Platelet Rich Plasma in Orthopaedics and Sports Medicine

Eduardo Anitua
Ramón Cugat
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Editors

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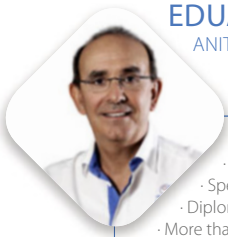

EDUARDO ANITUA

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· Diploma in Prosthodontics and Occlusion from the Pankey Institute (Florida, USA).
· More than 300 papers published in national and international journals.
· Author of 14 books and co-author of 7 books and chapters, being translated languages.
· 46 international patents in the fields of regenerative therapy and implant dentistry.
· Director of the programme "Continuing Education on Oral Implantology and Rehabilitation" given in Spain and various other countries for the last 25 years.
· More than 600 courses and conferences around the world on Tissue Regeneration, Implantology, Prosthodontics and Aesthetic Dentistry.

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Medical and Scientific Director of the Arthroscopic Surgery Unit (UCA), Hospital Vithas San José.

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· Specialized in Traumatology and Orthopedics at the University of the Basque Country, Spain. (1984).
· Head of the Arthroscopic Surgery Unit (UCA), Vitoria-Gasteiz, Spain (1995)
· Ph.D. in Medicine by the University of the Basque Country,, Spain (2017).
· Mikel Sánchez has been one of the pioneers in the advance of Arthroscopic Surgery in Spain.
· Part of Leeds-Keio teamwork (1986-1997), an Anglo-Japanese collaboration in order to boost developed prototypes of surgical equipment for the anterior and posterior cruciate ligament reconstruction and for the treatment of shoulder chronic instability.
· In 2000, he understood the therapeutic potential of PRP and its applications in traumatology.
· Since 2012 is a precursor in Spain of the use in surgery of 3D printing technology.
· Author of more than 250 national and international lectures, book chapters, international patents and more than 65 international scientific articles.

RAMÓN CUGAT

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· PhD in Medicine from the University of Barcelona (1978).
· Specialist in Orthopaedic and Trauma surgery (1979).
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· Associated Professor at the Medical School at Barcelona University and UIC.
· Active member of the Royal European Academy of Doctors.
· Member and honorary member of many national and international societies including ISAKOS, AAOS, AANA, ICRS, SLARD, ESSKA, AGA, SECOT, AEA, FEMEDE, HERODICUS SOCIETY, ASIAM PACIFIC INSTITUTE (Member of BOD) among others.
· Over 150 publications between specialised journal articles and book chapters.
· He has given lectures in congresses and collaborates in Teaching Courses of Arthroscopic Surgical Techniques and Sports Medicine around the world.

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PROLOGUE

It is not routine to be asked to write the prologue to a book on a topic somewhat removed from one's area of expertise. In trying to justify my acceptance to do this prologue I certainly took into account my long friendship with Eduardo Anitua, but thinking about reasons to do it I thought that having only little more than a layman knowledge about platelet rich plasma would give me a more unbiased view of this controversial subject.

PRP and its relative, stem cells, have been for some years at the forefront of innovative therapies for many medical conditions, especially musculoskeletal affections. And, as it has happened many times before with new techniques or therapeutics, they have been embraced enthusiastically by many, unfortunately including entrepreneurs and even charlatans. This has led to indiscriminate use and even abuse of these therapies before clinical evidence of their value was obtained. And both industry and individuals have benefitted greatly when basically no or minimal information about their real effect was available.

But with the passage of time more information is accumulating on the real importance of these substances and their unquestionable value in the treatment of many conditions. For example, there are now systematic literature reviews of randomized and prospective studies showing that injections of PRP into osteoarthritic knees secure better functional outcomes at 6 months than placebo or hyaluronic acid injections, although no difference in pain or patient satisfaction was shown.

This book represents a compendium of the knowledge available today on Platelet-rich plasma preparations, their formulations, methods of production, mechanism of action, different effects, and their applications to musculoskeletal conditions. It represents an attempt to "drain the swamp" and to provide evidence-based information in a field where that is painfully scarce.

In 16 chapters the authors have provided abundant information on the basic science of Platelet-rich plasma preparations, the already classical applications of these formulations to orthopedic conditions, primarily joints, tendons and muscle injuries, the use in dentistry and oral surgery (so the book extends beyond the realm of sports medicine), but there are also chapters that address other less common applications, such as nerve injuries or low back pain. One may frown at these novel uses of PRP, or at its intraosseous use in knee osteoarthritis. I would reason that background science for their use in these conditions appears sound and it seems reasonable that it should be up to the "developers" to first explore with well-designed studies the limits of these therapies.

The book is attractively produced, nicely illustrated and represents the authors long experience with PRP. It should be read by anybody who intends to use or has been using PRP in clinical settings. It will be therefore a valuable asset for orthopedists, oral surgeons, sport medicine physicians and all those interested in musculoskeletal conditions. The editors and authors deserve congratulations and thanks from all those of us that will benefit from reading this text.

Miguel E Cabanela, MD, MS (Orth Surg)

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INTRODUCTION

The adventure of the plasma rich in growth factors began in 1995 as a result of questioning ourselves about what were the biological mechanisms involved in the regeneration of the post extraction socket. I was deeply concerned to understand why a patient who underwent a tooth extraction healed in a few days and the process for other patients was instead slow and painful. The key to this question was in the blood clot and so we began to investigate what would be the clot's optimal characteristics in order to make it extendable to all patients and thus achieve an optimal healing.

We began investigating ways of anti-coagulating the blood and how to reverse the coagulation cascade, and as we closed fronts, others were opened. What was the effective concentration of platelets? Would it make sense that the plasma we prepared had white blood cells? At this point, I have to thank the extraordinary collaboration with Drs. Nurden, with whom we at our foundation have been tireless collaborators during all these years. Throughout these 25 years, we have studied many of the biological repair processes using different cellular phenotypes. We have also defined the release kinetics of proteins from the fibrin matrix, a fundamental process to be able to understand the effect of these molecular signals at the injury site. A pioneering work published in 1999 on the use of an autologous PRP from small volumes of blood was the key in the development of this biological system.

Following the path of the evolution of mammals, where the tooth was first and then bone and vertebrae, in 2001 and with the extraordinary collaboration of Dr. Mikel Sánchez, we began to investigate the possibilities of clinical application in the area of Orthopedics and sports medicine.

Everything was uncertain, and in the arduous path of intuition to evidence, a great effort had to be made, both in the laboratory and in the surgical experimental room, performing innumerable surgeries in animals that would eventually derive the gold standard in orthobiology in the clinical protocols that are currently used worldwide.

Thanks to Mikel and all his team, this path has been exciting and so much so that a 2003 article appears as the first work on the application of a PRP in the area of orthopedics and sports medicine in the world literature.

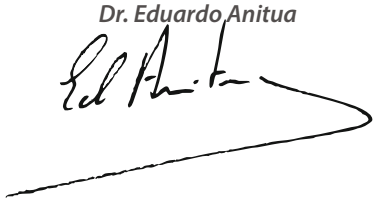
They have been years of hope and passion, where everything was yet to be discovered. There was nothing written on this subject and therefore the canvas was blank, which made the project even more interesting at the same time as challenging.

I believe that we have provided a new biological approach to orthopedic surgery where other teams have contributed to consider PRP as an irreplaceable tool in the therapeutic arsenal of the orthopedic surgeon and sports doctor.

Thanks to the extraordinary collaboration of my good friends, Drs. Mikel Sánchez and Ramón Cugat, as well as of all the authors, we offer the reader the most up-to-date information on the use of plasma rich in growth factors in orthopedics and sports medicine.

I would like to also express my gratitude to Dr. Miguel Cabanela for the preparation of the prologue. I hope that the reader will enjoy and be passionate about this book as much as we all have enjoyed working on it.

Dr. Eduardo Anitua



CHAPTER 1

Platelets at the Interface between Inflammation and Tissue Repair

AUTHORS

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SUMMARY

Blood platelets are produced in large numbers from megakaryocytes in the bone marrow. Anucleate, their principal role is to prevent blood loss on vascular injury and to promote tissue repair, and for this they adhere, aggregate and secrete a wide variety of metabolites and biologically active proteins. The latter are stored in organelles that undergo exocytosis when platelets are stimulated. Activated platelets may also become procoagulant, participate in thrombin formation and help constitute a stable fibrin-based clot. They liberate microparticles (MPs) that act as drones participating in hemostatic and pathologic events far from the parent thrombus. Platelets are also major players in angiogenesis, innate and adaptive immunity and participate in inflammation and host defense. For this, they possess membrane glycoproteins that include receptors for leukocytes and store or synthetize a multitude of adhesive proteins, coagulation and fibrinolytic factors, growth factors, chemokines and cytokines, anti-microbial proteins, proteases and protease inhibitors. On secretion, these components are vital in promoting such events as stem cell recruitment, tissue cell migration and maturation, blood vessel development, and DNA-NET formation. At the same time, platelets and MPs intervene in the progression of major illnesses including cardiovascular disease (atherosclerosis and thrombosis), cancer (tumor cell diffusion and metastasis) and inflammatory diseases (e.g. rheumatoid arthritis) and sepsis. Enigmatically, they often secrete proteins that have opposing roles (e.g. pro- and anti-angiogenic proteins). The challenge is to decipher the roles of secreted proteins and to adapt these natural processes for the therapeutic use of platelet-derived therapies in injury and disease.

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1. INTRODUCTION

Platelets are produced in vast numbers from megakaryocytes (MKs), a large multinucleate cell formed from hematopoietic stems cells (HSC) in a multistep process regulated by thrombopoietin (TPO) in the bone marrow. After initial mononuclear cell proliferation, MKs undergo polyploidy: when mature, they migrate to the endothelial barrier of the vascular sinus and extend long processes termed proplatelets into the blood stream (fig. 1)[1]. Platelets either bud off directly or proplatelets are released as large fragments that break up in the circulation, particularly in the lungs. Intermediate steps include the division of dumb-bell shaped preplatelets and even multiplication of platelets themselves[2]. Anucleate discoid platelets circulate in large numbers; the normal range is 150,000-400,000/μL of blood and their life span is 7 to 10 days. Their primary role is to assure hemostasis and to prevent bleeding (fig. 2). For this, they possess a unique range of receptors. For adhesion these include glycoprotein Ib (GPIb) that has matrix-bound von Willebrand factor (VWF) as ligand and GPVI that recognizes collagen, a major subendothelial matrix component. Platelet receptors for soluble agonists mostly belong to the seven transmembrane domain superfamily and include P2Y1 and P2Y12 that bind ADP while proteinase-activated receptor-1 (PAR-1) and PAR-4 coordinate the response to thrombin. A more complete list of surface receptors is found in figure 3. Multiple intracellular signaling pathways

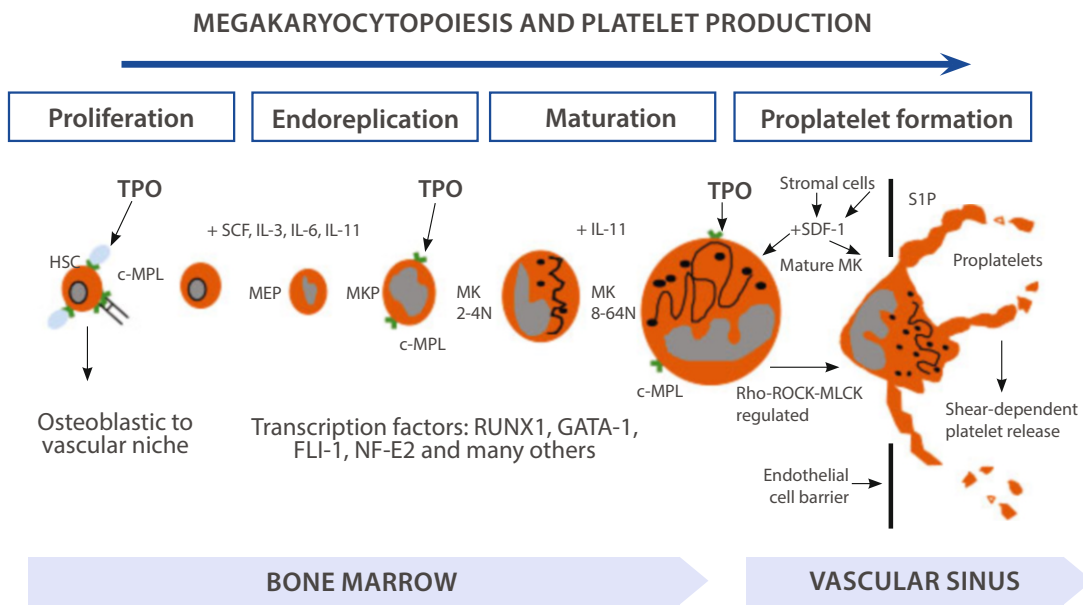

FIG. 1
Schematic representation of the essential steps of megakaryocytopoiesis and platelet production. The process starts with pluripotent hematopoietic stem cells (HSC) that migrate to the vascular niche within the bone marrow and first proliferate before undergoing a series of changes beginning with endoreplication and maturation, a process highly dependent on thrombopoietin (TPO). The chromosome content of mature megakaryocytes (MKs) can be as high as 64 or 128n, a step allowing the formation of the membrane systems and proteins required for platelet production. Many transcription factors are involved in MK maturation with multiple interactions between MKs and their environment (stromal cells, ECM proteins). Finally, mature MKs migrate to the vascular sinus where intracellular signaling pathways favour the formation of long projections termed proplatelets that penetrate across endothelial cell junctions into the blood stream and either bud off platelets directly from their ends or break off as larger structures under the influence of shear and which themselves divide into platelets in the circulation. MEP, MK-erythroid precursors; MKP, MK precursors.

lead to conformational changes in integrin αIIbβ3 enabling it to bind fibrinogen (Fg) or other adhesive proteins that form platelet-to-platelet bridges in the final common step of platelet aggregation. Platelet-to-platelet contacts allow other membrane GPs to interact and to consolidate the aggregate (fig. 3). Endothelial cells form a protective barrier to blood and limit platelet reactivity by secreting nitric oxide (NO) and prostacyclin (PGI2) that dampen down platelet activation; or by expressing enzymes that degrade ADP. But after the loss of ECs or their structural modification (such as during atherosclerosis or inflammation), platelets intervene. Attached and activated platelets spread on exposed extracellular matrix (ECM), particularly collagen, secrete metabolites and release the contents of storage organelles (dense granules, α-granules). These processes promote both flow-dependent thrombus formation and the ensuing tissue repair (fig. 2).

After strong platelet activation, transport of phosphatidylserine (PS) from the inner to the outer leaflet of the phospholipid bilayer makes the platelet membrane procoagulant. Platelets in the central core of the aggregate (or thrombus) are more tightly packed and undergo more extensive changes than those in the outer shell; thereby regulating intra-thrombus solute transport and local thrombin activity, fibrin formation and thrombus stability[3]. Fibrin is essential for blood clotting and wound repair, entrapping other blood cells while platelet aggregates act as hubs within the fibrin network ultimately mediating clot retraction. ADP-related formation of stable platelet aggregates, not fibrin, limits plasma extravasation and promotes tissue repair. In pathology, hyperactive platelets and spontaneous formation or uncontrolled embolization of platelet masses that severely perturb or occlude the circulation are at the origin of arterial thrombosis and stroke[4].

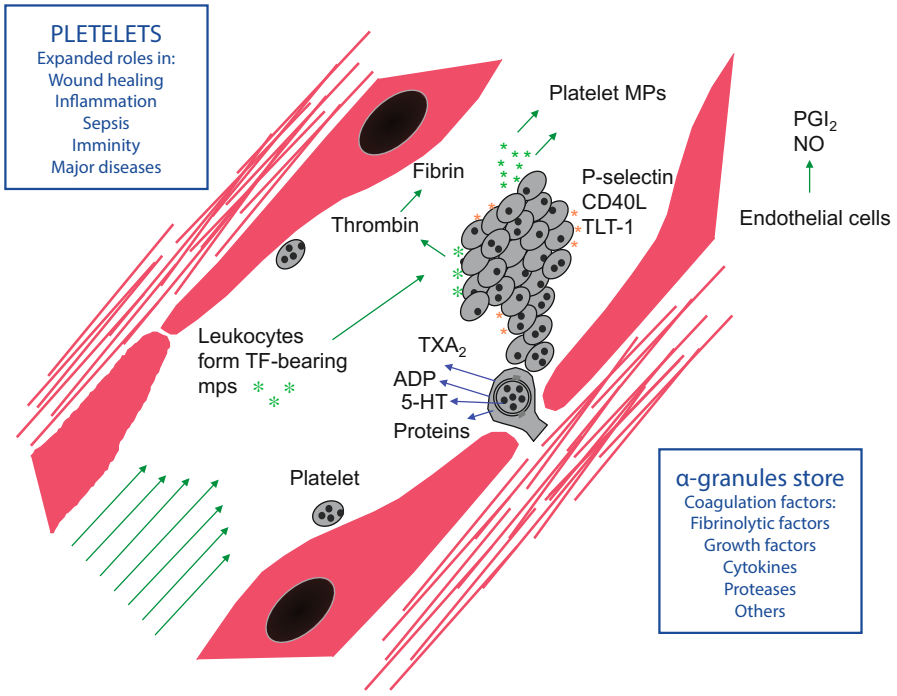

FIG. 2
Cartoon highlighting the biological roles of platelets. Platelets attach when they meet subendothelial elements, become activate and secrete metabolites and granule stores that promote stable aggregate formation at the injured site. Thrombus size is limited by blood flow and regulators secreted from endothelial cells (NO, PGI2). Thrombin generation within the aggregate promotes fibrin formation, consolidation of the hemostatic plug and the release of procoagulant MPs. Also highlighted are the α-granule secreted storage pools of biologically active proteins and the major non-hemostatic roles of platelets.

Platelets are either used up in hemostasis or, when aged undergo glycosylation changes that promote removal from the circulation in the liver, a process that stimulates TPO production in a feedback mechanism that masterminds platelet production[5]. Inherited or acquired defects (induced by certain drugs, chemotherapy, viral or bacterial infections, autoimmune-mediated destruction) that result in a dramatic fall in platelet numbers (i.e. below 30,000/μL) and/or a loss of platelet function favor bleeding. In addition to their essential hemostatic role, platelets also intervene in inflammation and infection, tissue repair, metastasis and tumor growth, and innate immunity[6-9]. This short review will now largely concentrate on describing the role of platelets in non-hemostatic events and to providing the background to their therapeutic use in healing and combatting disease.

2. PLATELETS AS A SOURCE OF BIOLOGICALLY ACTIVE PROTEINS AND METABOLITES

Certain features of the typical discoid anuclear platelet stand out (fig. 3A). These include an outer plasma membrane linked to an extensive intracellular open canalicular membrane system (OCS) that likens the platelet to a sponge. Under the membrane is a microtubular network that interacts with an actin-rich cytoskeleton, while the cytoplasm contains mitochondria and a series of secretory organelles. Platelet activation after adhesion and/or the binding of soluble stimuli results in Ca2+ fluxes and the generation of a plethora of second messengers. Important is the production of lipid metabolites such as thromboxane A2 (TXA2) that act in feedback mechanisms promoting platelet aggregation, a process

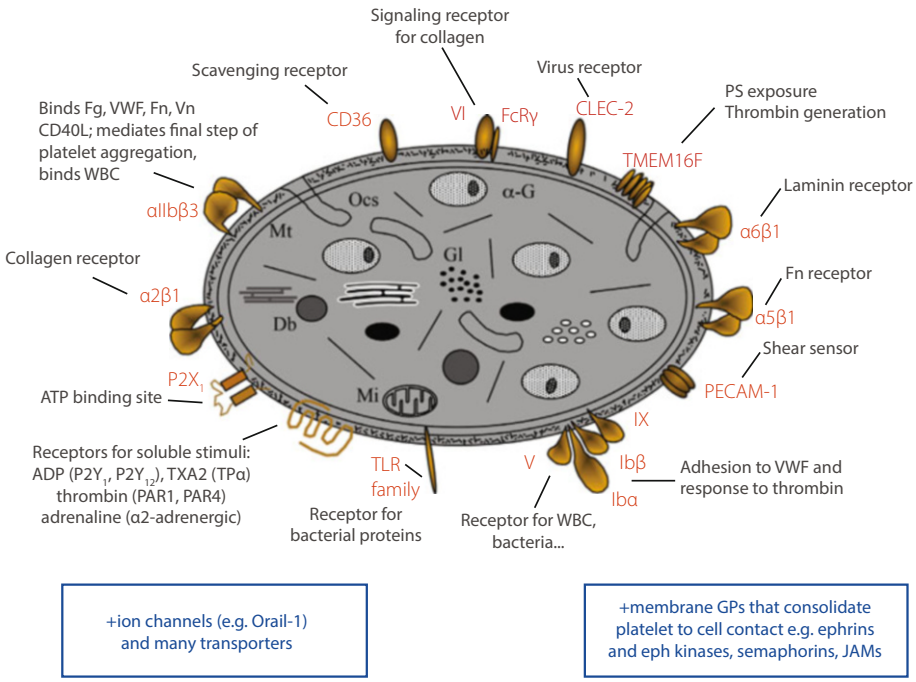

FIG. 3
Schema highlighting the surface structure of resting platelets showing many of the essential membrane GPs that mediate platelet adhesion and aggregation responses in hemostasis. By far the most abundant receptor is αIIbβ3 present at over 100,000 copies per platelet thereby reflecting its importance in platelet function. JAMs: junction adhesion molecules; α-G, α-granule; Db, dense body; Mio, mitochondria; Gl, glycogen store, Mt, microtubule ring; Ocs, open surface canalicular system.

inhibited by aspirin. Sphingosine 1-phosphate, a metabolite able to stimulate mitogenesis and cell proliferation, is released from platelets during clotting and favors fibronectin (Fn) matrix assembly, endothelial barrier integrity, and tissue factor (TF) expression in the vasculature[6]. Lysophosphatidic acid and platelet activating factor (PAF) are other released metabolites. A major early response of the platelet, and a primary subject of this review, is the release of the storage pools of biologically active agents from granules.

(i) Dense granules

These small lysosome-related organelles (3 to 8 per platelet) contain serotonin (actively taken up and stored by circulating platelets), ADP, ATP, polyphosphosphate, Ca2+ (itself a potential central regulator of wound healing) as well as small amounts of other amines such as histamine and dopamine. The dense granule membrane contains molecules associated with the uptake and storage of their contents such as two-pore channel 2 (for Ca2+ uptake), vesicular monoamine transporter 2 (serotonin) as well as membrane glycoproteins such as P-selectin that are shared with other organelles. Dense granule release from platelets requires a complex secretory mechanism involving SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) proteins and a series of proteins involved in vesicular trafficking and the late membrane fusion events required for exocytosis[10]. ADP has a universal role in assuring stable platelet aggregation by other agonists[3]. Highly charged polyphosphate promotes coagulation and enhances fibrin clot structure; in so doing, it provides an early link between platelets, coagulation and inflammation[11]. Released serotonin induces vasoconstriction while increasing vascular permeability. Although the subject of debate, release from dense granules is thought to occur faster than from α-granules.

(ii) α-Granules

These are the principal storage organelles of biologically active proteins (fig. 4, Table I). They are formed from intermediate multivesicular bodies (MVB) originating from the trans-Golgi network in maturing MKs and are numerous with 50-80 α-granules per platelet[10, 12]. Some MVB and α-granules may contain smaller vesicular struc-

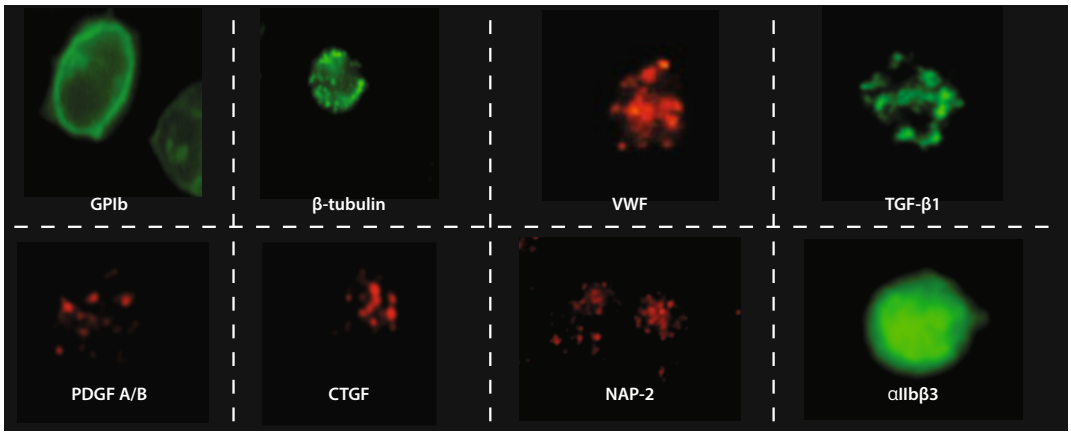

FIG. 4
Fluorescent detection of selected proteins in platelets. Parafarmaldehyde fixed and permeabilized resting human platelets were sequentially incubated with monospecific primary antibodies and fluorochrome-labeled anti-IgG subtype antibodies prior to visualization of bound antibodies by fluorescence microscopy [see 16 for Methods]. Note the presence of the adhesion receptor, GPIb, as primarily a plasma membrane component whereas the aggregation mediator, αIIbβ3, is abundantly distributed throughout the different membrane systems of the platelet. The microtubule component, β-tubulin, is revealed with a sub-membranous distribution whereas selected α-granule components, VWF, TGF-β1, PDGF A/B, CTGF and NAP-2 are localized to discrete organelles within the platelet ready to be released on platelet activation.

tures called exosomes that are enriched in CD63 and secreted intact; their significance is largely unknown. Proteomics show just how wide and diverse is the platelet protein content and several hundred secreted proteins have been identified[13]. Table I highlights a selection of the more prominent proteins that are somewhat arbitrarily grouped into functional categories. For the most part, stored proteins are synthesized in MKs and traffic in endosomes to developing granules; however, some are captured by MKs or platelets from their environment by endocytosis (e.g. Fg, factor V (FV), albumin, immunoglobulin G (IgG))[12]. Ca2+ and Mg2+ are enriched in α-granules that also contain acidic glycosaminoglycans (mainly chondroitin-4-sulphate) localized to distinct domains

Adhesive proteins	VWF + pro-peptide, Fg, Fn, Vn, TSP-1, TSP-2, laminin-8, osteonectin	Cell contact interactions, platelet function and clotting, wound healing, bone metabolism, inflammation
Clotting factors and their inhibitors	FV (+ multimerin), FXI, FXIII, TF*, prothrombin, HMWK, protein S, protease nexin-2 (amyloid β/A4 protein precursor (APP) (also see membrane glycoproteins)), C1 inhibitor, TFP1, protein C inhibitor, gas6**	Thrombin production and clotting, wound healing, inflammation
Fibrinolytic factors and their inhibitors	Plasminogen/plasmin, urokinase-PA, PAI-I, α2-antiplasmin, histidine-rich glycoprotein, thrombin-activatable fibrinolysis inhibitor (TAFI)	Plasmin production and fibrinolysis. Vascular modelling
Other proteases and anti-proteases	Metalloprotease (MMP)-1-4, -9, -14, ADAMTS-13, ADAM-10 (α-secretase), ADAM-17, TIMPs 1-4, α1-antitrypsin, α2-antitrypsin, α2-macroglobulin, granzyme B	Platelet function, angiogenesis, vascular modelling, regulation of coagulation, inflammation
Growth and mitogenic factors	PDGF (A, B and C), EGF, FGF, HGF, IGF, VEGF (A-D), bone morphogenetic proteins, IGFBP3, CTGF, connective tissue activating peptides	Chemotaxis, cell proliferation and growth, angiogenesis, wound healing, bone metabolism, cancer
Cytokines, chemokines and related compounds	TGF-β1, IL-1β□□□, IL-1α, Il-2, IL-4, TNF-α, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (MCP-3), CCL14, CCL15 (MIP-5), CCL17, CCL19 (MIP-3b), CCL20 (MIP-3a), CXCL1 (GROα), CXCL2 (MIP-2α), CXCL3 (MIP-2β), CXCL4 (PF4), CXCL4L1 (PF4alt), CXCL4L (PFAalt)CXCL5 (ENA-78), CXCL7 (β-thromboglobulin, platelet basic protein or CTAP-III), CXCL8 (IL-8), CXCL12 (SDF-1α), TPO*, angiopoietin-1	Regulation of angiogenesis, cellular proliferation and differentiation, chemotaxis, vascular modelling, cellular interactions, immunity, bone metabolism, immune-regulatory and inflammatory processes, cancer
Anti-microbial proteins	Several chemokines and truncated derivatives often grouped globally as thrombocidins (from CTAP-III or NAP-2) and kinocidins (from the PF4 family)****, human β-defensin-1, -2, -3*****, thymosin-β4, fibrinopeptides A/B	Bactericidal and fungicidal properties, chemoattractants, inflammation, infections (sepsis)
Others	Serglycin (secretory granule proteoglycan core), chondroitin 4-sulfate, albumin, IgG, IgA and IgM, C3 and C4 precursor, properdin factor D, Factor H, bile salt-dependent lipase, autotaxin, lysophospholipase-2, clusterin, (+ APP), PDI******, HMGB1*, dickkopf-1, osteoprotegerin (OPG), substance P, brain-derived neurotrophic factor (BDNF)*, endostatin (proteolytic fragment of collagen), angiostatin (proteolytic fragment of plasminogen), angiogenin	Various functions including tissue remodelling, inflammation, immunity and disease states including cancer
Membrane glycoproteins	αIIbβ3, αvβ3, GPIb, PECAM-1, ICAM-2, semaphorin 3A, semaphorin 4D, PLEXIN-B1, CD147, TLR-1-7, -9, Siglec-7, receptors for primary agonists, P-selectin, TLT-1, JAM-1, JAM-3, claudin-5, PSGL, CD40L, Glut-3, TRAIL (Apo2-L), TWEAK (Apo3-L (TNF), APP (amyloid beta (A4) precursor), gC1qR, Fas ligand (CD95), beta-2-microglobulin, hyaluronidase-2	Platelet aggregation and adhesion, endocytosis of proteins, thrombin generation, platelet-leukocyte and platelet-vascular cell interactions, inflammation, wound healing, immune modulation, disease states

TABLE 1

(or cores) where they concentrate basic proteins such as platelet factor 4 (PF4, chemokine CXC motif ligand 4 (CXCL4)). The granule membrane contains intrinsic GPs (e.g. P-selectin, Trem-like transcript-1 (TLT-1) and CD40L) as well as many of the plasma membrane receptors and the abundant presence of αIIbβ3. Their surface expression confers new properties to the activated platelet promoting platelet-leukocyte tethering or platelet interactions with other cells as well as consolidating platelet interactions within the thrombus.

Adhesive proteins are abundant in the α-granule storage pool (Table I); secreted VWF, Fg, Fn and vitronectin (Vn) all participate in platelet-to-platelet interactions even if Fg plays the major role. Fibrillar cellular Fn in the vessel wall is an excellent substrate for thrombus formation. Special mention should be made of thrombospondin-1 (TSP-1), one of the most abundant α-granule proteins; TSP-1 plays an important role in thrombus stability and clot retraction. Adhesive proteins may also act directly as mitogens or they may promote mitogen activity of growth factors. The α-granules are also a source of coagulation and fibrinolytic factors. However, they also contain inhibitors of coagulation (e.g. tissue factor pathway inhibitor (TFPI), protease nexin-2) and of fibrinolysis (plasminogen activator inhibitor type I, PAI-1). This illustrates the fundamental enigma of platelet α-granules that store proteins with contrasting effects and also of the corresponding roles of platelet as compared to plasma pools.

Questions were raised on how stimulators and inhibitors of angiogenesis were stored. Italiano et al[14] localized pro- (e.g. vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF)) and anti-angiogenic proteins (e.g. endostatin, TSP-1) to distinct granule sub-populations in platelets and in MKs; they also backed up earlier work that these cargos were released with different kinetics. Nevertheless, high-resolution and scanning transmission electron microscopy (STEM) suggested another explanation; that granule cargos are compartmentalized zonally but within the same organelle while three-dimensional images obtained by cryo-electron tomography showed α-granules with microvesicular and tubular internal structures consistent with structural heterogeneity[10, 15]. Tissue inhibitors of metalloproteases (TIMPs) were clearly stored separately from VWF as platelets from a donor with an inherited disorder of α-granule production failed to label for VWF while normally containing TIMPs that were organized in individual compartments[16]. Another granule cargo stored in specific granules (termed T-granules by some) is protein disulfide isomerase (PDI), a secreted protein that co-localizes with toll-like receptor-9 (TLR9) and which on secretion stabilizes a fibrin clot together with the cross-linking protein, FXIII[17]. The presence of TLR9 suggests a link with innate and adaptive immune responses as well as infectious inflammation.

Platelet release of α-granule constituents requires docking of the granule membrane with either the plasma membrane or the OCS followed by membrane fusion. Similarly to dense granules, exocytosis resolves around vesicle- and plasma membrane-bound SNARE proteins and their chaperones[10, 18]. STEM tomography further revealed how α-granules can liberate their contents through tubular extensions reacting directly with the plasma membrane while OCS membranes join independently with the plasma membrane thereby increasing platelet surface area[15]. Differential sorting of α-granules has also been shown, with granules labeling for VAMP-7 sorting to a more peripheral localization during platelet spreading as compared to those expressing VAMP-3 or VAMP-8[18]. Differentially packaged and segregated proteins may have different diffusion rates to the exterior while the spatial localization of the granules, determined by VAMP isoforms, and the size of the fusion pores may also influence secretion kinetics, as will the strength and nature of the stimulus initiating secretion.

(iii) Lysosomes

These contain enzymes such as cathepsins D and E, elastase, β-glucuronidase and acid phosphatase; while their membranes resemble dense granules in expressing CD63 and lysosome-associated membrane protein-2. Platelets also contain a constitutively active autophagy pathway[19].

3. OTHER PLATELET CHANGES ON ACTIVATION

PS expression on platelets allows the binding of coagulation factors (e.g. FVIII) and the rapid formation of an activated FXa/Va complex. The latter transforms prothrombin into thrombin in a Ca2+-dependent process. Thrombin itself is a powerful mitogen. However, its main immediate role is in the formation of the fibrin clot. PS expression is also essential for the release of membrane-bound MPs by platelets; these bud off from the platelet surface following calcium-dependent uncoupling of the underlying cytoskeleton from the plasma membrane. Procoagulant in nature, MPs intervene in thrombotic disease and inflammation being, for example, active mediators of rheumatoid arthritis[20]. MPs express P-selectin and 12-lipoxygenase and the release of 12(S)-hydroxyeicosatetranoic acid promotes their internalization by neutrophils. Quite surprisingly, platelets can also release mitochondria, both within MPs and as free organelles[21]. Degradation of the mitochondrial membrane by soluble phospholipase A2 leads to the release of inflammatory mediators while mitochondria themselves can bind to neutrophils.

4. PLATELETS AND WOUND HEALING

Platelets intervene at many stages of wound healing including restoring the integrity of blood vessels after injury or after atherosclerotic plaque rupture. Collagens, proteoglycans and adhesive proteins such as Fn are major constituents of the ECM; providing a molecular scaffold for incoming platelets and migrating cells such as fibroblasts[22]. Thrombus growth at the injured site concentrates platelets to participate in tissue remodeling by secreting a variety of growth factors, cytokines, chemokines and other factors (Table I). For example, VEGF, platelet-derived growth factor (PDGFa/b and c), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), connective tissue growth factor (CTGF) and insulin-like growth factor (IGF) form chemotactic gradients through binding to matrix components or to newly generated fibrin. Transforming growth factor-β1 (TGF-β1) recruits inflammatory cells into the wound area and stimulates fibroblasts to produce connective tissue and the ECM; Fg itself can enhance wound closure by increasing cell proliferation and migration while it forms matrix fibrils with Fn, a substrate for αvβ3 on fibroblasts[22]. PDGF particularly stimulates fibroblast migration. Fibrin is very important for wound healing, providing an additional meshwork for cells; but it is the platelet mass that limits plasma loss[3]. Fibrin degradation products also attract leukocytes and aid the transition between inflammation and tissue repair.

Platelets favor angiogenesis by recruitment, proliferation and differentiation of endothelial and other vascular cells. Growth factors such as VEGF, bFGF and PDGF, also enhance late events such as endothelial tube formation and sprouting of new vessels[23]. Yet we underline the apparent paradox that platelets also store and release anti-angiogenic factors such as endostatin, PF4, TSP1 and the TIMPs that may counterbalance the effect of the pro-angiogenic mediators[14]. PF4 is perhaps the best studied of these. It binds with high affinity to heparin and to heparin-like molecules on the endothelial cell surface and is a negative regulator of angiogenesis by inhibiting VEGF and FGF as well as blocking the cell cycle making it a molecule with anti-cancer properties[24]. Stromal cell derived factor 1 (SDF-1) is an α-granule stored chemokine that through binding to CXCR4 and CXCR7 on progenitor or mesenchymal stem cells enhances their recruitment to the site of vascular lesions[25]. Platelets also are capable of modulating the balance between cell survival and apoptosis. SDF-1 acts with serotonin, ADP and sphingosine-1 phosphate to favor cell survival. In contrast, a number of tumor necrosis factor-α (TNF-α) related apoptosis regulators secreted from platelets (e.g. CD40L, soluble Fas Ligand, TNF-related apoptosis-inducing ligand (TRAIL)) can induce inflammatory responses in fibroblasts, smooth muscle cells, neutrophils, monocytes and other cells as well as promoting apoptosis[23]. Not only biologically active proteins participate in wound healing. Serotonin plays an active role in liver regeneration[26].

Defining how platelets control the balance between cell proliferation and cell elimination at the wound site will be a key feature of future research. Tissue factor (TF) is the initiator of the extrinsic pathway of coagulation; it also plays a key role in angiogenesis and wound healing. Whether circulating platelets intrinsically possess TF is unclear; however, they can (i) take it up by transfer from monocytes and their MPs by a P-selectin dependent mechanism and (ii) on activation, can synthetize it from preformed mRNA via their spliceosome. Platelets are a rich source of metalloproteases (MMPs) possessing MMP1-4, -9, -14, ADAMTS-13 (a disintegrin and metalloprotease with thrombospondin type I repeats-13), ADAM-10 and -17 among others. MMPs have many biological roles that include tissue remodeling[27].

5. PLATELETS AND INFLAMMATION

A) Inflammatory proteins

Inflammation involves close interplay between platelets, leukocytes and cells of the immune system. It is critically linked with thrombosis in many major acquired diseases. Some secreted platelet metabolites are pro-inflammatory including TXA2 and PAF while dense granules are sources of serotonin and histamine[28]. Platelet α-granules contain many proteins able to influence inflammation[12, 28]. Activated platelets within the growing thrombus recruit and bind immune cells by secreting chemoattractants and expressing granule-derived P-selectin and other targets for leukocyte GPs. Monocytes, neutrophils and lymphocytes are all recruited and once present become activated as part of their inflammatory response[9]. Secreted chemokines and cytokines such as CXCL4, CXCL7 and CCL5 (chemokine C-C motif ligand 5 (regulated upon activation normally T-expressed and secreted (RANTES)) favor immune cell recruitment and activation; specifically, neutrophil-activating peptide-2 (NAP-2, a proteolytic derivative of CXCL7) induces immune cells to traverse the thrombus and enter the vessel wall[29]. Other chemokines of interest are interleukin-8 (IL-8), macrophage migration inhibitory factor (MIF), growth-regulated oncogene-α (Gro-α), epithelial activating protein-78 (ENA-78) and monocyte chemoattractant protein-3 (MCP-3) (Table I)[29]. Platelet expression of adhesive proteins, membrane GPs and the surface exposure of P-selectin helps stabilize the interaction between platelets and endothelial or immune cells via interplay between surface receptor pairs. Significantly, many of these mechanisms are involved in atherosclerotic plaque formation[22]. The importance of platelets is confirmed by the increased bleeding in inflammatory states when the platelet count is low[30]. The role of platelets extends well beyond the vascular system. For example, regardless of the blood-brain barrier, platelets influence central nervous system repair through leukocyte recruitment to inflammatory sites and by promoting regenerative processes in the nervous system including the incoming of stem/progenitor cells[31].

B) Antimicrobial proteins

A special and increasingly recognized function of platelets is in host defense both in the circulation and at sites of vascular lesions such as in infectious endocarditis[12, 32]. Bacteria can bind to platelets indirectly via adhesive proteins such as Fg or VWF that recognize receptors on platelets and the bacterial surface or they may even bind αIIbβ3 or GPIb directly. Platelets also contain FcγRIIA that recognizes IgG bound to bacteria and a host of specific receptors for bacterial proteins including TLR1-7; 9, whose occupancy leads to platelet release of microbicidal proteins and cytokines with recruitment of circulating inflammatory cells and bacterial destruction[33]. Bacteria can be internalized by platelets and they can promote apoptosis; platelet interactions with bacteria can modify platelet function with release of immunomodulators leading to falls in platelet count or even thrombosis. Taking a specific example, inflammation drives thrombosis in the liver after Salmonella infection and does so in a TLR4-dependent cascade via ligation of C-type lectin-like receptor-2 (CLEC-2) on platelets by the membrane glycoprotein, podoplanin, on monocytes and kupffer cells[34]. As well as bacteria, platelets can directly bind and internalize many types of virus including human immunodeficiency virus; capture involves multiple

platelet receptors including CLEC-2[35]. Some cytokines released from platelets have direct microbicidal activities (Table I) including CXCL4, CXCL7, thymosin-β4 and RANTES. Of particular importance are NAP-2 and thrombocidins (TC-1 and -2), small C-terminal proteolytic derivatives of CXCL7. Platelets also store and secrete from α-granules elements of the complement (C) cascade (C3, C4 precursor) as well as proteins that regulate complement activity. The ability of platelets to bind complement is another element in the interaction of activated platelets with bacteria[32].

C) Sepsis

An extreme condition combining infection, an uncontrolled immune response and inflammation, sepsis is associated with a high degree of mortality. Platelet accumulation in inflamed tissues accelerates immune cell recruitment and the excessive response can promote organ dysfunction. The onset of disseminated intravascular coagulation can lead to a fall in platelet count and increased vascular permeability (aided by platelet VEGF and serotonin release) with edema, shock and organ failure. Sepsis is a progressive systemic inflammatory condition and the kallikrein/kinin systems, elements of which can be secreted from platelets (Table I), can have a prominent role[36]. As discussed earlier, as well as secreting their granule contents, anucleate platelets entrapped in a clot can synthesize proteins such as Il-1β and TF from preformed mRNA. Il-1β can bind to fibrin where it retains its activity while TF favors thrombosis. Significantly, as well as producing inflammatory molecules, a major role for secreted ADP either from platelets and/or tissue cells, in systemic inflammation and sepsis, has been confirmed through the use of anti-platelet P2Y12 drugs in man[37]. In inflammatory states, hepatic TPO production is upregulated by IL-6 leading to an overproduction of platelets; at the same time, platelet clearance in the liver may be part of the acute phase response and help increase the chance of survival in sepsis[5]. Also, a highly inflammatory state can lead to an upregulation of platelet production by caspase-dependent direct fragmentation of MKs, a process promoted by IL-1α/IL-1 type I receptor signaling[38].

Wnt/β-catenin signaling has a major influence in lung repair and activated platelets are sequestered in pulmonary vascular beds. Modulation of Wnt/β-catenin signaling by platelet-derived Dickopf-1 (Dkk1) is a major factor in promoting neutrophil trafficking and the inflammatory response in the lungs; Dkk1 is another example of a relatively unknown α-granule protein[39].

6. INNATE AND ADAPTIVE IMMUNITY

Platelets are now known to act as sentinel innate immune cells[40, 41]. This role will now be illustrated with reference to three platelet α-granule proteins.

A) CD40L

A much-studied platelet cytokine is CD40 ligand (CD40L, CD154), first identified on activated helper T cells and a member of the tumour necrosis factor (TNF) family[41]. It binds to CD40 on antigen-presenting cells; other receptors include αMβ2, α5β1 and αIIbβ3 on platelets thereby also linking it to thrombosis. In the immune system, the CD40L/CD40 interaction drives B-cell proliferation and antibody production; it plays a primary role in immunoglobulin (Ig) class switching and it has been implicated in autoimmune disorders. Platelets constitute the major reservoir for CD40L in blood; present in the α-granule membrane, it is transported to the platelet surface on platelet activation. Here, it is available to bind vascular and immune cells and participate in inflammation, in stimulating interleukin and cytokine production and in the release of reactive oxygen species. Surface-expressed platelet CD40L is a substrate for MMP activity with release of the smaller but still biologically active soluble CD40L (sCD40L) that has become a plasma marker for inflammation.

B) TREM-like transcript-1

The triggering receptors expressed on myeloid cells (TREMs) contain a single V-set Ig domain, and are involved in cell activation within the innate immune system with a key role in sepsis. A GP with significant homology to the TREMs, TLT-1, is exclusive to the mouse and human megakaryocyte

(MK) lineages where it co-localizes with P-selectin in the α-granule membrane. It is translocated to the platelet surface when platelet activation leads to secretion and supports platelet aggregation thereby protecting against bleeding during inflammation. Like CD40L (and P-selectin), TLT-1 can be the object of cleavage by MMPs with liberation of a soluble form that has a regulatory role in sepsis by modulating platelet-neutrophil crosstalk[42].

C) High mobility group box 1 (HMGB1) protein
HMGB1, principally known as a nuclear protein, is also secreted by immune cells when it acts as a cytokine-mediator of inflammation. It was recently recognized to be stored in platelet α-granules from which it is translocated both to the platelet surface and secreted on platelet activation in multiple inflammatory diseases[43]. As repeatedly stated by us, thrombosis and inflammation are inseparably linked and in this context HMGB1 appears as a critical player in both processes. Mice specifically lacking HMGB1 in their platelets have increased bleeding risk, reduced thrombus formation and platelet aggregation, and reduced inflammation and organ damage during experimental trauma/hemorrhagic shock[43]. HMGB1 offers yet another excellent example of a previously unrecognized platelet protein with multiple functions in health and disease. Activated platelets commit neutrophils to form neutrophil extracellular DNA traps (NETs) with released HMGB1 playing a key role by binding to neutrophils and through the induction of autophagy[44]. Platelets play a key role in NET formation. NETs are important for the host response to infection and inflammation but can have harmful effects such as promoting microvascular and deep vein thrombosis).

7. CONCLUDING REMARKS

Space restrictions have necessitated that we make our review highly selective. Platelets participate in many major illnesses. For example, circulating tumor cells may bind platelets and even aggregate them; an interaction that can protect tumor cells from the immune system and also help deposit them within the vasculature by way of platelet adhesive receptors (e.g. GPIb, integrins, P-selectin)[8]. Release of ADP and ATP, the expression of P-selectin after platelet activation and the generation of thrombin on the now procoagulant platelet surface may all favor metastasis within the vessel wall and help tumor stability. The release of α-granule proteins may promote angiogenesis and vascularization of the tumor; a novel enzyme secreted from platelets that liberates lysophosphatidylcholine and stimulates tumor cell mobility is autotaxin[45].

By acting as a major source of secretable pools of amyloid-β precursor, a substrate for α-secretase (ADAM10), and by being activated by amyloid-β in the walls of cerebral vessels leading to thrombus formation and granule release, platelets participate actively in the progression of Alzheimer's disease, an age-related neurodegenerative disorder[46, 47]. Amyloid-β binds directly to αIIbβ3 integrin and stimulates release of ADP and the chaperone protein clusterin from platelets. The latter promotes the formation of fibrillar amyloid-β aggregates while ADP further promotes αIIbβ3 activation and clusterin release in a feedback mechanism. The pro-inflammatory potential of platelets and MPs lead to roles in acute lung injury, asthma, inflammatory bowel disease (with elevated levels of RANTES) and migraine (through IL-1 and β-thromboglobulin) among many examples[9].

Yet in this context, studies using platelet-rich plasma derivatives therapeutically confirm many in vitro studies showing how platelets stimulate the growth of many types of cell including osteogenic cells, brain and nerve cells and various cellular constituents of muscles and tendons [see chapters 4 and 14 in this book]. It will be exciting to see how these therapies advance and how the active players are identified. It will also be interesting to see the progression of alternative approaches such as using genetically modified progenitor cells or MKs so that platelets are produced with α-granules containing proteins of therapeutic benefit such as FVIII as a treatment for hemophilia; an approach that may also ultimately be of benefit in cardiovascular disease, cancer and Alzheimer's disease[48].

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CHAPTER 2

Characterization of Plasma Rich in Growth Factors (PRGF): Components and Formulations

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SUMMARY

Platelet-rich Plasma (PRP) is a set of autologous platelet products used to reduce pain and speed up recovery from injury while maintaining the tissue function. Its basic rationale is to mimic yet enhance the natural processes of healing by bringing to the injury site a set of molecules that will accelerate functional recovery, and even regenerate the tissue. In the array of PRP-products, Plasma Rich in Growth Factors (PRGF)-Endoret is a pioneering autologous regenerative technology with multiple therapeutic potentials. It can be produced in at least four different formulations, depending on coagulation and degree and type of activation. PRGF-Endoret technology is safe and versatile, and has a wide range of applications.

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1. POTENTIAL OF PLASMA RICH IN GROWTH FACTORS (PRGF-ENDORET): MIMICKING THE NATURAL HEALING PROCESS

The increasing number of musculoskeletal injuries has produced a concurrent stimulus in both the number and the effectiveness of different treatments of these lesions, especially in the search for minimally invasive procedures or adjuvants[1-3]. One of these cutting-edge technologies is Plasma Rich in Growth Factors (PRGF-Endoret)1. This biological treatment mimics the natural pathways of wound healing[4] by driving to the injury site the whole protein array of PRGF that is involved in the repair of damaged tissues. In this way, all the bioactive molecules (including growth factors and other proteins) necessary for tissue repair are efficiently and locally released.

The tissue repair process occurs naturally in a staged fashion[5] and includes removal of dead cells, proliferation, migration of cells to the injury site, production of new vascular structures, and other events. The organization of all these elements influences healing in a given injury, preventing fibrotic elements that cause loss of functional capacity in that tissue[6,7] i Growth factors play an important role coordinating the whole process in an orchestrated fashion in all tissues of the musculoskeletal system, including muscle[8], tendon[9], bone[10,11], and cartilage[12]. Growth factors act on other tissues as well, including skin[13], oral soft tissue[14,15], and cornea[16] among others.

PRGF-Endoret technology mimics the natural healing mechanisms, but with two special features: avoiding loss of functionality (fibrous tissue) and shortening healing times. This is achieved in part by adjusting the PRGF-Endoret formulation and dosage to the type of tissue and injury.

PRGF-Endoret therapy accelerates and improves tissue healing by local delivery of autologous bioactive molecules and hence, contributing a first line provisional scaffold[1]. This autologous therapeutic toolbox consists of platelets as both reservoir and vehicle of a large repertoire of proteins[17,18.] Recently, a proteomic dissection of PRGF scaffold was performed[19]. In this research, the authors studied those proteins that remained most closely bound to the fibrin network and that were therefore retained by the mesh itself, rather than being released into the supernatant. The high-throughput proteomic techniques used in this characterization allowed us to produce a catalogue of these proteins and subsequently to classify them into families on the basis of their function and gene ontology. The results of this process showed that the fibrin network is enriched in proteins specifically involved in tissue regeneration and wound healing. Interestingly, there was found to be an enrichment in certain lipoproteins, which are involved in regenerative processes, particularly by delaying degradation (fibrinolysis) of the fibrin network, thereby extending the controlled release of other molecules. Similarly, an important family of proteins involved in the acute phase reaction was found to be present. These proteins form the first line of defence in the immune system[19].

In the last decade, several systems have been developed to produce a biologically active product, both commercially and in-house, but they differ in the presence of white blood cells, growth factors concentration, and architecture of fibrin scaffold[20-24]. The different PRP commercial systems can be certified for various medical applications, but the therapeutic outcome will depend on the type of platelet-rich plasma used and the dosage employed. Establishing a proper classification of PRPs and identifying the biological differences among them is absolutely necessary to understand some of the controversial results obtained with these types of technologies so far[25].

One of the most relevant and controversial issues is the presence of leukocytes in the platelet-rich plasma. In order to distinctly define the PRGF technology, and thus be able to compare other PRPs, PRGF can be categorized according to three of the most cited classifications that have been proposed for PRPs. The first and most widely used[26] classifies PRGF as pure-PRP (P-PRP) since it does

not contain WBC. The PRGF is classified as type 4-B (Minimal WBCs, activated with $CaCl_2$, and platelet concentration below 5x) as has been proposed[27] for sports medicine classification. Finally, PRGF would fit in the P2-x-Bβ category (platelet count greater than baseline levels to 750,000 platelets/µL, exogenous activation with $CaCl_2$, with WBC -and specifically neutrophils- below to baseline levels) according to the PAW (platelets, activation and WBC) classification[28].

2. UNDERSTANDING THE PROPERTIES OF PLATELET-RICH PLASMA PRODUCTS

Several key biological mediators are present in a PRP. The more studied growth factors contained in platelet-rich plasma that are important during tissue repair include IGF-I (Insulin-like Growth Factor type I), TGF-β1 (Transforming Growth Factor β type 1), PDGF (Platelet Derived Growth Factor), HGF (Hepatocyte Growth Factor), VEGF (Vascular Endothelial Growth Factor), EGF (Epithelial Growth Factor) and bFGF (basic Fibroblastic Growth Factor) among others (Table 1)[29,30]. Some of them (IGF-I and HGF) are plasmatic proteins, and their concentration does not depend on the platelet enrichment. However, most of the growth factors are indeed platelet proteins, both synthesized and adsorbed, and thus their quantity does depend on the platelet concentration. To understand the properties of platelet-rich plasma products, it is necessary to detail the different roles of molecules that it contains:

- IGF-I: This protein circulates in plasma as a complex with binding proteins (IGFBP). This determines the bioavailability and regulates the interaction between this IGF-I and its receptor[31,32]. IGF-I is involved in keratinocyte migration and wound healing[33,34], stimulates bone matrix formation and maintenance[35] by promoting preosteoblast proliferation[36,37], and also is involved in striated muscle myogenesis[38]. Furthermore, knockout mice for IGF-IR in muscle exhibited impaired muscle regeneration and deficient myoblast differentiation[39]. Recently, It has been observed that IGF-1 promotes tissue repair of skeletal muscle without scar tissue formation by increasing fibre size and muscle size hypertrophy[40]. Also, and related to this, IGF-1 is considered a potent enhancer of tissue regeneration, and its overexpression in muscle injury leads to hastened resolution of the inflammatory phase[41].

- TGF-β1: The role of TGF- β family proteins in wound healing has been recently reviewed[42]. TGF-β has different effects, depending on the tissue and the cell type[6]. The release and posterior bioactivation of latent TGF-β contributes to the early cellular reparative responses, such as migration of cells and neovascularization and angiogenesis[43] into the wound area. In bone, TGF-β1 induces osteogenic differentiation of mesenchymal cells of the bone marrow, upregulating osteoblast differentiation markers[44]. TGF- β plays a crucial role in maintaining homoeostasis of both articular cartilage and subchondral bone[45].

- PDGF: This growth factor is a mitogen and chemotactic factor for all cells of mesenchymal origin[46]. It is important in the repair of joint tissue, including cartilage and meniscus[47,48]. Bone is also a target of PDGF, influencing its metabolism and acting in repair mechanisms[49,50], including the recruitment of pericytes to stabilize new blood vessels[51].

- HGF: Also called scatter factor, it regulates cell growth, migration and morphogenesis[52] and plays an important role in wound-healing through an epithelial-mesenchymal interaction[53]. HGF modulates central inflammatory and immune events that are common to many diseases and organ systems[54]. The antifibrotic effect of HGF has been shown in various tissues[55,56], through induction of Smad[7], and thus regulates the myofibroblast phenotype, allowing the initial contraction of the wound, but gradually making the myofibroblast disappear[57].