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Platelets and wound healing

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1. ABSTRACT

Platelets help prevent blood loss at sites of vascular injury. To do this, they adhere, aggregate and form a procoagulant surface favorizing thrombin generation and fibrin formation. In addition, platelets express and release substances that promote tissue repair and influence processes such as angiogenesis, inflammation and the immune response. They contain large secretable pools of biologically active proteins, while newly synthesized active metabolites are also released. Although anucleate, activated platelets possess a spliceosome and can synthesize tissue factor and interleukin-1beta. The binding of secreted proteins within a developing fibrin mesh or to the extracellular matrix can create chemotactic gradients favoring the recruitment of stem cells, stimulating cell migration and differentiation, and promoting repair. The therapeutic use of platelets in a fibrin clot has a positive influence in clinical situations requiring rapid healing. Dental implant surgery, orthopaedic surgery, muscle and tendon repair, skin ulcers, hole repair in eye surgery and cardiac surgery are situations where the use of autologous platelets accelerates healing. We now review the ways in which platelets participate in these processes.

2. INTRODUCTION

Blood platelets are produced in large numbers from megakaryocytes (MKs), polyploid cells that differentiate and mature in the bone marrow (1). Anucleate, platelets circulate for 7 to 10 days and mediate primary hemostasis. Much is known about how platelets fulfil this function. Briefly, under the influence of shear, the GPIb-IX-V complex assures transient adhesion to von Willebrand factor (VWF) in exposed subendothelium, an attachment stabilised not only through the involvement of the collagen receptors alpha2beta1 and GPVI but also the alphaIIbbeta3 integrin (2, 3). Integrin alphaIIbbeta3 is the principal mediator of platelet aggregation acting through its ability to bind multivalent adhesive protein ligands after platelet activation (3, 4). Fibrinogen (Fg) and 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 adhering platelets, and newly generated thromboxane A2 (TXA_2), react with receptors of the seven transmembrane domain family ($P2Y_1$ and $P2Y_{12}$ for ADP; TPalpha for TXA_2) and act in synergy to promote platelet plug formation. Platelets become procoagulant by (i) exposing anionic phosphatidylserine on

Table 1. Platelet α-granule contents and their functional categories

Category	Protein	Function
Adhesive proteins	VWF + pro-peptide, Fg, Fn, Vn, TSP-1, laminin-8 (alpha4- and alpha5-laminin subunits), SCUBE1	Cell contact interactions, hemostasis and clotting, extracellular matrix composition
Clotting factors and associated proteins	Factor V/Va, Factor XI-like protein, multimerin, protein S, high-molecular weight kininogen, antithrombin III, tissue factor pathway inhibitor (TFPI)[1]	Thrombin production and its regulation
Fibrinolytic factors and associated proteins	Plasminogen, PAI-I, u-PA, alpha2-antiplasmin, histidine-rich glycoprotein, TAFI, alpha2-macroglobulin	Plasmin production and vascular modelling
Proteases and anti-proteases	Tissue inhibitor of metalloprotease 1-4 (TIMPs 1-4), metalloprotease-1, -2, -4, -9, ADAMTS13, TACE, platelet inhibitor of FIX, protease nexin-2, C1 inhibitor, serpin proteinase inhibitor 8, alpha1-antitrypsin	Angiogenesis, vascular modelling, regulation of coagulation, regulation of cellular behaviour
Growth factors	PDGF, TGF-beta1 and -beta2, EGF, IGF-1, VEGF (A and C), bFGF (FGF-2), HGF, BMP-2, -4, -6, CTGF	Chemotaxis, cell proliferation and differentiation, angiogenesis
Chemokines, cytokines and others	RANTES, IL-8, MIP-1alpha, ENA-78, MCP-3, GRO-alpha, angiopoietin-1, IGF-BP3, IL-6sR, PF4, beta-TG, platelet basic protein, NAP-2, connective-tissue-activating peptide III, HMGB1, FasL, LIGHT, TRAIL, SDF-1alpha, endostatins[1], osteonectin[1], bone sialoprotein	Regulation of angiogenesis, vascular modelling, cellular interactions, bone formation
Anti-microbial proteins	Thrombocidins	Bactericidal and fungicidal properties
Others	Chondroitin 4-sulfate, albumin, immunoglobulins, disabled-2, semaphorin 3A, PrP^C	
Membrane glycoproteins	alphaIIbbeta3, alphavbeta3, GPIb, PECAM-1, most plasma membrane constituents, receptors for primary agonists, CD40L, tissue factor, P-selectin, TLT-1, furin?	Platelet aggregation and adhesion, endocytosis of proteins, inflammation, thrombin generation, platelet-leukocyte interactions

[1]This list of proteins is as complete as possible but does not include the results of proteomic analyses (64). Although most proteins are mentioned in the text, space restrictions did not allow the addition of references for them all.

their surface and (ii) releasing tissue factor (TF)-enriched microparticles (a process involving cross-talk with leukocytes) (5). In so doing, they form catalytic surfaces accelerating thrombin formation. The newly generated thrombin further stimulates platelet aggregation and transforms plasma Fg into a fibrin network around the plug in which leukocytes and red cells become trapped. Platelets permit retraction and consolidation of what is now a blood clot.

Recent evidence suggests that platelets may also have a new and previously unsuspected role in tissue repair and vascular remodelling as well as being active players in the inflammatory and immune responses (6-8). By liberating biologically active proteins and other substances they are able to influence a range of process promoting the recruitment, growth and morphogenesis of cells. Such substances are either released or presented on the activated platelet surface. Components of the extracellular matrix (collagens, glycosaminoglycans, adhesive proteins), or the fibrin network of the clot, bind platelet-derived growth factors and cytokines establishing chemotactic gradients facilitating cell recruitment, as well as constituting a storage pool that can be secondarily released by metalloproteases (MMPs) active in the matrix. The ability of platelets to release within a growing clot makes the latter a natural autologous source of growth factors and cytokines that can be used therapeutically to accelerate natural healing (7, 9).

3. HOW PLATELETS PARTICIPATE IN TISSUE HEALING

We first briefly detail the proteins and other substances that are provided by platelets and which can participate in repair and tissue healing. Many are stored in alpha-granules easily distinguishable by electron microscopy and by immunofluorescent staining (Figures 1 and 2). Proteins are secreted by exocytosis by the formation of secretory vesicles that fuse with the plasma membrane allowing the liberation of their contents to the milieu (10). Newly synthesized 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 by a process that has been related to apoptosis (5).

3.1. Dense granules

Substances stored in and released from dense granules are not only important cofactors of platelet aggregation but are also involved in other biologic processes. 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 (11). Ca^{2+} is a necessary cofactor for platelet aggregation and fibrin formation. It is also a potential central regulator in wound healing (12). Serotonin has receptors on vascular cells and its release from platelets leads to vasoconstriction and increased capillary permeability. Recently, platelet-derived serotonin has also been shown to mediate liver regeneration (13). Histamine can have pro- and anti-inflammatory effects while the roles of small pools of stored catecholamines such as noradrenaline and dopamine need further investigation.

3.2. Alpha-granules

Table 1 details many of the proteins stored in and secreted from alpha-granules. Where possible, we have subdivided the proteins according to their functional properties. Abundant are the adhesive proteins: Fg, fibronectin (Fn), vitronectin (Vn), and thrombospondin-1 (TSP-1). A subpopulation of these adhesive proteins becomes attached to platelet receptors during secretion, and this is shown for Fg in Figure 1. They participate directly in thrombus growth. Platelets will therefore supplement the plasma source of these proteins at injured sites. Fg may act as a mitogen, being first shown to potentiate the effect of interleukin-3 (IL-3) on human haematopoietic progenitor

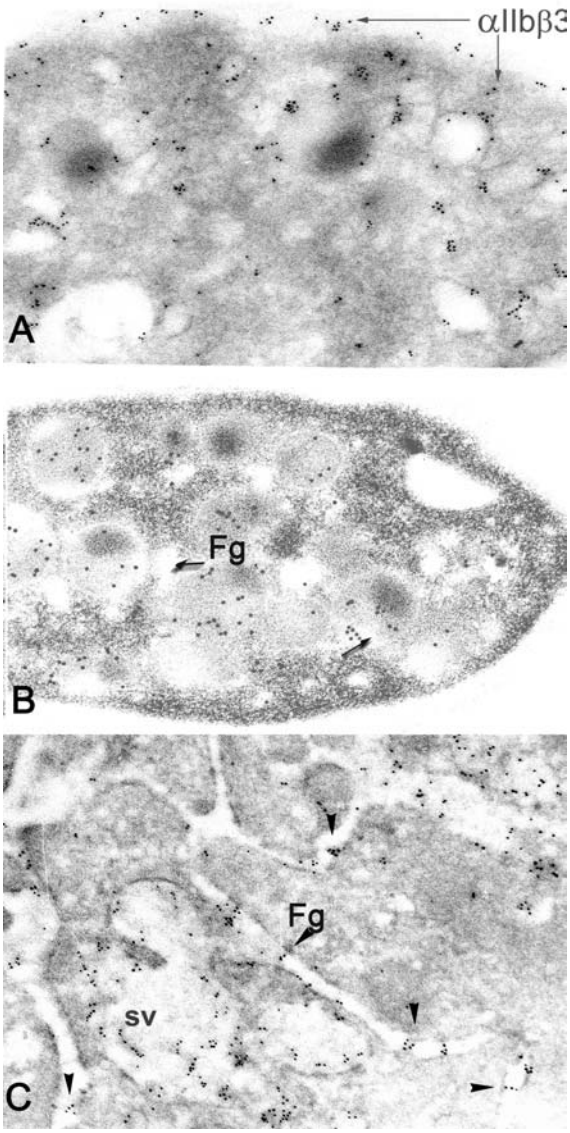

Figure 1. Electron microscopy showing secretion and the formation of protein bridges between aggregated platelets. Immunolabeling with rabbit antibodies was performed on sections of Lowicryl-embedded platelets, with bound IgG visualized with species-specific anti-IgG antibodies adsorbed on gold beads. In A) is shown the immunolocalization of the alphaIIbbeta3 integrin (GPIIb-IIIa complex) on sections of unstimulated human platelets. Gold beads representing bound IgG clearly identify surface and internal integrin pools (arrows). In B) is shown the localization of fibrinogen within the alpha-granules of unstimulated platelets. Note the typical morphology of the alpha-granules often with an electron-dense core. Fg is only found within the granules. In C) platelets have been allowed to aggregate by stimulation with thrombin. The alpha-granules have undergone fusion to form secretory vesicles (sv) that open to the surface. Both soluble exogenous Fg and secreted Fg binds to the activated alphaIIbbeta3 integrin; and the bound Fg links the platelets together by forming protein bridges clearly identified by the arrow heads.

cells (14). Fn also participates in wound repair and it promotes the mitogenic activity of PDGF (15). Among fibrinolytic proteins, plasminogen activator inhibitor type I (PAI-1), as well as regulating fibrinolysis, can bind to Vn, promoting multimer formation and enhancing cell-to-matrix interactions (16). Both soluble and cellular components of the fibrinolytic system (PAI-1, uPAR) are recognized regulators of cell adhesion and migration mechanisms through their interaction with integrins (17). Another protein from platelets able to form a complex with plasminogen and anchor it to collagen is osteonectin, a protein also secreted by osteoblasts (18).

Among the stored growth factors essential for wound repair are platelet-derived growth factor (PDGF) with the -AB and -C isoforms predominating in platelets; also present are transforming growth factor beta1 (TGF-beta1), vascular endothelial growth factor (VEGF, essentially VEGF-A), basic fibroblast growth factor (bFGF, also known as FGF-2), hepatocyte growth factor (HGF), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) (7, 19). Some examples of their alpha-granule localization are shown in Figure 2. PDGF is a powerful chemoattractant and stimulator of cell proliferation (20). Like other secreted factors, PDGF may not work alone, for example its effect on vascular smooth muscle cells may be promoted by TSP-1 (21). TGF-beta1 is a 2-chain polypeptide and is abundant in platelets as well as in bone. TGF-beta family members are important in wound repair and scarring. TGF-beta1 function is regulated by its activation from a secreted latent form; it may negatively influence angiogenesis although it promotes production of matrix proteins (22). In fact, platelets are able to activate TGF-beta1 in a time-dependent fashion and the involvement of a furin-like enzyme has been proposed (23). TSP-1 is a strong promoter of latent TGF-beta1 activation during fibrogenesis (24). TGF-beta1 recruits inflammatory cells into the wound area, apparently at the expense of a delay in re-epithelialisation. TGF-beta1 can then promote connective tissue production by fibroblasts. It has been shown to stimulate tendon cells to produce VEGF (25). Whereas most growth factors are synthesised by MKs, IGF-1 and a modulating bone protein, IGFBP-3, resemble Fg in that they are captured by endocytosis prior to storage (26). IGF-1 is a single chain protein that binds to a specific cell surface receptor (IGF-1-R) and directly stimulates bone matrix formation and replication of osteoblasts and their precursors. VEGF constitutes a family of proteins that act through a cognate receptor kinase family expressed in endothelial cells to stimulate blood vessel formation (27). VEGF exerts trophic effects on endothelial cells (28). It can also be pro-inflammatory and stimulate the adhesion of leukocytes to endothelial cells, a function suppressed by HGF acting through the NF-kB transcription factor (29). Yet again this shows interplay between secreted factors. An interesting family of proteins belonging to the TGF-beta family are the bone morphogenetic proteins (BMPs), three members of which (BMPs-2, -3, and -4) are synthesised by MKs and stored in the alpha-granules (30).

There is functional organisation in alpha-granules, for example proteoglycans such as chondroitin 4-

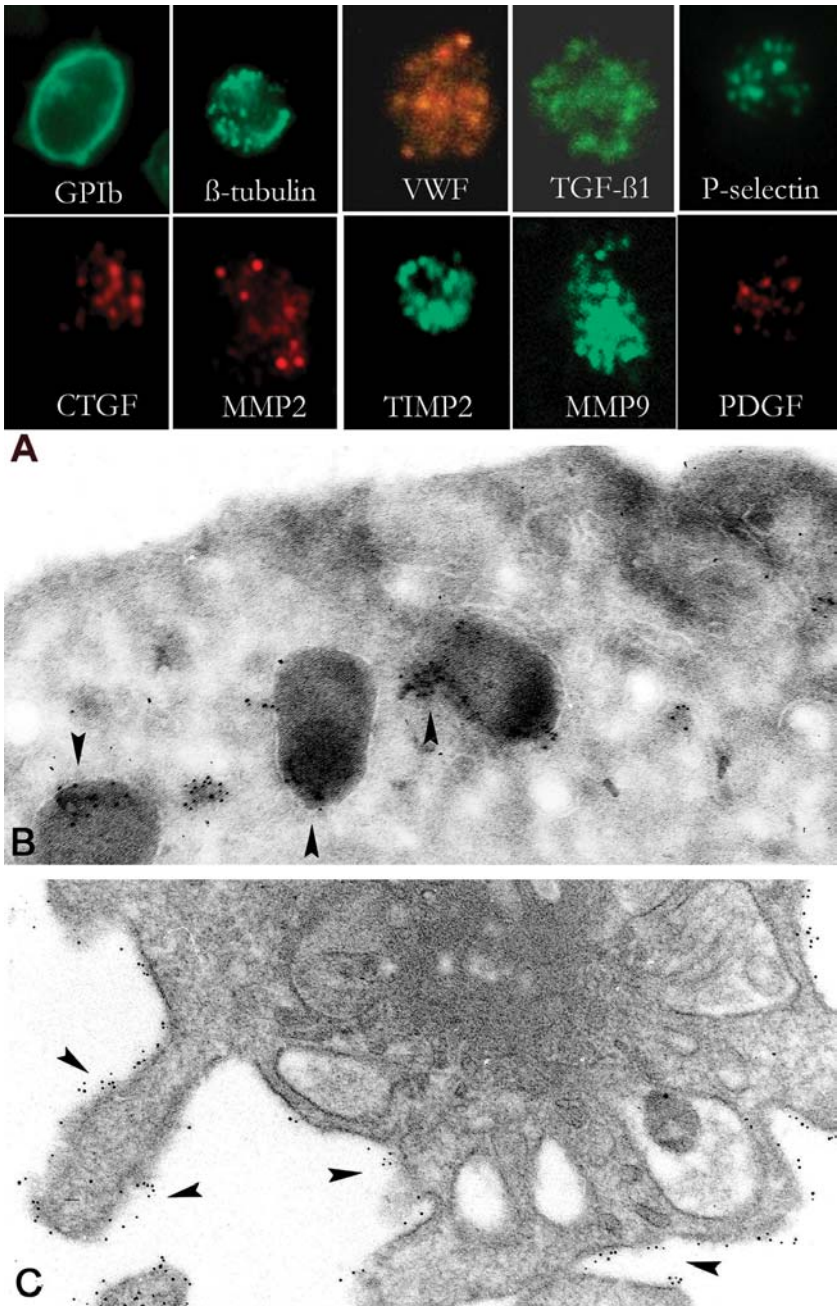

Figure 2. Detection of alpha-granule proteins in human platelets. A) Immunofluorescence detection of proteins in permeabilized cells. Unstimulated platelets in PRP were fixed in 1% paraformaldehyde and permeabilised using 0.1% Triton X-100 to ensure antibody access to internal protein pools. After blocking non-specific binding sites with bovine serum albumin, proteins were detected using predetermined optimal concentrations of specific monoclonal or polyclonal antibodies to the target protein followed by species-specific secondary antibodies to mouse or rabbit IgG. The latter were conjugated with Alexa468 (red) or Alexa488 (green) and bound antibody revealed in a fluorescence microscope. Note the annular surface fluorescence of membrane GPIbalpha, and the submembranous localization of beta-tubulin. The proteins VWF, TGF-beta1, CTGF and PDGF are clearly localised in alpha-granules, also identified by their labeling with P-selectin. Whereas the bulk of MMP2 and MMP9 are probably also alpha-granule-bound, that of TIMP2 resembles in part that of beta-tubulin suggesting a different localization. B) Electron microscopy used to show the localization of P-selectin in unstimulated platelets. Immunogold labeling with a mouse monoclonal antibody was performed on sections of Lowicryl-embedded sections. P-selectin is shown associated with alpha-granule membranes (arrow heads) and small secretory vesicles. C) Electron microscopy used to show the surface localization of P-selectin after secretion. The platelets have been stimulated with thrombin, and P-selectin is now evenly distributed on the platelet surface (arrow heads) where it can serve as a receptor for PSG-L on neutrophils and monocytes.

sulphate are localised in discrete domains. A family of basic proteins including platelet factor 4 (PF4) and beta-thromboglobulin (beta-TG) (CXC family members) are packaged in close association with the proteoglycans (7). PF4 is a negative regulator of angiogenesis and a powerful inhibitor of endothelial cell proliferation (31). PF4 can interact with cells by binding to proteoglycans on their surface or to specific CXC receptors, although it also binds to and inhibits growth factors such as VEGF and FGF. PF4 shares anti-angiogenic properties with other stored proteins including TSP-1 and the endostatins (see below) (see (14) and Table 1). PF4 is a chemotactant for neutrophils and fibroblasts; it can also participate in stem cell recruitment (32, 33). Beta-TG, neutrophil activating peptide (NAP-2) and additional beta-TG variants are generated from a precursor, platelet basic protein. Platelets store antibacterial and fungicidal proteins that help prevent infection. Two such proteins, termed thrombocidins, are C-terminal deletion products of CXC chemokines being variants of NAP-2 and connective tissue-activating peptide-III (34). 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 (35).

Concordant with a role in healing, platelets are a rich source of cytokines and chemokines (Table 1). 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 also involving PF4 that promotes monocyte arrest (32). Proteoglycans recognise RANTES through its heparin-binding motifs. Other released chemokines of the CXC family include IL-8, MIP-1alpha, growth-regulated oncogene-alpha, ENA-78 and MCP-3 (reviewed in (36)). 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, CD154) (37). Known for its role in the immune response, binding of platelet-bound CD40L to its receptor, CD40, on vascular cells leads to inflammation and integrin production, as well as to the synthesis of interleukins and chemokines. CD40L is present in the alpha-granule membrane, and is translocated to the platelet surface on platelet activation. Here it becomes a substrate for a platelet MMP (probably ADAM-10 and/or ADAM-17), being released from activated platelets in a soluble form (sCD40L). CD40L mediates IgM to IgG isotype switching, it can also drive the activation of autoreactive B lymphocytes in immune thrombocytopenic purpura (38). Platelet-derived CD40L of patients with systemic lupus erythematosus can activate mesangial cells giving an increased production of TGF-beta1 (39). Interestingly, sCD40L can rebind to alphaIIbbeta3 through a KGD sequence and integrin clustering can present zones of CD40L with high avidity that themselves intervene in maintaining the stability of platelet aggregates as well as promoting activation of other cells (40).

TF, the initiator of the extrinsic pathway of blood coagulation, is also a natural regulator of angiogenesis (41). Interestingly, in other cells the TF-factor VIIa complex promotes developmental angiogenesis by signalling through the PAR-2 receptor with the TF cytoplasmic domain negatively regulating PAR-2 signalling (42). Monocytes are a source of TF in circulating blood, and platelets accumulate leukocyte-derived microparticles by a P-selectin-dependent mechanism (43). As well as promoting thrombin generation, platelet-bound TF may also contribute to wound healing by, for example, directly inducing migration of cultured smooth muscle cells (44). The most well-studied of alpha-granule membrane glycoproteins is P-selectin, which after secretion becomes evenly distributed over the platelet surface (Figure 2). This surface expression now allows platelet-leukocyte interactions, with P-selectin binding to PSGL-1 on PMNs and monocytes. Through this function, P-selectin also indirectly participates in TF generation (45). Other membrane glycoproteins such as CD63 and TLT-1 also participate in the surface properties of activated platelets. The alpha-granule membrane also contains receptors present in the plasma membrane of unstimulated platelets. Present is the alphaIIbbeta3 integrin, which recycles to and from the surface and assures the endocytosis of Fg.

MMPs are recognized pro-angiogenic proteases and their expression on tissue cells can be regulated by platelet-released growth factors, cytokines or chemokines. For example, MMP-2 expression on vascular smooth muscle cells is stimulated by PDGF-BB and IGF-1 despite their different abilities to induce proliferation, while platelet releasate induces MMP-2 release by endothelial cells (46, 47). Platelets themselves are a potential source of MMPs possessing amongst others MMP-2, MMP-9, ADAM-10, ADAM-17 (TACE), and ADAMTS-13 as well as tissue inhibitors of metalloproteases (TIMPs 1-4). Although alpha-granules may constitute the source of the bulk of the MMPs, cytoplasmic pools and even membrane pools may also be present (especially for the TIMPs). It is not as yet known if pro- and anti-angiogenic factors co-localize to the same population of granules.

3.3. Lysosomal granules

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

3.4. Newly synthesized active metabolites

Platelets provide eicosanoids synthesised from arachidonic acid released from membrane phospholipids. TXA_2 is a powerful vasoconstrictor that is also involved in the injury-induced vascular proliferative response, a process regulated by prostacyclin (48). Sphingosine 1-phosphate, a novel active metabolite able to stimulate mitogenesis, is liberated from activated platelets during clot formation. It also stimulates fibronectin matrix assembly through a Rho-dependent signalling pathway (49). As well as promoting endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement, this sphingolipid can induce TF expression on endothelial cells (50, 51). Sphingosine 1-phosphate participates with PDGF and TGF-beta1 in potentiating the proliferative response of

fibroblasts mediated in part by an increased expression of 5-lipoxygenase (52). It is also a regulator of osteoclast differentiation and osteoclast-osteoblast coupling in bone marrow-derived macrophage (BMM) single and BMM/osteoblast coculture systems (53). Lysophosphatidic acid (LPA) induces endothelial cell migration, a process regulated by extracellular matrix molecules (54). In another model, platelet-derived LPA supported the progression of osteolytic bone metastases in breast cancer where tumour cells induced LPA release from platelets and in turn the tumor cells responded by proliferating (55). Platelet-activating factor (PAF) is another platelet-derived bioactive lipid; it can play a role in mediating leukocyte arrest and intriguingly this inflammatory property is enhanced in platelets showing signs of apoptosis (56). PAF has also been shown to induce the migration of endothelial and other tissue cells.

3.5. Thrombin generation

Activation-dependent transport of phosphatidylserine (PS) to the outer surface of the platelet plasma membrane is accompanied by microvesicle release. Both processes result in procoagulant surfaces and thrombin generation (5, 57). 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 alpha-granules store factor V associated with an abundant large protein termed multimerin (7), and provide a source of subsequently activated factor V for the prothrombinase complex. Thrombin is a powerful mitogen, while factor Xa and other coagulation factors can also elicit specific cellular responses (58). Taking just one example, thrombin modulates the expression of a set of genes including that encoding TSP-1 in endothelial cells through activation of the PAR-1 receptor (59).

3.6. Protein synthesis

Although anucleate, activated platelets are able to synthesize proteins by processing preformed pre-RNA due to the presence of a functional spliceosome (60, 61). This was first shown for interleukin-1beta where the activated platelets are able to excise introns from the interleukin 1beta pre-RNA yielding a mature message that is translated into protein. Interestingly, interleukin-1beta binds to Fg and fibrin and the bound form has enhanced activity as shown by increased activation of endothelial cell nuclear factor kB, greater monocyte MCP-1 secretion and greater nitric oxide synthesis (62). More recently, platelets have been shown to possess Clk-1 splicing pathways able to generate TF in response to cellular activation (63). Whether other biologically active proteins are synthesized by this mechanism is so far unknown, but the presence of a spliceosome leaves open the possibility that integrin-bound platelets can provide a continuous source of biologically active proteins when incorporated in a fibrin clot.

4. THE PLATELET SECRETOME STUDIED BY PROTEOMICS AND MASS SPECTROMETRY

In the previous sections, we have dealt with proteins that have largely been identified individually by well-characterized procedures. The application of proteomics to the platelet secretome has widely extended the list of proteins tentatively shown as being secreted from platelets. Coppinger et al (64) have identified 146 proteins in the platelet releasate by peptide tandem mass spectrometry. Although many proteins already known as being released from platelets were identified, there were some surprises. The significance of the apparent secretion of cytoskeletal proteins such as filamin A and alpha-actinin remains to be shown. The value of proteomics though is the identification of new proteins and Coppinger et al (64) have shown a series of cytokines and growth factors whose expression was further quantified using antibody arrays. Among those proteins previously unknown to be present in platelets were oncostatin M, angiogenin, growth-regulating growth factor and MCP-2. Understanding the importance of the biological activity of these proteins in the releasate will require further studies. The effect of anti-platelet drugs such as aspirin that modulate and moderate the platelet release reaction in response to ADP, collagen and thrombin was also underlined in this study.

5. MATRIX MOLECULES AND THE FIBRIN CLOT

Collagens, proteoglycans and adhesive glycoproteins such as Fn are the major constituents of the extracellular matrix (ECM), and collectively provide cells with biological information and a protein-based scaffold for adhesion and migration. As well as providing ECM structure, these constituents bind cytokines, growth factors and proteases. Not be forgotten as modulators of wound healing are TSP-1, TSP-2, SPARC, tenascin-C and osteopontin as well as cytokines such as connective tissue growth factor (CTGF) (65). TSP-1 is secreted in large amounts from activated platelets and upregulates expression of cell adhesion molecules (VCAM-1, ICAM-1, E-selectin) and promotes monocyte binding to endothelium (66). TSP-1 is an antiangiogenic molecule and may act as a scavenger for some growth factors such as FGF-2 (67). Studies on a model of thrombopoiesis have confirmed that release of TSP-1 and TSP-2 functions as a major anti-angiogenic switch by controlling both the numbers of platelets produced and the revascularization of myelosuppressed bone marrow and ischaemic limbs (68). Mice deficient in TSPs showed enhanced angiogenesis with activation of MMP-9 and release of SDF-1.

Fg itself can enhance wound closure by increasing both cell proliferation and migration (69). In a dermal fibroblast model, both processes depended more on the exposure of the beta15-21 epitope of Fg than on the additional presence of growth factors such as PDGF and FGF-2. Fg assembles with Fn into matrix fibrils of fibroblasts independently of the formation of fibrin. An involvement of the fibroblast alphavbeta3 receptor was also shown for matrix fibril formation (70). Fibrin is an important participant in wound healing. As reviewed elsewhere, the outcome of healing is influenced by fibrin structure (thickness of the fibres, number of branch points, the porosity and permeability of the clot) at the wound site (71). The addition of an exogenous source of platelet-rich plasma at the wound site will accelerate the natural healing

process and provide additional support for the binding not only of platelets, but also among others of endothelial cells, smooth muscle cells, fibroblasts, leukocytes, keratinocytes and incoming stem cells. As already underlined, the binding of growth factors to the fibrin will facilitate the formation of chemotactic gradients. FGF-2 binding to fibrin sustains endothelial cell growth (72). Cell binding to fibrin may be aided by the presence of Fn and Vn that crosslink fibrin and cell integrin receptors. Thrombin-induced release of fibrinopeptides and plasmin-induced fibrinogen degradation products attract leukocytes and participate in the transition between inflammation and tissue repair. Significantly, there is a marked delay in cutaneous wound repair in plasminogen-deficient mice (73).

6. ANGIOGENESIS

The total effect of platelet-released proteins will depend not only on the environment in which they are released, but also on the degree of secretion that has been achieved. Proteins can act synergistically, but also in competition. The following examples will illustrate just how complex is the interplay. For example, TGF-beta1 has been shown to decrease the proliferation of human fibroblasts by inhibiting PDGF binding (74). VEGF while secreted from platelets, is induced in other cells by platelet releasate (25). In apparent contradiction, TGF-beta1 stimulates monocyte chemoattractant protein-1 (MCP-1) as a target gene in endothelial cells and the latter mediates the angiogenic effect of TGF-beta1 by recruiting vascular smooth muscle cells. In so doing, this promotes the maturation of new vessels (75). Thrombin-activated platelets stimulate ILK-1 synthesis by human skin fibroblasts (76). Interestingly, TSP-1 and dermal fibroblasts have been shown to cooperate for stimulating endothelial cell tubulogenesis (77). As well as promoting TGF-beta1 activation from its latent form, TSP-1 potently reduced the expression of PAI-1, a crucial negative regulator of tubulogenesis. Endostatins are derived from the C-terminal domain of collagen XVIII and they inhibit endothelial cell migration, vascular morphogenesis, and perivascular cell recruitment (19, 78). How they come to be part of the platelet releasable pool remains to be shown but as they are proteolytic products (~ 20 kDa) of collagen XVIII then it is highly probable that they are endocytosed. Most interestingly, Ma et al (79) have shown that the PAR-1 and PAR-4 thrombin receptors counter-regulate endostatin and VEGF release from human platelets. Not least, this finding promotes the idea that factors that promote (such as VEGF) or inhibit (e.g. endostatin) angiogenesis are stored in different subpopulations of granules although other intra-cytoplasmic pools are envisageable. Such studies show how agonist strengths may effect healing and underline how changes in the balance of secreted products from platelets may influence their pro- or anti-angiogenic effect.

7. Influence on hematopoietic stem cells (HSC) and/or progenitor cells

PF4 enhances the adhesivity of HSCs to endothelial cells (80). In fact, adherent platelets were shown to be potential mediators of endothelial progenitor cell chemotaxis and homing. A role has been proposed for the chemokine stromal-derived factor-1alpha (SDF-1alpha, also secreted by platelets, and previously shown to recruit bone-marrow-derived progenitor cells to arterial thrombi in vivo (81). Activated human platelets recruit CD34+ progenitor cells via specific adhesion receptors P-selectin/PSGL-1 and integrins of the beta1, beta2 and/or beta3 families. Under certain culture conditions this is followed by the differentiation of the progenitor cells into foam cells and endothelial cells (82). Recently, more evidence has been obtained showing a role for hemostatic components in the recruitment of CD34+ cells to sites of injury (83). Platelet-expressed P-selectin permitted the initial tethering of CD34+ cells in flowing blood. This was followed by firm adhesion of the CD34+ cells to tissue factor-expressing endothelial cells or to fibrin-containing thrombi. The latter supported migration of the stem cells to the sites of injury, a step followed by their differentiation into endothelial cells. A role for VEGF should also be underlined. VEGF signals through a family of receptor tyrosine kinases (known as VEGFR 1, 2 and 3) essential for the functions of endothelial cells and lymphoendothelial cells as well as acting as regulators of the migration of hematopoietic precursors and the migration of monocytes and macrophages (84).

8. THERAPEUTIC USES OF AUTOLOGOUS PLATELETS IN HEALING AND TISSUE REGENERATION

It was in the early 1990s that fibrin glue (fibrin sealant or fibrin gel) was developed as a biomaterial with hemostatic and adhesive properties. Use of platelets in association with the fibrin came later (reviewed in 7, 85). Preparations have since been variously described as PRP-gel, platelet gel, PRP-clot or plasma-rich in growth factors (PRGF). The wound-healing process is typically divided into three phases (inflammatory, proliferative and remodelling). As discussed in the first section of this review, platelets and their released cytokines and growth factors are pivotal elements of each of these processes. We now show how the therapeutic uses of platelet-derived growth factors are widespread. Selected clinical situations where guided secretion of autologous platelet products can promote healing and wound repair are listed in Table 2 and illustrated in Figure 3.

8.1. Bone regeneration in dentistry and maxillofacial surgery

An early and widespread use of PRP-clots was in dentistry and oral-maxillofacial surgery (reviewed in 7, 9). Platelets are activated at tooth extraction sites as a natural consequence of vascular disruption and there is evidence that platelet-released substances can stimulate the proliferation of osteoblastic cells (86). Bone regeneration requires the recruitment, proliferation and maturation of osteoblasts that are derived from mesenchymal stem cells (MSC). Although MSC are mainly localised in the bone marrow, a subpopulation is present in peripheral blood. 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 the BMPs,

Table 2. Some uses of autologous platelets and platelet-rich clots in wound healing

Clinical situations
Dentistry and maxillofacial surgery
Consolidation of titanium implants
Maxillary sinus augmentation
Bone remodelling
Orthopaedic Surgery
Knee arthroplasty
Lumbar spinal fusion
Intervertebral disc degeneration
Tendon and ligament repair
Anterior cruciate ligament reconstruction
Tendon tear repair
Muscle repair (sports medicine)
Facial plastic and reconstructive surgery
Rhytidectomy
Other cosmetic surgery (breast size changes)
Skin wound healing
Superficial non-healing ulcers
Ophthalmology
Dormant corneal ulcers
Retinal repair (macular holes)
Facial nerve regeneration
Cardiac and bariatric surgery

themselves members of the TGF-beta superfamily, whose activity is potentiated by $1,25(OH)_2D_3$ (30, 87). Also involved are IGF-1, FGF and TGF-beta and in vitro studies suggest that platelet releasates also promote the migration of bone cells (88). When the effects of PRP on the proliferation and differentiation of two human osteoblast-like cell lines were examined, the PRP was shown to enhance the levels of procollagen type I, osteopontin, osteoprotegerin and core binding factor alpha 1 mRNA (89). Studies using BMP-deficient mice have shown that BMP-2 activity, although indispensable for bone formation, is required for the initiation of fracture healing (90). The application of tissue engineering in regenerative medicine is of actuality. An example comes from periodontology (91). MSCs were isolated from a patient's iliac crest marrow aspirate and mixed in a PRP gel. Full thickness periodontal flaps were elevated and the gel applied to the root surface and adjacent defect space. Growth of new bone was enhanced. The use of MSCs in PRP gel may therefore be helpful in many settings. Interestingly, PRP has also been shown to be a valid preclinical tool for obtaining an effective, rapid and safe ex vivo expansion of BMSCs prior to their clinical utilization in bone engineering (92).

Autologous platelet gel was first used by Whitman et al (93) in reconstructive oral and maxillofacial surgery and as an adjunctive procedure in the placement of dental titanium implants. Marx et al (94) evaluated the effect of autologous PRP during bone graft reconstruction of mandibular continuity defects. 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 (95, 96). 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 (97). In another study, PRP was beneficial when added with freeze-dried bone allograft to promote bone formation during subantral maxillary sinus augmentation (98).

We 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 (7, 99). Subsequent implant placement was another precondition. In most of the patients with PRP-clot, bone regeneration was accelerated and the bone tissue compact with well-organised trabeculae. In contrast, in the control group the cavity was mainly filled with connective tissue and little mature bone was found. 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 (7, 9). 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 (100). Interestingly, the alphaIIbbeta3 integrin mediates an initial platelet adhesion to titanium surfaces probably via adsorbed fibrinogen (101). An improved platelet interaction is seen with titanium surfaces that are micro-roughened rather than smooth. A direct osteoblast adhesion to titanium surfaces has also been described with integrin-mediated intracellular signalling and activation (102). Osteoblasts appeared to proliferate and differentiate on the titanium surface while the role of integrins suggests that adsorbed plasma proteins such as fibronectin serve as intermediates for their attachment. Also of relevance is the possible cross-talk between growth factor receptors and the alphavbeta3 integrin. Both VEGF-R2 (flk-1) and PDGFRbeta have been shown to interact directly with beta3 via their extracellular domains (103).

Bone remodelling involves forming a balance between the resorbing activity of osteoclasts and the matrix-generating capacity of osteoblasts (reviewed in 104). 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 (105). 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 (106, 107). TGF-beta family members may regulate the response of cells to PDGF and PDEGF and it has been known for some time that TGF-beta is able to initiate bone formation (108). 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 (see 7). In some ways platelets mimic and up-regulate the osteoblast's own activation machinery.

Not to be forgotten among the advantages in using a platelet-rich clot is the safety provided by the anti-bactericidal affects of the platelets (109). Not only do

Figure 3. Therapeutic use of PRP in three different clinical situations. A) Image showing PRGF application in dentistry: hydroxyapatite combined with PRP (left) is used as a biomaterial in oral reconstruction (right). B) Autologous platelet gel (PRP clotted ex-vivo) (left) is applied to promote skin ulcer healing (right). C) Image showing the arthroscopic procedure for anterior cruciate ligament reconstruction using PRP: Growth factors are transferred to the substitute graft by injecting PRP (left). Subsequently the tendon graft is inserted from the tibia to the femur tunnels and secured with the bone plugs and PRP (right).

platelets secrete bacteria-neutralizing proteins (Table 2) (34), they also participate directly in the elimination of bacteria during sepsis (110). The possession on platelets of toll-like receptor 4 promotes a series of events that activate neutrophils to ensnare bacteria. Quite surprising is the recently recognized ability of platelets to reduce pain (see also the Section on Orthopaedic Surgery). The molecular basis of how platelets can influence pain is unknown; one possible explanation is protease-induced release of PAR-4 peptides from platelets that have anti-nociceptive properties (111). Interestingly, the use of PRP as an adjunct to dental surgery was shown to significantly accelerate mucosal wound healing by providing a strong stimulant effect on capillary regeneration (112).

8.2. Cosmetic surgery

Autologous platelets are especially useful for the soft tissue and bony reconstruction encountered in facial plastic and reconstructive surgery (113). 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 (114) who used autologous platelet-fibrin glue in 14 patients with skin and soft tissue losses caused by recent trauma or chronic pathology. This confirms useful bactericidal properties as well as cell proliferation promoting properties, proteins capable of both are present in platelet releasates (Table 2). Anti-inflammatory properties with reduced oedema and ecchymosis was associated with the use of autologous platelet gel in 8 women after deep plane rhytidectomy (115). 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) (116). An increased recovery time is often observed. Autologous platelet gel sealant has also been noted to result in significantly lower surgical drain fluid after rhytidectomy (117).

8.3. 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 (118). 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 on average 10 weeks after the application of HPDWHF. 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 (119) to conclude that use of platelet releasate is of proven efficacy, especially for patients with more severe wounds. A similar conclusion was reached by Driver et al (120) who studied a group of 72 patients with non-healing diabetic foot ulcers. Platelets provide not only autologous growth factors for tissue formation and epithelialization of chronic cutaneous wounds, it was again noted that they provide both infection-fighting and pain-reducing properties (121). Apart from ulcers, application of autologous platelet gel will also accelerate the closure of acute human skin wounds (122). Recently, PRP was combined with a bioengineered skin substitute for chronic wound healing (123). A dramatic reduction in wound dimensions was seen for a patient with a severe decubitus ulcer of the sacrum.

8.4. Orthopaedic surgery

Autologous growth factor concentrate (AGF) prepared by ultraconcentration 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 MSCs and osteoblasts and therefore accelerate bone healing. Lowery et al (124) 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. Human intervertebral disc (IVD) degeneration is a problem in aging. Chen et al (125) have shown that PRP and TGF-beta1 can provide a growth factor cocktail to induce human nucleus pulposus proliferation and differentiation, and also to promote tissue-engineered nucleus pulposus formation. Real-time-PCR analysis of mRNA showed that Sox9, type II collagen and aggrecan were all up-regulated by PRP. A combination of platelet-rich plasma with engineered scaffolds (polycaprolactone-tricalcium phosphate) has been proposed for segmental bone defect repair (126). Transplantation of cultured expanded bone marrow cells together with platelet-rich plasma has been shown to accelerate femoral healing in distraction osteogenesis thereby shortening the treatment period and reducing associated complications (127).

8.5. Eye surgery

A novel 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 (128). 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. Gehring et al (129) showed anatomic closing of Stage II to IV macular holes in 18 of 19 patients treated with autologous platelet concentrates.

8.6. Soft-tissue disorders: tendon and ligament repair

A case report involving our laboratory showed that injecting calcified autologous PRP may facilitate reattachment of knee articular cartilage (130). A role for

endogenously released growth factors including IGF-1, TGF-beta, VEGF, PDGF and bFGF in tendon and ligament healing is well documented (see 131). They variously participate in each of inflammation, cell proliferation and tissue remodelling. Studies performed in our laboratory on human tendon cells in culture have shown that platelet-rich factors induce both VEGF and HGF production (132). In contrast, exogenous TGF-beta1 increased the synthesis of VEGF and simultaneously abolished the production of HGF (25). It seems that the balance between TGF-beta1 and the pools of platelet-secreted molecules may determine the nature of the response. Platelet injection was thought to act at an early stage of tendon regeneration and this allows mechanical stimulation to start driving neo-tendon development at an earlier time point (133). Platelet-rich plasma-derived fibrin clot formation is capable of up-regulating collagen synthesis in periodontal ligament and osteoblastic cells in vitro. The application of autologous platelet gel has been shown to be beneficial in total knee arthroplasty (134). As well as assuring a more complete haemostasis and lower blood loss, addition of the gel resulted in accelerated repair and decreased post-operative pain. Recently, the role of PDGF release in tendon repair in promoting cell proliferation and collagen remodelling has been emphasized (135). Current, promising applications from our group involve the use of calcified autologous platelet preparations in Achilles tendon and muscle repair in athletes (136).

8.7. Facial nerve regeneration

Studies on a rat model have shown a potential new application of PRP in peripheral nerve regeneration (137). The model involved the repair of facial nerves and PRP was compared with PPP and fibrin sealant, all with or without suture after nerve transection. A distinct neurotrophic effect was seen with PRP and the best result was achieved for a combination of PRP with suture.

8.8. Cardiac and bariatric surgery

The standard transfusion of platelets during bypass or other forms of cardiac surgery is to reduce blood loss. Cardiac pathologic events including myocardial infarction may trigger maladaptive remodelling of the myocardium that in turn leads to distolic and systolic dysfunction, myocyte loss, and malformation of the extracellular matrix. Application of platelet gel to the injured site has been proposed to provide growth factors, cytokines and chemokines that may stimulate adaptive remodelling (138). A lower blood loss was also seen. The topical use of a platelet-rich concentrate (platelet gel) in cardiac surgery to enhance wound healing at the sternum has also been shown to afford an increased protection against infection and to lower post-operative pain (139, 140). Gastric bypass or bariatric surgery is another situation where autologous platelet gel is widely used (141).

9. 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 diffusible compounds and release large numbers of microparticles that carry proteins such as TF or IL-1beta and which are prothrombotic (5). Therefore, care may be required in using this procedure in the vicinity of large blood vessels, especially in patients with inherited or acquired thrombotic risk factors (such as high blood pressure, Factor V Leiden). The parallel use of anti-platelet medications could theoretically limit efficacy. The taking of aspirin, an anti-platelet drug may be unavoidable in some conditions, but experience of studies on stomach ulcers is interesting to consider. Ma et al (142) showed that ulcer induction in rats was associated with increases in the serum levels of VEGF and decreases in endostatins (an antiangiogenic factor) (Table 1). 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. As we have discussed earlier, aspirin will reduce platelet secretion and thus should be avoided in the days prior to autologous PRP preparation. The presence of leukocytes in the PRP-gel can be beneficial or not and further studies are still required to ascertain the best optimal mixture of platelets and neutrophils and other blood cells.

10. CONCLUDING REMARKS

Unravelling the roles of individual platelet proteins will require the study of transgenic mouse models and the use of microarray systems capable of revealing the gene response of specific cell types to stimulation with platelet releasates. Backtracking along signalling pathways and knockout of specific receptors or secreted proteins will help identify primary response signals. It is clear that platelet-rich fibrin clots constitute a bioactive reservoir. Although gene transfer with modified expression of growth factors and/or cytokines may become a potential alternative in, for example, periodontal tissues (143), the accompanying risk may be incompatible with routine dental implant surgery. A more realistic approach to improving results may be to combine the use of recombinant proteins with autologous PRP. But even this may be difficult to determine due to cross-talk between cells and receptors. For example, VEGF can stimulate the migration of human adult mesenchymal stem cells by binding directly to PDGF receptors (144).

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Abbreviations: MK, megakaryocyte; GP, glycoprotein; VWF, von Willebrand factor; Fg, fibrinogen; ADP, adenosine diphosphate; TXA_2, thromboxane A2; TF, tissue factor; MMP-2 or -9, metalloprotease-2 or -9; Fn, fibronectin; Vn, vitronectin; TSP-1 or -2, thrombospondin-1 or -2; IL-3, -8 or -1beta, interleukin-3, -8 or -1beta; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PAI-1, plasminogen activator inhibitor type I, uPAR, urokinase-type plasminogen activator; TGF-beta1, transforming growth factor beta1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; IGFBP-3, insulin growth factor binding protein-3; NF-kB, nuclear factor-kappa B; BMP, bone morphogenetic protein; PF4, platelet factor 4; beta-TG, beta-thromboglobulin; NAP-2, neutrophil activating peptide; RANTES, regulated upon activation, normal T-cell expressed and secreted; MIP-1alppha, macrophage inflammatory protein-1alpha; ENA-78, epithelial-derived neutrophil activating peptide-78; MCP-3, monocyte chemoattractant protein-3; CD40L, CD40 ligand; ADAM-10 or -17, a disintegrin and metalloprotease-10 or 17: PAR-1, -2 or -4, proteinase activated receptor-1, -2 or -4; P-selectin, platelet selectin; PSGL-1, P-selectin glycoprotein ligand-1; E-selectin, endothelial cell selectin; PMN, polymorphonuclear leukocytes; TLT-1, trem-like transcript-1; ADAMTS-13, a disintegrin and metalloprotease with a thrombospondin type 1 motif-13; TIMP, Tissue inhibitor metalloprotease; BMM, bone marrow-derived macrophage; LPA, lysophosphatidic acid; PAF, platelet-activating factor; PS, phosphatidylserine; MCP-1 or -2, monocyte chemotactic protein-1 or -2; Clk-1, CDC-like kinase; ECM, extracellular matrix; SPARC, secreted protein, acidic and rich in cysteines; CTGF, connective tissue growth factor; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intracellular adhesion molecule-1; FGF-2, fibroblast growth factor-2; SDF-1, stroma cell-derived factor-1; ILK-1, integrin-linked kinase-1; HSC, hematopoietic stem cell; MSC, mesenchymal stem cells; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PRGF, plasma-rich in growth factors; OPG, osteoprotegerin; PGE_2, prostaglandin E_2; HPDWHF, human platelet-derived wound healing factors; AGF, autologous growth factor concentrate; IVD, human intervertebral disc; SCUBE1, signal peptide CUB domain EGF-like 1; TFPI, tissue factor pathway inhibitor; TAFI, thrombin activatable fibrinolysis inhibitor; TACE, tumor necrosis factor-converting enzyme; GRO-alpha, growth-regulated oncogene alpha; HGMB1, high-mobility group box-1; LIGHT, homologous to lymphotoxin; exhibits inducible expression and competes with HSV glycoprotein D for herpes virus entry mediator; a receptor expressed on T cells; TRAIL, TNF-related apoptosis-inducing ligand; PECAM-1, platelet endothelial cell adhesion molecule-1.

Key Words: : Platelet, Secreted Proteins, Fibrin Clot, Wound Healing, Inflammation, Review

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