A recent case report involving our laboratory has suggested that injecting calcified autologous PRP may facilitate anterior cruciate ligament reconstruction and reattachment of knee articular cartilage in man (98). A role for endogenously released growth factors including IGF-1, TGF-β, VEGF, PDGF and bFGF in tendon and ligament healing is well documented (99). They variously participate in each of inflammation, cell proliferation and tissue remodelling. Autologous platelets would therefore provide an additional source of the above factors. We subsequently reported their use in arthroscopic surgery (100). Current and promising applications of our group involve the use of calcified autologous platelets in achilles tendon and muscle repair (Sanchez M, Anitua E and Andia I, unpublished data). Interestingly, growth factor-dependent proliferation and invasion of muscle satellite cells require the cell-associated fibrinolytic system (101). Sports medicine is a new potential field for this procedure.

Potential problems and risks

So far we are unaware of major health problems that have arisen through the therapeutic use of autologous PRP-clots. Obviously, high haematocrits or low platelet counts may be a limiting factor and further research is required to establish the optimum number of platelets to apply. As well as secreting proteins, platelets release small molecular weight diffusable compounds (Fig. 1) and release large numbers of microparticles that carry proteins such as TF or Il-1β and which are prothrombotic (102, 103). Therefore, care may be required in using this procedure in the vicinity of large blood vessels, especially in patients with known thrombotic risk factors. The parallel use of anti-platelet medications could theoretically limit efficacy. The taking of aspirin, an anti-platelet drug would appear unavoidable for some conditions, but experience of studies on stomach ulcers is interesting to consider. Ma et al. (104) showed that ulcer induction in rats was associated with increases in the serum levels of VEGF and decreases in endostatins (an antiangiogenic factor) (Table I). Interestingly, the anti-platelet drug, ticlopidine (an ADP receptor antagonist), impaired gastric healing and angiogenesis as well as reversing the changes in circulating levels of both class of protein, an effect mimicked by immunodepletion of circulating platelets. Endostatins are derived from the C-terminal domains of collagens XV and XVIII and bind to heparan sulfate proteoglycans and matrix metalloproteases which they inhibit (105). How they come to be part of the platelet releasable pool remains to be shown but as they are proteolytic products (~ 20 kDa) of a matrix protein, it is highly probable that they are endocytosed. Such studies show how drugs may effect healing and underline how changes in the balance of secreted products from platelets may influence their pro- or anti-angiogenic effect.

Concluding remarks

The promotion of bone healing by PRP-clots has interested orthopaedic surgeons while in dentistry they are used as an aid

to implantation as well as in oral and maxillofacial surgery (106). In this latter context, their use is becoming common particularly to our knowledge in the USA, Spain, Germany, Portugal and Argentina. Our experience now extends to the use of PRP-clots in around 4000 extraction sites and 2500 implants. Their use accelerates significantly the stabilisation of the implants in solid bone and virtually eliminates the risk of implant failure. Nevertheless, despite these advances, it is too early to provide a complete molecular explanation for the role of the platelet. Healing is a complex process in which a myriad of cellular and humoral components interact. Release of growth factors, cytokines and chemokines will provide a stimulus for nearby cells both in terms of chemotaxis, proliferation and maturation. For example, in terms of bone regeneration, PDGF and TGF-β have a strong and synergistic chemotactic effect on human osteoblasts, responses also aided by BMP-2 (55). A thick matrix rapidly surrounded Millipore filters that were soaked in TGF-β and placed in exposed dental pulps in dogs, matrix formation being lost in the presence of a TGF-β neutralizing antibody (107). Fibrin or other adhesive proteins in a clot actually retain growth factors and present them to migrating cells. This is an advantage in situations where occlusive dressing therapy or topical administration of recombinant growth factors as practised in cutaneous wound healing is impractical (108). PDGF can bind to extracellular proteins, while TGF-β and other factors can bind to Vn either in the matrix or in soluble form (109). Vn and Fn are widely distributed in tissues. VEGF is retained in fibrin clots where it maintains its ability to induce endothelial cell proliferation, monocyte migration with enhanced secretion of IL-6 and IL-8 (110). FGF-2 (present in platelets) binds fibrin and supports prolonged endothelial cell growth with the endothelial cells actually attaching through VE-cadherin (18, 111). Overall, fibrin can be considered as a matrix reservoir both protecting and presenting specific growth factors to support cell growth at sites of injury. It also acts as a physical barrier around a wound site

Integrins are required for cell survival and proliferation during development (112). Platelet contact interactions mediated by integrins may have an active role in healing; for example, platelet surface membranes are highly mitogenic for smooth muscle cells and act through a PDGF-independent mechanism (113). Unravelling the roles of individual platelet will require the development of mouse models and the use of transgenic in culture models deficient in one or more of the players as well as the use of microarray systems capable of revealing the response of specific cell types to contact with platelet releasates. Backtracking along signalling pathways and knockout of specific receptors or secreted proteins will help identify primary response signals. Also, more research is required to establish the optimum number of platelets that need to be applied.

As well as possessing classical pathways of granule secretion and eicosanoid generation, platelets have recently been shown, somewhat surprisingly for anucleate cells, to contain mRNA for interleukin 1β precursor (102). The mRNA was localised in polysomes and platelet activation was accompanied by a rapid and sustained synthesis of pro-IL-1β protein, a response that was inhibited by translational inhibitors. IL-1β is an inflammatory cytokine and IL-1β mRNA is translated into protein by activated platelets during fibrin clot formation and was shown to be associated with the fibrin lattice (102). Its synthesis will create a continuous stimulus over a longer duration; the newly formed IL-1β is able, for example, to react with endothelial cells and induce PMN adhesion via P-selectin. The synthesis of IL-1β appeared to require signalling via β3 integrins. Interestingly, integrin-mediated cell attachment and growth factor stimulation often act synergistically on cell proliferation, differentiation, migration and survival. An example is the demonstrated physical interaction between αvβ3 through the β3 extracellular domain and two receptor tyrosine kinases (PDGF-Rβ and VEGF-R2) (114). Activation of the kinases by their ligands promoted cell proliferation and migration when cells are attached to Vn. Cross-talk between receptors may be a key element of the healing process. Similarly, crosstalk between secreted components may also occur. For example, plasma or secreted fibronectin may potentiate the mitogenic activity of PDGF and complement its wound healing effects (115).

It is clear that platelet-rich fibrin clots constitute a bioactive reservoir. It is interesting that platelets are a source of proinflammatory proteins such as CD40L, yet it is our experience that applying a platelet-rich clot around a titanium implant actually decreases inflammation (Anitua E, personal observation). The molecular basis for this will be interesting to elucidate. It should not be forgotten either that clot formation is not the end point of a vessel's response to injury but a major step that is followed by activation of the complement cascade and the fibrinolytic pathway with release of other biologically active molecules only some of which have been covered by this review. Plasminogen dependent activation of latent TGF-β has been shown in growing cultures of osteoblast-like cells showing a potential regulating role of the fibrinolytic system (116). In fact, it is the complexity of the healing process that may make the use of an autologous platelet-rich clot preferable to the use of recombinant proteins either alone or in combination. Although gene transfer with modified expression of growth factors and/or cytokines may become a potential alternative in, for example, periodontal tissues (117), the accompanying risk would seem incompatible with routine dental implant surgery. MSC transplant therapy is also potentially an exciting future evolution but will require a source of compatible osteoblast progenitors.

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