Tissue regeneration and in loco administration of platelet derivatives: clinical outcome, heterogeneous products, and heterogeneity of the effector mechanisms

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BACKGROUND: In loco administration of platelet (PLT) derivatives is a relatively new auxiliary treatment for tissue regeneration to be hastened. Enthusiastic reports are faced by more critical ones. The more obvious rationale for the in vivo administration of PLT derivatives resides in their growth factor content.

STUDY DESIGN AND METHODS: The relevant literature was systematically reviewed. Close scrutiny of the technical details was carried out to find out the procedural differences accounting for conflicting results.

RESULTS: An impressively vast heterogeneity of conduct was found in both in vitro and in vivo studies. Major outcome-affecting variables were recognized such as those associated with PLT preparation; growth factor measurement; proliferation test; dose, timing, and administration of the PLT derivatives; study design; and primary endpoints.

CONCLUSIONS: So many variables were found making standardization or confrontation of the in vitro and the in vivo studies barely conceivable or manageable. The mechanisms of action are very complex. The attribution of tissue regeneration capacity of PLT derivatives solely to the PLT-derived growth factors is simplistic. The results obtained through in vitro experiments are indicative for general mechanisms. Their simplistic hold to the complex in vivo environment may be misleading.

Appreciable effects have been obtained by in loco administration of platelet (PLT) derivatives such as PLT gel, PLT-rich plasma (PRP), and similar products to treat tissue lesions.[1-3] Although medicine and transfusion medicine are both science-based disciplines,[4] empiric therapeutic practice may sometime precede the full scientific comprehension of the biologic mechanisms underlying the therapeutic effect of the administrated treatment. "Medicine profits from the fact that clinicians often provide a first impulse and then the basic scientists take over the background research into mechanisms"[5] dissecting the true *noumenic* biologic order underlying the *phenomenic* appearance.

PLTs release growth factors (GFs) upon activation. GFs are a driving force for tissue regeneration. Based on these data, concentrated PLTs have been proposed to enhance soft and bony tissue repair providing in loco GF delivery in the assumption that PLT and GF concentrations correlated.[6-8]

Studies were carried out in vitro to measure the amount of GFs in the PLT concentrates and to test the proliferation effect of these factors upon cultured cells from a variety of tissues. The common denominator of

ABBREVIATIONS: b-FGF = basic fibroblast growth factor; ERK = extracellular signal-regulated kinase; HUVEC(s) = human umbilical vein cells; GF(s) = growth factor(s); MMP(s) = matrix metalloproteinase(s); PDGF = PLT-derived growth factor; PRP = PLT-rich plasma; VEGF = vascular endothelial growth factor.

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these studies was to define the product characteristics and to assess the predictability for its clinical value. In vivo studies were carried out to verify the hypothesis of the clinical usefulness of the PLT derivatives in a clinical and an experimental (animal) setting.

To understand if and how PLT-derived products work in clinical conditions, several questions should be answered. How must PLTs be prepared and handled to achieve and maximize their putative tissue regeneration effect? Is their presumed healing capacity solely mediated by PLT-derived GFs or other factors are engaged for tissue regeneration to occur likewise? In this review, we will navigate through several experimental reports trying to find the Ariadne's thread leading to common elements of certainty.

MATERIALS AND METHODS

Articles relevant to the production, the study, and the use of PLT products were reviewed to disclose the details that entailed the nature of the results, whichever the conclusion. In particular, we considered the description of specific features related to the preparation of the PLT concentrates; the GF dosage and their selection for dosage; the selection of cells used for the proliferation study; the aim of the study; the amount, the timing, and the route of administration of PLT products in clinical studies; the lesions and the patients selected for treatment; the tissue and the cells assumed to be responsive to PLT-derived factors; and the endpoints chosen to ascertain the clinical efficacy. In doing so, we dissected the variables of the experimental conduct aiming to find elements to disclose some consensus among the heterogeneity of the studies and to answer some of the questions previously asked. Because results are thought to be strongly determined by methods, methodologic details are given in the next section.

RESULTS

PLT preparation

PLTs were collected with either continuous-flow[9,10] (COBE, Gambro BCT Inc., Lakewood, CO; COM.TEC, Fresenius HemoCare GmbH, Schweinfurt, Germany) or discontinuous-flow[6,11] (MCS-3p, Haemonetics, Munich, Germany; MCS+, Haemonetics, Braintree, MA) blood cell separators. Alternatively, PLTs were obtained by differential centrifugation of whole blood collected in acid citrate dextrose (ACD)-containing tubes,[12,13] citrate phosphate dextrose–containing bags,[14] or ACD-containing microbags[8] (PCCS, 3i Implant Innovations Inc., Palm Beach Gardens, FL). In one case, the PLT collection system was not specified.[15] In one report, leukodepleted PLTs were used.[9] In another one, both leukodepleted and white blood cell (WBC)-rich PLTs were used.[10] In other reports, no detail on leukodepletion was given.[6,8,11-15] Human PLTs were used thoroughly with a unique exception where equine PLTs were used.[11] In most studies, no detail was given on donor selection criteria. Just one study reported on the selection of nonsmoker donors.[12] In one study, PLTs were collected from both donors and patients.[13] The number of subjects who were actually investigated was reported only in two studies, 213 and 5, respectively.[6,8] The concentration of the PLT suspension was reported in all cases but one. Most authors have used PLTs at the concentration of 1×10^9 to 2×10^9 per mL.[6,8,10,13,14] Others have used PLTs at a lower concentration, ranging from 1.0×10^6 to 6.53×10^8 per mL.[9,11,12] In a single case, instead of PLT concentration, the authors described the protein content of the PLT releasate.[15]

The analytical measurement of PLT GFs and the proliferation tests were performed after different kinds of PLT manipulation. In one case, PLTs were used fresh without any prior manipulation.[12] In two cases, washed PLTs (phosphate-buffered saline and PBS-ethylenediaminetetraacetate, respectively) were used.[9,13] One report described the use of 4 percent paraformaldehyde-fixed PLTs.[9] Frozen PLTs (from –30°C to –80°C) were used by several authors.[6,10,11,13,15] In these cases, PLTs were centrifuged at high speed to remove debris before testing. In two experimental settings, PLT extracts were obtained by multiple freeze (–20°C and –80°C, respectively) and thaw (37°C) treatment followed by final ultracentrifugation of the samples.[9,13] In a single case, the PLT extract was obtained by solvent treatment (0.5 percent Triton X-100, Serva Electrophoresis GmbH, Heidelberg, Germany) before freezing the mixture at –70°C.[10] In some experiments the PLT releasate was obtained treating PLTs with calcium and thrombin at different concentrations: the releasate was then used after storage at temperatures between –70°C and –85°C.[8,11,14]

GF measurement

In most cases, GFs were measured with a quantitative sandwich enzyme immunoassay (Quantikine, R&D Systems, Minneapolis, MN).[6,8-12] In one article, transforming growth factor-β1 (TGF-β1) was measured by means of an enzyme-linked immunosorbent assay kit (OptEIA, PharMingen, San Diego, CA) and vascular endothelial growth factor (VEGF) was measured by means of a human VEGF ELISA development kit (Peprotech Inc., Rocky Hill, NJ).

The concentration of different PLT-derived GFs was measured: PLT-derived growth factor (PDGF)-AB,[6,9-12] PDGF-BB,[6,8,10] TGF-β1,[6,8–12] TGF-β2,[6] insulin growth factor-I,[6] basic fibroblast growth factor (b-FGF),[8] and VEGF.[8] Statements on the rationale for the selection of the GF to be measured were usually lacking. Among the authors

TABLE 1. GF released from different kinds of preparation of PLT concentrates*

Reference and relevant results	PDGF-AB	PDGF-BB	TGF-β1	TGF-β2	Insulin growth factor-1	b-FGF	VEGF
Weibrich et al.[6]							
Whole PLTs	8.80	0.986	15.57	0.028	6	X	X
Whole freeze-stored PLTs	8.38	0.6	12.46	0.03	6.53	X	X
Martineau et al.[8]							
Thrombin-activated PLTs	X	1.95	0.62	X	X	0.004	0.007
Zagai et al.[9]							
Cocultured (fibroblasts) whole PLTs	0.453	X	1.9	X	X	X	X
Fixed PLTs	0.004	X	0.258	X	X	X	X
Cocultured (fibroblasts) lysed PLTs	1.061	X	3.561	X	X	X	X
Zimmermann et al.[10]							
Thermic shock							
One cycle	12.0	0.7	22.7	X	X	X	X
Two cycles	24.1	1.6	22.9	X	X	X	X
Lysed PLTs	47.7	2.2	24.0	X	X	X	X
Thrombin-activated PLTs	29.7	1.9	9.3	X	X	X	X
One cycle plus WBCs	18.4	1.6	33.2	X	X	X	X
Two cycles plus WBCs	27.3	3.5	33.1	X	X	X	X
Lysed PLTs plus WBCs	46.2	4.9	38.1	X	X	X	X
Thrombin-activated PLTs plus WBCs	29.9	2.6	13.8	X	X	X	X
Carter et al.[11]							
Thrombin-activated PLTs (equine)	0.152	X	1.526	X	X	X	X
Okuda et al.[12]							
Whole fresh PLTs	25.43	X	19.36	X	X	X	X

* Preparation and cell treatment before GF measurement are summarized the first column. Results of GF measurement are all reported as pg per 10^5 PLTs.

who have measured TGF-β1, only two described the requirement for its acidic conversion from the latent to the immunoreactive form.[6,10]

GFs were measured by almost all authors in the PLT releasate.[6,8,10-12] In a single experimental setting, PDGF-AB and TGF-β1 were measured in the supernatant of human fetal lung fibroblast HEL1 (American Type Culture Collection, Rockville, MD) in serum-free culture medium D-MEM (Gibco BRL/Life Technologies, Rockville, MD) cocultured with PLTs or with PLT lysate in the presence of 0.75 mg per mL type I rat-tail tendon collagen.[9]

The amount of the GFs was reported in different units of measure: pg per mL,[9,11] ng per mL,[6,12] either pg per mL or ng per mL,[8] and pg per 10^5 PLTs.[10] The vast heterogeneity of conduct of the experimental settings drove the disparity of the results. Evidence of disparity is given in Table 1, which briefly reports the most relevant results of the measurement of the GFs releasable from the PLT preparations. To make the comparison of the original results feasible these were converted in a single unit of measure (pg released from 10^5 PLTs).

In general, the measurements that resulted were rather coherent. GFs were poorly released from fixed PLT and they become more and more releasable going from fresh PLTs to frozen-thawed PLTs and to thrombin-activated PLTs. WBC-containing PLT concentrates released a greater quantity of GFs. Nevertheless, although with some degree of coherence, remarkable differences were found. Figure 1, a computational interpretation of the PDGF-AB measurements, depicts that the amount of PDGF-AB released from PLTs lysed either by thermic shock or by tensioactives were quite narrow, whereas that released from whole PLTs and that released from thrombin-activated PLTs were quite sparse. The dispersion of these results was wide, some data scattering up to 80 times from the mean value.

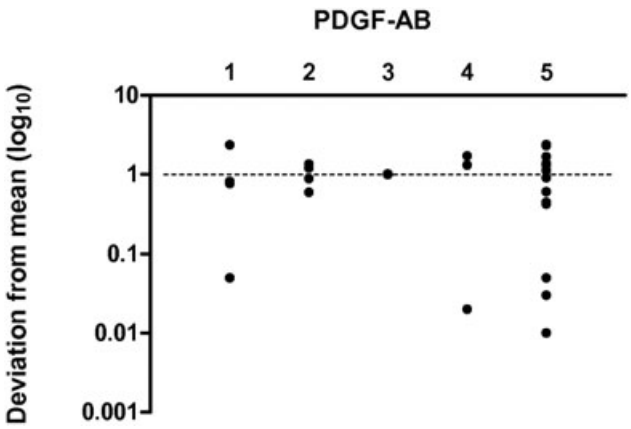

Fig. 1. Results of PDGF-AB measurement in the supernatant from different experimental settings. The deviation from the mean is reported (dotted line, log = 1). Lane 1, whole PLTs; Lane 2, PLTs lysed by thermic shock; Lane 3, PLTs lysed by tensioactives; Lane 4, thrombin-activated PLTs; Lane 5, overall results.

Cell proliferation studies

The target cells used to probe the capacity of PLTs, PLT releasate, or PLT lysate to enhance the cell proliferation rate in vitro were quite heterogeneous: human fetal lung fibroblast HEL1 (American Type Culture Collection, Rockville, MD);[9] human gingival fibroblast Gin-1, osteoblasts MG63, squamous epithelial SCC25, rat osteoblast UMR106 cells (Dainippon Pharmaceutics Co., Osaka, Japan), and human periodontal ligament cells from patients;[12] human umbilical vein cells (HUVECs, Clonetics/Bio-Whittaker, Walkersville, MD);[8] human fibroblasts from patients;[13,16] human stromal stem cells from healthy marrow donors;[14] chondrocytes from bovine and human cartilage tissue specimens;[15] and human fibroblasts from operative normal and irradiated tissue specimens.[17]

The culture conditions were even more heterogeneous. Variability concerned the presence and the amount of fetal bovine serum (FBS), the source and the amount of PLT-derived GFs, the duration of the culture, and the means by which the proliferation of the target cells was measured. Table 2 summarizes such heterogeneity.

In vivo studies

The in vivo surveys covered both animal[3,18,19,21-23,36-38,40] and human studies.[1,20,24-35] Animal studies included rats,[19] rabbits,[21,37,38] sheep,[23,36] and pigs.[18,22,40] Among these animal models, pigs are thought to have human-like osteoregeneration dynamics. A variety of tissues were treated such as maxillary bone,[18-24,33,35-38] long bones,[25] vertebral bones,[34,40] skin,[1,26,27,32] and ocular tissues.[28-31] Several PLT-derived products were used: PLT gel,[1,25-27] PLT lysate,[32] PRP,[19,21,22,34] PRP and autogenous cellular bone,[18,20,23,24,36-39] PRP and anorganic bone or bioceramics,[33,40] PDGF,[35] and hyperconcentrated PLTs.[28-31] The medication consisted of repeated doses[1,26,27,32] or single application[18-25,28-31,33-40] according to the kind of lesion to be treated. The primary aims of the studies differed according to the kind of lesion. The endpoints selected to evaluate the clinical results were quite heterogeneous. Last but not least, a few clinical reports were managed as case-control studies.[18,20-24,32,36-38,40] Given these premises, the clinical outcome results were quite heterogeneous (Table 3).

DISCUSSION

A vast heterogeneity of conduct was evident through PLT gel–related literature. Variables had to do with the methods for PLT preparation, the conditions to assay the PLT-derived GFs, the type and the origin of the cells selected for the in vitro study, the culture conditions, and the aim of the studies. The only unambiguous fact resulting from literature was that PLTs do contain GFs and that these factors have a primary role in cell proliferation (nothing new so far). Table 1, Table 2, and Fig. 1 summarize the span of the results. Several preanalytical factors affected such variability. PLT activation, aggregation, and stress might occur across PRP preparation. GF-containing releasate may be obtained with a variety of chemical and physical means: different amount of Ca^{2+} and thrombin, batroxobin, freezing-thawing cycles, and tensioactives. The quantity of GF-containing WBCs varied according to the PRP preparation. Poor correlation was found between the PLT count and the recovery of PLT-derived GFs.[6,8,12] High interindividual and inter-GF kinetics were demonstrated as well[8,10] making the picture, if possible,

TABLE 2. Heterogeneity of the in vitro experimental conditions

Reference	FBS content (%)	Cultured cells	Source and amount of GFs	Culture length (days)	Measurement of proliferation	Incremental proliferation (fold over the control value)
Martineau et al.[8]	1	HUVECs, 2.5×10^4/mL	10 percent PLT releasate from activated PLTs	5	Thymidine incorporation	8-32
Zagai et al.[9]	10	HEL1, 3×10^5/mL	PLTs, 1×10^8/mL	2	Fluorochromatic assay*	1.5
Okuda et al.[12]	1	Gin-1, MG63, SCC25, UMR106, PDL 1×10^5-2×10^5/35-mm dish	2-5 percent PRP	1	BrdU	1.4
Borzini et al.[13]	10	Human fibroblasts, 1×10^4/well	5-30 percent PLT releasate	7	MTT	2-5
Lucarelli et al.[14]	0-20	HSS, 2×10^4/cm^2	10 percent PLT releasate	9	MTT	2
Kaps et al.[15]	0-20	Human and bovine chondrocytes, 3×10^4/cm^2	PLT releasate paired (mg/mL) with FBS	1	BrdU	1.5
Loots et al.[16]	0.2	Human fibroblasts, 1×10^4/well	0.05-80 ng/mL recombinant	1	Thymidine incorporation	1.7-5
Lonergan et al.[17]	0	Human fibroblasts, 6×10^4/mL	50-100 ng/mL recombinant	7	Tetrazolium salt†	5

* Hoechst dye 33258 (Sigma-Aldrich, St Louis, MO).
† WST-1 assay (Roche Molecular Biochemicals, Indianapolis, IN).

TABLE 3. Summary of reference clinical studies performed with PLT derivatives as adjuvant treatment

Reference	Tissue/lesion	Species	PLT-derived product	Recovery or regeneration
Mogan and Larson[3]	Heart	Animal and human	PLT gel	Hypothetical
Schlegel et al.[18]	Maxillary bone	Animal—pig	PRP and autogenous bone graft	Not significant
Fontana et al.[19]	Maxillary bone	Animal—rat	PRP	Significant
Yazawa et al.[21]	Maxillary bone	Animal—rabbit	PRP	Significant (early phase)
Thorwarth et al.[22]	Maxillary bone	Animal—pig	PRP	Significant (early phase)
Fennis et al.[23]	Maxillary bone	Animal—sheep	PRP and autogenous bone graft	Significant
Grageda et al.[36]	Maxillary bone	Animal—sheep	PRP and autogenous bone graft	Weak
Weibrich et al.[37]	Maxillary bone	Animal—rabbit	PRP and autogenous bone graft	Weak
Aghaloo et al.[38]	Maxillary bone	Animal—rabbit	PRP and autogenous bone graft	Weak
Li et al.[40]	Spinal bone	Animal—pig	PRP and tricalcium phosphate	Poor
Mazzucco et al.[1]	Dehiscent wound and chronic ulcer	Human	PLT gel	Significant
Senet et al.[32]	Chronic leg ulcer	Human	PLT lysate	Weak
Marx et al.[20]	Maxillary bone	Human	PRP and autogenous bone graft	Significant
Oyama et al.[24]	Maxillary bone	Human	PRP and autogenous bone graft	Significant
Froum et al.[33]	Maxillary bone	Human	PRP and anorganic bone	Dubitative
Freymiller and Aghaloo[39]	Maxillary bone	Human	PRP and autogenous bone graft	Dubitative
Giannobile and Somerman[35]	Periodontal tissue	Human	GFs	Insufficient
Rughetti et al.[25]	Bone (pseudoarthrosis)	Human	PLT gel	Significant
Castro[34]	Spinal bone	Human	PRP	Poor
Crovetti et al.[26]	Chronic ulcer	Human	PLT gel	Significant
Caloprisco and Borean[27]	Chronic ulcer	Human	PLT gel	Significant
Gehring et al.,[28] Wachtlin et al.,[29] Vote et al.[30]	Retina	Human	Hyperconcentrated PLTs	Significant
Hartwig et al.[31]	Cornea	Human	Hyperconcentrated PLTs	Helpful

much more complicated. The recovery and the release of PLT-derived GFs is barely predictable at the individual level. In spite of this variability and lack of predictability, lysed PLTs were demonstrated to release GFs at a larger extent than unlysed fixed PLTs.[9,10] Taken together, these data provide an adequate body of evidence supporting the idea that those methods that preserve GFs from being released from PLTs throughout the preparation, and those that make GFs to be releasable in loco from PLTs through PLT lysis or PLT activation, must be definitely preferable for clinical administration. The variability described above hinders a mechanistic transfer of the results obtained in vitro to the clinical perspective. Some more expectancy was placed in in vitro proliferation studies with PRP-derived GF-conditioned culture medium.

Many studies claimed that all kind of cells, except squamous epithelial cells,[12] were stimulated by PLT GFs both in vitro and in vivo.[1,8,9,11,12,14,20,33] Clear-cut dose dependency was shown in most articles. The proliferation rate of HUVECs was dependent on GF concentration, mostly VEGF.[8] Fibroblasts, chondrocytes, and stromal stem cells were sensitive to the dose of PLT-derived GFs.[13-15] In contrast, irradiated fibroblasts were not influenced by b-FGF, hence confirming that GF-mediated cell proliferation needs well-functioning machinery for chromosome replication. As shown in Table 2, GF-inducible proliferation of somatic cells was predictable as a general biologic phenomenon but the extent of such proliferation was variable and far from being predictable. In fact, the results summarized in Table 2 were barely comparable to one another because of the heterogeneity of conduct. FBS or fetal calf serum, which are known to contain GFs, were used in various amounts, from 0 to 20 percent final concentration. Other influential variables included the cell origin and their concentration, the GF origin and its concentration, the length of the culture, and the means to measure the cell proliferation. The only indisputable generalization emerging from these data was that GFs, whatever their source, enhanced the cell proliferation in vitro, whatever their kind. Again, this is not particularly new. It must be stressed that as yet there is no evidence that cell proliferation such as that induced under in vitro experimental conditions might be comparable with that occurring in vivo under GF stimulation. In addition, wound healing takes quite a long time resulting from the combination of chemotaxis, proliferation, and tissue remodeling. In vitro studies cannot cover these dynamics.

Some reports describing the clinical results obtained through the PLT derivatives were quite impressive.[1,3,18-31] Nevertheless, the actual role of PLT-derived GFs leading to such healing progression was just inductive. Others did not find clinical evidence supporting the topical use of PLT derivatives.[32-40] These conflicting results are summarized in Table 3. The technical variables described above might have accounted for the different outcome. Poor clinical outcome might have derived from a series of circumstances: insufficient concentration of the PLT products, loss of GFs across the preparation of the PLT concentrate, local conditions (e.g., infections, metabolic disturbance) that impaired or counteracted the GF action,

and poor GF-receptor availability in the target cells. Some of these circumstances may be kept under the investigator's control whereas others cannot.

Clinical data suggest that the topical administration of PLT derivatives induced a quite durable healing process, although alternate phases of proliferation and quiescence are observable in long-term treatments (personal observation). In short, as the treatment is started, the healing process—mainly granulation tissue formation—goes on vigorously for some weeks than decelerates nearly to stop its progression. More weeks, or a short course interruption of treatment, are necessary for the healing process to restart again vigorously. We have also observed that cells pulsed in vitro with PLT-derived GFs exhibited strong proliferation and nucleic acid synthesis for up 7 to 11 days, then returned to resting phase after this short period. This behavior is consistent with the hypothesis that self-limiting feedback pathways modulating cell proliferation intervene after the GF-induced proliferation phase.[41] This accumulating mass of evidence about cell response to PLT-derived GFs is not so far helping predictability of the clinical outcome. Such inability is made more evident considering that GFs are just some of the actors playing in the scene of wound healing. Next we will discuss PLT derivatives as a source of locally deliverable GFs and the role of GF-independent mechanisms implicated in tissue remodeling.

Most authors, including ourselves, focused on the PLT-derived GFs emphasizing their wound-healing effect hence underestimating the role of other molecules that do play a not secondary role in the wound-healing process. Among these molecules thrombin, fibrin, fibrinogen, thrombospondin, and zinc are known to have relevant functions.

Following thrombin-mediated activation, PLTs release lysophosphatidic acid, which is a potent chemotactic factor for keratinocytes, but it is also involved in the cell growth arrest of proliferating keratinocytes through activation of Smad3, an effector for transducing TGF-β signals from the cell membrane to the nucleus.[42] Similar modulation was previously described on proliferating epithelial cells.[43] Along its indirect modulating activity upon cell growth, thrombin enhances cell proliferation directly. We have experimentally found (data not shown) that thrombin is responsible for some 40 percent of fibroblast proliferation and that thrombin acts in synergy with PLT-derived GFs. Similar results were obtained also by other authors.[44] Thrombin and PLT-derived GFs exert their proliferation-inducing action by different metabolic pathways. Thrombin-activated cells have an increase in phosphorylated extracellular signal-regulated kinase (ERK) and enhanced synthesis of matrix metalloprotease-9. Cells activated by PLT-derived GFs have an increase in p38MAPK phosphorylation. Cells activated with either thrombin or PLT-derived GFs have an increase of both phosphorylated ERK and p38MAPK phosphorylation.[45-47] Finally, fibrin and fibrinogen enhance collagen synthesis in the extracellular matrix, PLT gel, and fibrin glue providing optimal scaffolds for cell spreading and proliferation.[48-52] We have demonstrated (data not shown) that fibroblasts seeded on the bottom of culture wells and brought at G_0 phase by serum-free culture conditioning, when covered by gel-forming PLT releasate and thrombin, began quick proliferation and migrated through the entire volume of the gel. This demonstrated that PLT lysate–containing fibrin gel combines proliferation, spreading, and chemotaxis messages for cultured fibroblasts. Similar results were obtained on marrow-derived mesenchymal progenitor cells.[53]

PLT-derived thrombospondin was shown to induce proliferation of human fibroblasts and bovine aortic smooth muscle cells and to depress the proliferation of bovine aortic endothelial cells.[54] This dated study also showed that zinc increased the stimulatory effect of thrombospondin and counteracted the inhibitory effect of thrombospondin on the endothelial cell growth. This sounds nowadays of great interest taking into account that zinc-dependent matrix metalloproteinases (MMPs), in particular MMP-2 and MMP-9, play a relevant plastic role modulating the wound repair and the angiogenesis processes. PLTs accumulate zinc in their cytosol and into the α-granules.[55] Hence, concentrated PLT derivatives act as a reservoir providing zinc, which in turn up regulates the synthesis and the availability of metalloproteinases.[56] Fibroblast migration, keratinocyte resurfacing, wound contraction, and granulation tissue organization are dependent on extracellular matrix remodeling exerted by MMP-2 and MMP-9.[57,58] In addition MMPs may also be induced by trace elements such as boron and manganese,[59] the latter being found in PLTs in substantial amounts.[60] Metalloproteinase also sustain a proliferation-inducing autocrine loop triggering the secretion of amphiregulin, which in turn activates ERK phosphorylation.[61,62] At last, cross-talking of different GFs acting on the same target cell through specific membrane receptors has been demonstrated to control the activation and the inhibition of common effector pathways hence modulating cell functions such as survival, migration, proliferation, and differentiation.[63]

To attribute to the sole PLT-derived GFs the healing properties of PLT-gel is reductive and simplistic. GFs are expected to exert a pivotal role in cell proliferation but the underlying mechanism of wound healing promoted by in loco administration of PLT derivatives is much more complex than that. Many molecules act in synergy through different mechanisms and metabolic pathways stimulating or modulating chemotaxis, cell proliferation, extracellular matrix deposition, tissue reorganization, and remodeling. It follows therefore that the pharmacokinetics of PLT derivatives is much too complex. Its clinical efficacy

can only be grossly predicted, provided that PLT derivatives from high-concentrated PLT products are used.

In some clinical settings, treatment consists of a single PLT gel administration such as those aiming to facilitate bone graft, bone regeneration, and osseointegration in maxillary sinus grafts. In these cases, the clinical endpoints are easily evaluated, the therapeutic efficacy of the treatment can be evaluated in large-scale statistical analysis, and clinical predictability may be assessed consequently. In some other clinical settings, treatment consists of repetitive treatment for weeks or months such as those for chronic ulcers. Also in these cases, if proper case-control studies are conducted, the efficacy of the treatment may be statistically evaluated. Nevertheless, no element is still available to predict the efficacy and the length of the treatment at the individual level. This is due to the extreme individual variability and to the complex set of variables described in this review. Much more biologic and clinical study is necessary to determine predictive elements of this relatively new therapeutic treatment.

To achieve more enlightening evidence it might be highly desirable to reduce the study-related variable as far as possible. We recommend three major points: 1) PLTs must be stressed as little as possible during preparation; a consensus about the preparation methods should be found among the hematologists and transfusion medicine specialists. 2) PLTs should be administrated with narrow variation of concentration (e.g., 1.5×10^9-2×10^9/mL); indications, lesion-related treatment schedules, and clinical endpoints should be provided by scientific societies through a wide specialists' consensus. 3) Three-dimensional in vitro studies will be preferable to two-dimensional proliferation assays, providing more "physiologic" conditions.

REFERENCES

1. Mazzucco L, Medici D, Serra M, et al. The use of autologous platelet gel to treat difficult-to-heal wounds: a pilot study. Transfusion 2004;44:1013-8.
2. Yamada Y, Ueda M, Naiki T, et al. Autogenous injectable bone for regeneration with mesenchymal stem cells and platelet-rich plasma: tissue-engineered bone regeneration. Tissue Eng 2004;10:955-64.
3. Mogan C, Larson DF. Rationale of platelet gel to augment adaptive remodeling of the injured heart. J Extra Corpor Technol 2004;36:191-6.
4. Borzini P, Nembri P, Biffoni F. Evolution of transfusion medicine as a stand-alone discipline. Transfus Med Rev 1997;11:200-8.
5. Hescheler J, Weltz A, Fleishman BK. Searching for reputability: first randomized study on bone-marrow transplantation in the heart. Lancet 2004;364:121-2.
6. Weibrich G, Kleis WK, Hafner G, Hitzler WE. Growth factor levels in platelet-rich plasma and correlations with donor age, sex, and platelet count. J Craniomaxillofac Surg 2002;30:97-102.
7. Zimmermann R, Jakubietz R, Jakubietz M, et al. Different preparation methods to obtain platelet components as a source of growth factors for local application. Transfusion 2001;41:1217-24.
8. Martineau I, Lacoste E, Gagnon G. Effects of calcium and thrombin on growth factor release from platelet concentrates: kinetics and regulation of endothelial cell proliferation. Biomaterials 2004;25:4489-502.
9. Zagai U, Fredriksson K, Rennard SI, et al. Platelets stimulate fibroblast-mediated contraction of collagen gels. Respir Res 2003;17:4-13.
10. Zimmermann R, Arnold D, Strasser E, et al. Sample preparation technique and white cell content influence the detectable levels of growth factors in platelet concentrates. Vox Sang 2003;85:283-9.
11. Carter CA, Jolly DG, Worden CESr., et al. Platelet-rich plasma gel promotes differentiation and regeneration during equine wound healing. Exp Mol Pathol 2003;74:244-55.
12. Okuda K, Kawase T, Momose M, et al. Platelet-rich plasma contains high levels of platelet-derived growth factor and transforming growth factor-beta and modulates the proliferation of periodontally related cells in vitro. J Periodontol 2003;74:849-57.
13. Borzini P, Mazzucco L, Panizza R, Rivara G. Regarding randomized trial and local biological effect of autologous platelets used as adjuvant therapy for chronic venous leg ulcers. J Vasc Surg 2004;39:1146-7.
14. Lucarelli E, Beccheroni A, Donati D, et al. Platelet-derived growth factors enhance proliferation of human stromal stem cells. Biomaterials 2003;24:3095-100.
15. Kaps C, Loch A, Haisch A, et al. Human platelet supernatant promotes proliferation but not differentiation of articular chondrocytes. Med Biol Eng Comput 2002;40:485-90.
16. Loots MA, Kenter SB, Au FL, et al. Fibroblasts derived from chronic diabetic ulcers differ in their response to stimulation with EGF, IGF-I, bFGF and PDGF-AB compared to controls. Eur J Cell Biol 2002;81:153-60.
17. Lonergan DM, Mikulec AM, Hanasono MM, et al. Growth factor profile of irradiated human dermal fibroblasts using serum-free method. Plast Reconstr Surg 2003;111:1960-8.
18. Schlegel KA, Donath K, Rupprecht S, et al. De novo bone formation using bovine collagen and platelet-rich plasma. Biomaterials 2004;25:5387-93.
19. Fontana S, Olmedo DG, Linares JA, et al. Effect of platelet-rich plasma on the peri-implant bone response: an experimental study. Implant Dent 2004;13:73-8.
20. Marx RE, Carlson ER, Eichstaedt RM, et al. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;85:638-46.
21. Yazawa M, Ogata H, Kimura A, et al. Basic studies on the bone formation ability by platelet rich plasma in rabbits. J Craniofacial Surg 2004;15:1-8.

22. Thorwarth M, Rupprecht S. Falk, et al. Expression of bone matrix proteins during de novo bone formation using bovine collagen and platelet-rich plasma (PRP)—an immunihistochemical analysis. Biomatherials 2005;26: 2575-84.
23. Fennis JP, Stoelinga PJ, Jansen JA. Reconstruction of the mandible with an autogenous irradiated cortical scaffold, autogenous corticocancellous bone-graft and autogenous platelet-rich-plasma: an animal experiment. Int J Oral Maxillofac Surg 2005;34:158-66.
24. Oyama T, Nishimoto S, Tsugawa T, Shimitsu F. Efficacy of platelet-rich plasma in alveolar bone grafting. J Oral Maxillofac Surg 2004;62:555-8.
25. Rughetti A, Flamini S, Colafarina O, et al. Closed surgery: autologous platelet gel for the treatment of pseudoarthrosis. Blood Transfus 2004;2:37-43.
26. Crovetti G, Martinelli G, Issi M, et al. Platelet gel for healing coutaneous chronic wounds. Transfus Apheresis Sci 2004; 30:145-51.
27. Caloprisco G, Borean A. Chronic skin ulcers: a regenerative stimulation by topical hemotherapy. Int J Artific Org 2004; 27:816-7.
28. Gehring S, Hoerauf H, Laqua H, et al. Preparation of autologous platelets for the ophthalmologic treatment of macular holes. Transfusion 1999;39:144-8.
29. Wachtlin J, Jandeck C, Potthofer S, et al. Long-term results following pars plana vitrectomy with platelet concentrate in pediatric patients with traumatic macular hole. Am J Ophthalmol 2003;136:197-9.
30. Vote BJ, Membrey WL, Casswell AG. Autologous platelets for macular hole surgery: the Sussex Eye Hospital experience. Clin Experiment Ophthalmol 2004;32:472-7.
31. Hartwig D, Harloff S, Liu L, et al. Epitheliotrophic capacity of growth factor preparation produced from platelet concentrates on corneal epithelial cells: a potential agent for the treatment of ocular surface defects? Transfusion 2004;44:1724-31.
32. Senet P, Bon FX, Benbunan M, Bussel A, et al. Randomized trial and local biological effect of autologous platelets used as adjuvant therapy for chronic venous leg ulcers. J Vasc Surg 2003;38:1342-8.
33. Froum SJ, Wallace SS, Tarnow DP, Cho SC. Effect of platelet rich plasma on bone growth and osseointegration in human maxillary sinus grafts: three bilateral case reports. Int J Periodont Restor Dent 2002;22:45-53.
34. Castro FP Jr. Role of activated growth factors in human spinal fusions. J Spinal Disord Tech 2004;7:380-4.
35. Giannobile WV, Somerman MJ. Growth and amelogenin-like factors in periodontal wound healing: a systematic review. Ann Periodontol 2003;8:193-204.
36. Grageda E, Lozada JL, Boyne PJ, et al. Bone formation in the maxillary sinus by using platelet-rich plasma: an experimental study in sheep. J Oral Implantol 2005;31: 2-17.
37. Weibrich G, Hansen T, Kleis W, et al. Effect of platelet concentration in platelet-rich plasma on peri-implant bone regeneration. Bone 2004;34:665-71.
38. Aghaloo TL, Moy PK, Freymiller EG. Investigation of platelet-rich plasma in rabbit cranial defect: a pilot study. J Oral Maxillofac Surg 2002;60:1176.
39. Freymiller EG, Aghaloo TL. Platelet-rich plasma: ready or not. J Oral Maxillofac Surg 2004;62:484-8.
40. Li H, Zou X, Qingyun X, et al. Anterior lumbar interbody fusion with carbon fiber cage loaded with bioceramics and platelet-rich plasma: an experimental study on pigs. Eur Spine J 2004;13:354-8.
41. Minami T, Horiuchi K, Miura M, et al. VEGF- and thrombin-induced termination factor, down syndrome critical region-1, attenuates endothelial cell proliferation, and angiogenesis. J Biol Chem 2004;279:50537-54.
42. Sauer B, Vogler R, Zimmermann K, et al. Lysophosphatidic acid interacts with transforming growth factor-beta signaling to mediate keratinocyte growth arrest and chemotaxis. J Invest Dermatol 2004;123:840-9.
43. Liu X, Sun Y, Costantinescu SN, et al. Transforming growth factor β-induced phosphorilation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. Proc Natl Acad Sci U S A 1997;94:10669-74.
44. Seher A, Axmann D, Geis-Gerstorfer J, et al. Stimulation of cell proliferation by platelet rich plasma (PRP). Biomaterialen 2003;4:149. Available from: http://plateletgel.net/Abstracts/PRP%20Cell%20Proliferation.pdf
45. Fernandes DJ, Ravenhall CE, Harris T, et al. Contribution of the p38MAPK signaling pathway to proliferation in human cultured airway smooth muscle cells is mitogen-specific. Br J Pharmacol 2004;142:1182-90.
46. Tsopanoglou NE, Maragoudakis ME. Role of thrombin in angiogenesis and tumor progression. Semin Thromb Hemost 2004;30:63-9.
47. Gruber R, Jindra C, Kandler B, et al. Proliferation of dental pulp fibroblasts in response to thrombin involves mitogen-activated protein kinase signaling. Int Endod J 2004;37:145-50.
48. Sahni A, Francis CW. Stimulation of endothelial cell proliferation by FGF-2 in the presence of fibrinogen requires $\alpha_v b_3$. Blood 2004;104:3635-41.
49. Leddy HA, Awad HA, Guilak F. Molecular diffusion in tissue-engineered cartilage constructs: effects of scaffold material, time, and culture conditions. J Biomed Mater Res 2004; 70B:397-406.
50. Rybarczyk BJ, Lawrence SO, Simpson-Haidaris PJ. Matrix-fibrinogen enhances wound closure by increasing both cell proliferation and migration. Blood 2003;102: 4035-43.
51. Kawase T, Okuda K, Wolff LF, Yoshie H. Platelet-rich plasma-derived fibrin clot formation stimulates collagen synthesis in periodontal ligament and osteoblastic cells in vitro. J Periodontol 2003;74:858-64.

52. Bensaid W, Triffitt JT, Blanchat C, et al. A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. Biomaterials 2003;24:2497-502.
53. Gruber R, Karreth F, Kandler B, et al. Platelet-released supernatants increase migration and proliferation, and decrease osteogenic differentiation of bone marrow-derived mesenchymal progenitor cells under in-vitro conditions. Platelets 2004;15:29-35.
54. Kaji T, Fujiwara Y, Yamamoto C, et al. Stimulation of cultured smooth muscle cell proliferation by thrombospondin is potentiated by zinc. Biol Pharm Bull 1995;18: 1264-6.
55. Marx G, Korner G, Mou X, Gorodetski R. Packaging zinc, fibrinogen, and factor XIII in platelet α-granules. J Cell Physiol 1993;156:437-42.
56. Agren MS. Matrix metalloproteinases (MMPs) are required for re-epithelialization of cutaneous wounds. Arch Dermatol Res 1999;291:583-90.
57. Mirastschijski U, Haaksma CJ, Tomasek JJ, Agren MS. Matrix metalloproteinase inhibitor GM 6001 attenuates keratinocyte migration, contraction and myofibroblast formation in skin wounds. Exp Cell Res 2004;299:465-75.
58. Gillard JA, Reed MW, Buttle D, et al. Matrix metalloproteinase activity and immunohistochemical profile of matrix metalloproteinase-2 and -9 and tissue inhibitor of metalloproteinase-1 during human dermal wound healing. Wound Repair Regen 2004;12:295-304.
59. Chebassier N, El Houssein O, Viegas I, Dreno B. In vitro induction of matrix metalloproteinase-2 and martix metalloproteinase-9 expression in keratinocytes by boron and manganese. Exp Dermatol 2004;13:484-90.
60. Milne DB, Sims RL, Ralston NV. Manganese content of the cellular components of blood. Clin Chem 1990;36:450-2.
61. Stoll SW, Kansra S, Elder JT. Metalloproteinases stimulate ErbB-dependent ERK signaling in human skin organ culture. J Biol Chem 2002;277:26839-45.
62. Kansra S, Stoll SW, Johnson JL, Elder JT. Autocrine extracellular signal-regulated kinase (ERK) activation in normal human keratinocytes: metalloproteinase-mediated release of amphiregulin triggers signaling from ErbB1 to ERK. Mol Cell Biol 2004;15:4299-309.
63. Schlessinger J. Common and distinct elements in cellular signaling via EGF and FGF receptors. Science 2004;306: 1506-7.