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Flow cytometric and morphological characterization of platelet-rich plasma gel

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Abstract

Background of problems: Platelet-rich plasma (PRP) gel is derived from an autogenous preparation of concentrated platelets and is widely used in implant dentistry as a vector for cell growth factors. However, limited data are available on its structure and composition. The present study was aimed at providing a flow cytometric and ultrastructural characterization of PRP gel.

Materials and methods: Twenty PRP gel samples were obtained from healthy volunteers. These PRP gel specimens were prepared for transmission (TEM) and scanning electron microscopy (SEM) examination of their morphological ultrastructure. Flow cytometry with CD41-PE monoclonal antibody was used to detect platelet cells, as this antibody recognizes human-platelet-specific antigen CD41.

Results: Both SEM and TEM showed that PRP gel contains two components: a fibrillar material with striated band similar to fibrin filaments, and a cellular component that contains human platelet cells. Both techniques indicated that no morphological elements were bound between the cellular component and the fibrillar material. The cells were confirmed as platelet cells by flow cytometric study after incubation with specific monoclonal antibody CD41-PE.

Conclusion: PRP gel contains a fibrillar and a cellular (largely human platelet cell) component. This unique structure may be capable of acting as a vehicle for carrying of cells that are essential for soft/hard tissue regeneration.

Platelet-rich plasma (PRP) is defined as a platelet pool found in non-coagulated and centrifuged blood (Marx et al. 1998). PRP is widely used to enhance tissue regeneration, especially after oral and maxillofacial surgery (Froum et al. 2002; Lekovic 2002, 2003; Della Valle 2003; Jakse et al. 2003; Maiorana et al. 2003; Sánchez et al. 2003; Wiltfang et al. 2003; Fennis et al. 2004; Thorn et al. 2004), based on the premise that autogenous PRP contributes large quantities of mitogenic polypeptides such as platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor and others, thereby enhancing osteogenesis (Marx et al. 1998; Anitua 1999; Schmitz & Hollinger 2001; Okuda et al. 2003). However, controversy remains over its possible oncogenic role (Schmitz & Hollinger 2001), and some authors have even questioned its activity in osseous-epithelial regeneration (Soffer et al. 2003; Weibrich et al. 2003a, 2003b; Arpornmaeklong 2004; Choi et al. 2004; Roldán et al. 2004).

Various protocols have been used to obtain PRP, but many of these techniques have been criticized for their inability to obtain an adequate concentration of platelets (Anitua 1999; Marx 2001; Gonshor

2002). Clinically, it is difficult to apply PRP as a mere pellet after the centrifugation of whole blood, because it lacks cohesion, hence it is difficult to maintain the platelet concentrate in the desired surgical site. As a result, many researchers have obtained a PRP gel by incubating the centrifuged material with calcium chloride, trapping these cell elements for the subsequent release of growth factors involved in osseous-epithelial regeneration (Anitua 2001). However, limited information is currently available on the elements that constitute PRP gel or their possible interactions. Therefore, the aim of the present study was to use electronic techniques to study the morphological structure of PRP gel and to analyze the interactions among the constitutive elements visualized.

Material and methods

Preparation of PRP

Venous blood was obtained from healthy volunteers who gave their informed consent and who had taken no medications for at least 2 weeks. To minimize platelet activation during blood collection, a 19-gauge butterfly needle with a light tourniquet was used and the first 2 ml of blood was discarded. The procedure proposed by Anitua (1999) was followed to produce the PRP gel. Briefly after drawing 20 cm^3 of blood, it was placed into four tubes, of 5 cm^3 each one, that contains 0.1 volume of 3.8% (w/v) sodium citrate. These tubes were then placed in a centrifuge machine to spin at 1500 r.p.m. for 6 min in order to separate the blood fractions. The top of the red fraction was collected and 0.05 cm^3 of calcium chloride was added for each 0.5 cm^3 of plasma. Finally, the tubes were placed in 37°C warm water for 20 min to produce PRP gel.

Monoclonal antibody

CD41-PE, purchased from Caltag Laboratories (Burlingame, CA, USA), a mouse monoclonal antibody to human-platelet-specific antigen CD41 [(GpIIa–IIIb ($a_{11b}B_3$)], was used to detect and confirm the presence of platelet cells in PRP.

Flow cytometric analysis (FACScan)

Briefly, 10^5 platelet cells were transferred to universal screw cap tubes containing sterile PBS, then washed and centrifuged at 225 *g* for 5 min, discarding the supernatant. The washing and centrifugation steps were then repeated. Cells were permeabilized with methanol for 10 min, washed three times in PBS, and once in distilled water. These cells were incubated for 30 min at 4°C with the monoclonal antibodies. They were then washed twice in cold PBS and re-incubated with FITC-conjugated anti-mouse IgG (1 : 50) for FACScan analysis (Becton Dickinson, Mountain View, CA, USA).

Transmission electron microscope (TEM) analysis

Twenty PRP concentrate gels obtained were processed for electron microscopic study as described by Fernández et al. (1998). Briefly, they were fixed *in situ* with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. The gel was post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature, dehydrated in ethanol, then detached from the culture vessel by rapid treatment in propylene oxide and embedded in Epon 812. After polymerization, the plastic was removed and ultrathin sections were cut parallel and perpendicular to the surface of the flask. The ultrathin sections were stained with uranyl acetate–lead citrate and examined by a Hitachi H 7000 TEM (Hitachi Ltd, Tokyo, Japan).

Scanning electron microscopy (SEM) analysis

Twenty gel samples were immediately immersed in a sodium cacodylate-buffered formaldehyde–glutaraldehyde fixative for 24 h at room temperature and post-fixed in 20% osmium tetroxide for 2 h. Subsequently, samples were dehydrated by serial transfers in ascending concentrations of acetone (50–100%) and infiltrated with liquid carbon dioxide before the critical drying point. Finally, the samples were made electrically conductive by mounting on aluminum slabs with a silver point, followed by sputter coating with gold/palladium to a thickness of approximately 250 Å. The above specimens were attached to an acrylic plate with glue tape. Subsequently, half of the plate bottom was cut with a diamond disc and vertically divided into two pieces with a chisel and hammer. Finally, the cut face of each specimen was examined with a scanning electron microscope (JXA-840; JEOL, Tokyo, Japan) at 5–10 kV.

Results

Flow cytometry

The flow cytometric study showed that the platelet cells in the PRP gel were strongly positive for CD41 when incubated with CD41-PE (Figs 1–3).

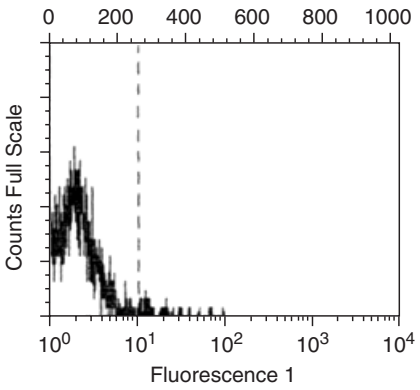

Fig. 1. Negative control.

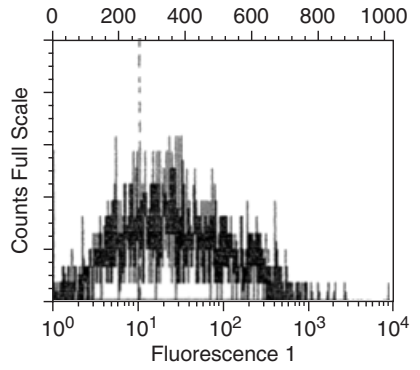

Fig. 2. HLA-II positive.

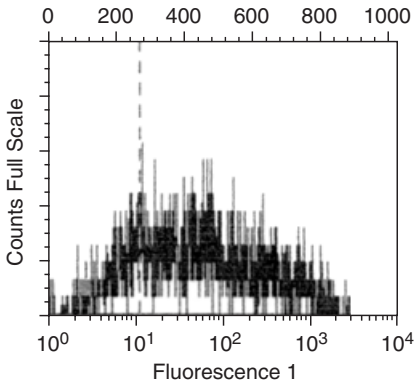

Fig. 3. CD-41 positive.

TEM

Ultrastructural analysis by TEM confirmed that the cell component of the PRP gel was constituted by platelet cells, visualizing the following: dense, rounded and elongated granules in the cytoplasm, surrounded by membrane; small, ovoid mitochondria; peripheral microtubule and microfibril bundles; rough endoplasmic reticulum of variable morphology and a small proportion of free ribosomes; and some siderosomes (Figs 4 and 5).

SEM

The SEM study yielded clear images of the elements that constituted the PRP gel, which had a breadcrumb-like appearance (Fig. 6). It contained randomly arranged fibrillar elements, of homogeneous thickness throughout their length, with platelet cell elements arranged among them (Fig. 7). The platelets did not show the typical morphology of their resting state but rather morphologic signs of their activation: ovoid platelet cell elements with exocytosis phenomena, evidenced by the presence of vesicular formations on the cell surface (Fig. 8). No binding elements were observed between the cells and filaments in the matrix.

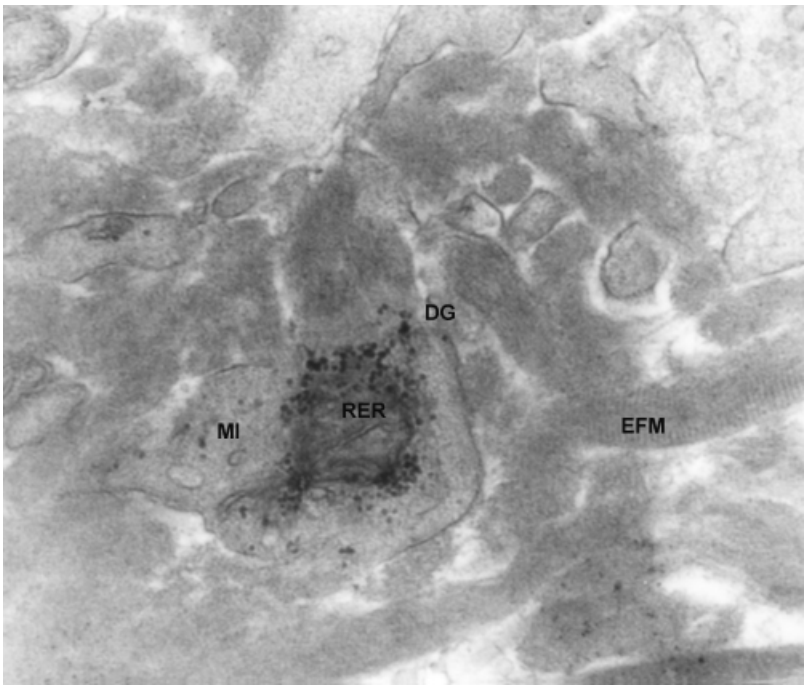

Fig. 4. Transmission electron microscopic image of human platelet-rich plasma. Platelet cells can be observed that contain dense granules (DG), mitochondria (MI) and microtubules, rough endoplasmic reticulum (RER) with ribosomes, siderosomes and section of the extracellular fibrin matrix (EFM).

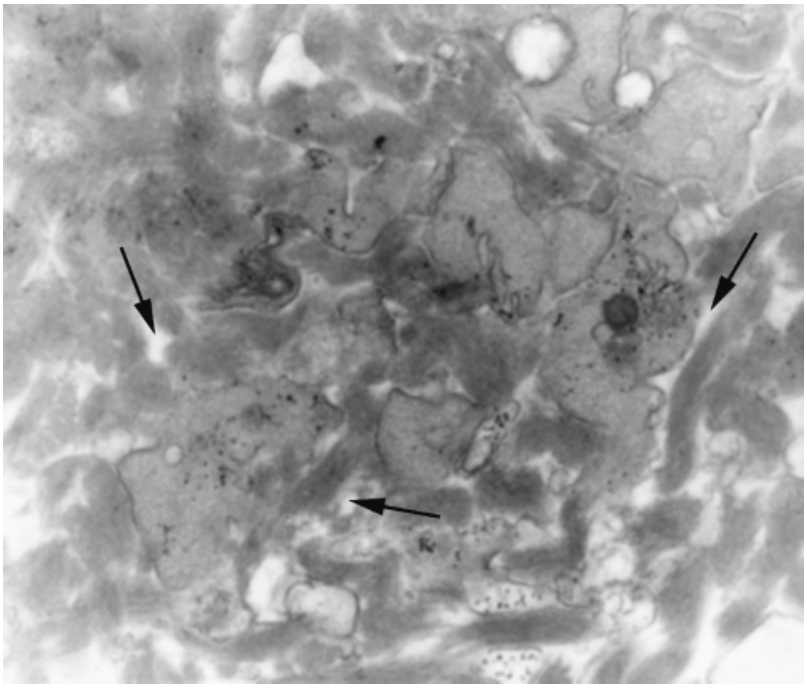

Fig. 5. Transmission electron microscopic image of human platelet-rich plasma. No physical relationship could be observed between the platelet cells and extracellular fibers (arrows showing spaces). The cells appear trapped in the fibrin matrix as if by a filter.

Discussion

This study used SEM and TEM techniques to characterize a PRP gel produced *in situ* during the therapeutic procedure. Many different techniques have been proposed to obtain a PRP concentrate (Marx et al. 1998; Anitua 1999; De Obarrio et al. 2000; Landesberg et al. 2000; Rosenberg & Torosian 2000; Lozada et al. 2001; Shanaman et al. 2001; Sonnleitner et al. 2000; Vanassche & Defranq 2001; Gonshor 2002; Tischler 2002). The method of Anitua (1999), one of the most frequently adopted protocols, was employed in the present study.

Although the techniques to obtain PRP have been widely described and debated (Marx et al. 1998, 2001; Landesberg et al. 2000; Appel et al. 2002; Tozum & Demiralp 2003; Weibrich et al. 2003a, 2003b), limited information is available on the morphological ultrastructure of PRP gels. The literature has described how molecular factors derived from the gel may influence bone remodeling and regeneration of oral structures (Aghaloo et al. 2002; Carlson & Roach 2002; Fürst 2003a, 2003b; Kawase 2003; Zechner et al. 2003; Wiltfang 2004).

Our flow cytometric study showed that the most of cells in the PRP gel were platelet cells. Flow cytometry is a simple laboratory technique with multiple uses, including the identification and quantifica-

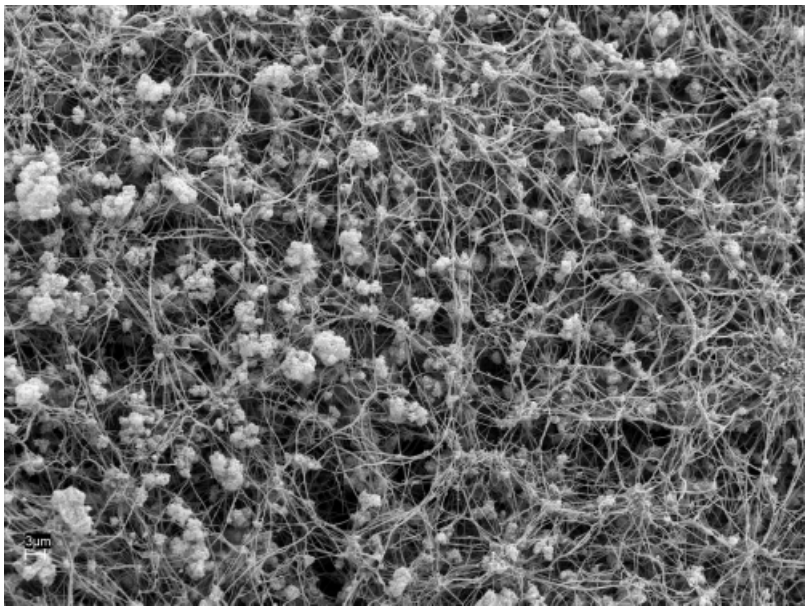

Fig. 6. Scanning electron microscopy of human platelet-rich plasma. A matrix can be observed of bread crumb-like appearance, comprising randomly arranged fibrillar elements and platelet cells.

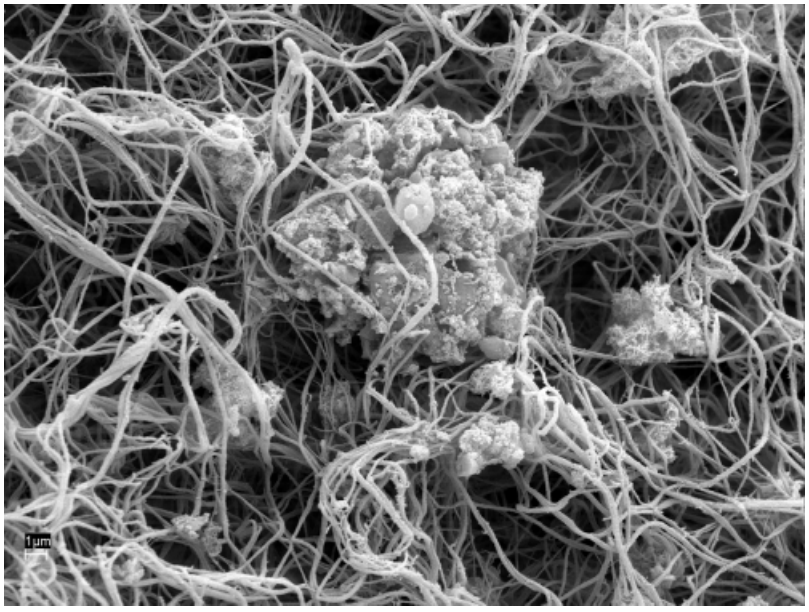

Fig. 7. Scanning electron microscopy of human platelet-rich plasma. Multiple platelet cell elements can be observed, forming a cell conglomerate trapped among fibrillar elements (fibrin). The platelet activation was illustrated by these cells, via their spherical-ovoid morphology and presence of exocytic vesicles.

tion of different cell populations within the same sample by the detection of specific monoclonal antibodies. This technique has been widely used by different authors in research on cell-based diseases (Morita et al. 1998; Thomas et al. 2002) and, more specifically, in human and animal studies of platelets and other blood cells (Leytin et al. 1996; Michelson 2000; Matyus et al. 2001; Pichler et al. 2002; Chapman et al. 2003; Moritz et al. 2003).

The microscopic images obtained clearly demonstrate that the PRP gel is composed of fibrillar and cellular (essentially platelet cells) elements that are essential for the soft/hard tissue regeneration. Nonetheless, it has been shown the biologic effect of PRP is largely, contributed by the variety of growth factors that it possesses (e.g. PDGF, TGF-β, IGF, etc.) (Bennett & Schultz 1993a, 1993b; Okuda et al. 2003). The TEM study revealed a wide filamentous network with striations, reminiscent of descriptions of fibrin in blood clots (Diaz-Flores et al. 1974). It is possible that these filaments are involved in directing osteogenic cell movements during the bone generation process. Moreover, the fibers that make up this biological mesh are randomly arranged with no given orientation, indicating that a pre-determined arrangement does not appear to be essential for the migration and subsequent bone growth.

Therefore, the particular structure of PRP gel described here may enhance tissue regeneration, via its ability of carrying undifferentiated mesenchymal cells. Kawase et al. (2003) studied the action of PRP in different cell cultures and suggested that a fibrin clot in combination with growth factors can promote tissue regeneration in situations of periodontal damage. Furthermore, Anitua (2004) described platelets as cell elements capable of participating in tissue regeneration because of their high content of proteins that can enhance this process, especially growth factors.

On the other hand, no physical binding was found between the fibers and the trapped platelet elements, suggesting that the extensive network of filaments of the gel may act as a filter for the cell elements within it. This appears inconsistent with the known presence in these small non-erythrocyte cells of membrane glycoproteins responsible for establishing molecular bonds between the platelets and fibrillar elements. Nonetheless, it is agreed that one end of glycoproteín Gp1b-IX is bound to the cytoskeleton with filamin molecules and the other acts as a receptor for two blood-clotting proteins (Coller 1990; Kieffer & Phillips 1990; Godet et al. 1995). In addition, the platelet complex GpIIb–IIIa is able to bind with fibrinogen, producing the cross-linking of platelet into an aggregate (Hawiger et al. 1978; Leytin et al. 1996), and can then act as main receptor for platelet adhesion to inert surfaces of glass, titanium, and different biomaterials (Broberg et al. 2002).

With this background, it would be reasonable to assume molecular interactions occurred between fibrillar and cellular elements of the gel. Although they do not produce complex binding structures such as those described for cell–cell interactions, which are visible with SEM and TEM (Bloom et al. 1994).

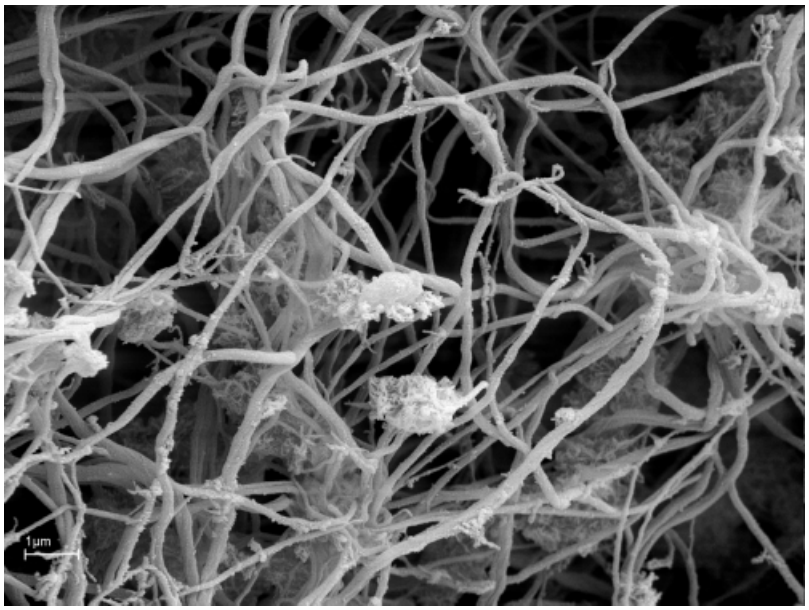

Fig. 8. Scanning electron microscopy of human platelet-rich plasma. Platelet cell elements can be observed trapped among fibrillar elements (filter-type trapping).

Taken together, the above results indicate the formation *in vitro* of a biological matrix that allows not only osteogenic cell migration but also the trapping of platelet elements that may induce proliferation and cell growth.

The SEM images showed a lumpy, breadcrumb-like surface of the gel at low magnification. At higher magnification, these lumps were seen to be composed of clumps of platelet cells, with exocytic vesicles on their surface that have been related to platelet activation mechanisms (Rotllan et al. 1993). We believe this observation to be of great interest, because it demonstrates that platelet activation is possible not only during the formation of a blood clot *in vivo* but also in a blood clot formed *in vitro*.

Further studies to characterize the PRP gels used in patients are warranted to establish the morphological and molecular mechanisms of the induced oral osteogenesis, the end-point of numerous procedures in oral and maxillofacial surgery. The ability to develop artificial clots and vary their fibrillar and cellular composition by means of molecular biology and tissue engineering would allow their use as a carrier in novel genetic therapies for the oral cavity.

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要旨

問題の背景：インプラント治療において自己血の濃縮血小板から得られる多血小板血漿（PRP）ゲルは、細胞増殖因子のベクトルとして広く用いられているが、その構造と組成に関するデータは限られている。本研究は、流動細胞測光法と超微細構造の評価によって PRP ゲルの特性指摘を行った。

材料と方法：健常ボランティアから２０の PRP ゲル検体を得た。これらの PRP ゲル検体は、透過電子顕微鏡（TEM）と走査電子顕微鏡（SEM）によって形態学的な超微細構造を調べた。CD41-PE モノクローナル抗体はヒト血小板に特異的な抗原 CD41 を認識するので、これを用いて流動細胞測光法によって血小板細胞を検出した。

結果：SEM でも TEM でも、PRP ゲルはフィブリン・フィラメントに似た横紋帯を伴う原繊維性成分とヒト血小板細胞を含む細胞成分という２つの構成要素を含んでいることが示された。また両方のテクニックは、細胞成分と原繊維成分の間に形態学的な要素が結合していないことを示した。細胞を特異的モノクローナル抗体 CD41-PE で培養した後に流動細胞測光法の試験を行ったところ、血小板細胞であることが確認された。

結論：PRP ゲルは、原繊維性成分と細胞成分（主としてヒト血小板細胞）を含んでいる。このユニークな構造は、硬・軟組織の再生に必須の細胞を運ぶ媒体として作用する可能性がある。

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