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Platelet Rich Fibrin Matrix Improves Wound Angiogenesis via Inducing Endothelial Cell Proliferation

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Abstract

The economic, social and public health burden of chronic ulcers and other compromised wounds are enormous and rapidly increasing with the aging population. The growth factors derived from platelets play an important role in tissue remodeling including neovascularization. Platelet-rich plasma (PRP) has been utilized and studied for the last four decades. Platelet gel and fibrin sealant, derived from PRP mixed with thrombin and calcium chloride, have been exogenously applied to tissues to promote wound healing, bone growth, hemostasis and tissue sealing. In this study we first characterized recovery and viability of as well as growth factor release from platelets in a novel preparation of platelet gel and fibrin matrix, namely, platelet rich fibrin matrix (PRFM). Next, the effect of PRFM application in a delayed model of ischemic wound angiogenesis was investigated. The study for the first-time shows the kinetics of the viability of platelet embedded fibrin matrix. A slow and steady release of growth factors from PRFM was observed. The VEGF released from PRFM was primarily responsible for endothelial mitogenic response via ERK activation pathway. Finally, this preparation of PRFM effectively induced endothelial cell proliferation and improved wound angiogenesis in chronic wounds, providing evidence of probable mechanisms of action of PRFM in healing of chronic ulcers.

Keywords

platelet rich plasma (PRP); wound healing; angiogenesis; autologous platelet gels; ischemic wounds

INTRODUCTION

The economic, social and public health burden of chronic ulcers and other compromised wounds are enormous and rapidly increasing with the aging population(1). Of the over 20 million individuals with diabetes in the U.S., 15% or more can be expected to develop diabetic ulcers, while over 2 million individuals currently suffer from venous leg ulcers. The numbers of patients with arterial ulcers and pressure ulcers are equally staggering. Managing these wounds represents \$5–10 billion each year(1). Consequently, there has been heightened interest in developing new advanced therapies to address the compromised wound.

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Healing in acute wounds follows a progression through a series of complex but orderly physiological and molecular processes. These processes include coagulation, inflammation, cell recruitment, migration, proliferation, and connective-tissue production followed by matrix remodeling and maturation(2). Chronic, compromised wounds are characterized as having stalled somewhere in this progression to healing due to a variety of systemic and local factors including poor perfusion and low oxygen tension, excess microbial burden, necrotic tissue, chronic venous insufficiency, wound bed cells that are unresponsive to normal cell signaling, (3) and decreased growth-factor production and response. Even with optimal wound-bed preparation including adequate debridement, infection control, restoration of blood supply and establishment of a moist healing environment, significant progress toward healing is still not achieved in 30% or more of ulcers seen(4). In these cases, advanced therapies must be considered(4). Current advanced therapies available include therapy to replace deficient components (autologous epidermis, allografts, and living skin equivalents), complimentary therapies(hyperbaric oxygen, negative pressure, ultrasound, and electrical stimulation), dermal matrix equivalents, and exogenous growth factors (purified single growth factors, autologous growth factor, and growth factor/fibrin preparations) (3). Much renewed interest has recently been seen in this last category of autologous platelet/fibrin biologics (5). However, there still remain questions concerning their effectiveness compared to other advanced therapies, the extent of variability inherent in autologous biologics and, ultimately, questions concerning their mode of action in compromised, non-healing wounds.

The role of platelets in wound healing is well described. However, there are few studies on platelet-induced changes in wounded skin at the molecular level. There is a need to characterize key platelet-derived factors in terms of levels of expression, viability and functionality in order to better understand their role in the healing process. There are several animal wound models reported in the literature (6, 7). Since compromised blood supply and local tissue ischemia is often of paramount importance in chronic ulcers, the comparison of the wound healing process in an ischemic and non-ischemic wound models is of particular relevance(8). Growth factors derived from platelets play an important role in tissue remodeling including neovascularization(9). Two complimentary studies are presented in this article. First, we identified and characterized representative factors present in an autologous human platelet-rich fibrin matrix that impact endothelial cell function in an endothelial cell culture system. Secondly, we examined the action of platelet-derived factors on wound healing coupled with an approach to a potential mechanism of action at the tissue molecular levels in an ischemic porcine wound model.

MATERIALS AND METHODS

Human subjects and sample collection

Blood samples were collected from healthy adult male and female donors aged 20–50 years. The subjects were not pregnant and/or on immunosuppressants. Protocols were approved by the Ohio State University's Institutional Review Board. Declaration of Helsinki protocols was followed and patients gave their written, informed consent.

Platelet-Rich Fibrin Matrix (PRFM) Preparation

PRFM was prepared using the FIBRINET® Platelet Rich Fibrin Matrix(PRFM) system (Cascade Medical, New Jersey, USA) as per protocol as shown in Fig 1A. In brief, a small amount of blood (18 ml) was drawn into two collection tubes (yellow top tube), containing tri-sodium citrate as an anticoagulant and a proprietary separator gel. The tubes were centrifuged at 1100 x g for 6 minutes at room temperature. The platelet rich plasma layer (PRP) was transferred to a 30 ml vial containing CaCl₂. The clotting process was triggered

by the presence of CaCl_2 and the vial was immediately centrifuged at $4500 \times g$ for 25 minutes at room temperature. The PRFM membranes were recovered and were used for either in vitro culture or porcine wound studies. For porcine wound studies, PRFM were prepared from autologous blood obtained from pigs used for the study.

Percent platelet recovery

Percent recovery of platelets in PRP was determined by counting platelets in whole blood and in PRP using Beckman Coulter LH 755. The Beckman Coulter LH 755 Workstation is an automated quantitative hematology analyzer that utilizes a refined electronic particle counting principle to quantitate white cells, red cells, and platelets. Percent platelet recovery was calculated using the following formula:

$$= \frac{(\text{Whole blood platelet count} \times \text{Whole blood collected volume})}{(\text{Platelet count in PRP} \times \text{Total PRP volume})} \times 100$$

Cell viability assay for platelets in PRFM

PRFM were placed in 6-well plates with 2 ml of RPMI 1640 media supplemented with antibiotics solution (100 IU/mL penicillin and 0.1 mg/mL streptomycin) and were maintained in a standard cell culture incubator at 37°C and 5% CO_2 . The media was collected from the wells at designated times. Lactate dehydrogenase (LDH) leakage from PRFM platelets to the media was assessed using CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI). We have observed that presence of even very minute amounts of hemoglobin interferes with most colorimetric assays available to assay LDH based cell viability. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay(a) is a fluorometric method where hemoglobin does not interfere. This assay is routinely used for estimating the number of nonviable cells present in culture plates. The CytoTox-ONE™ Assay measures the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. LDH released into the culture medium is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into a fluorescent resorufin product. The amount of fluorescence produced is proportional to the number of lysed cells. The assay was performed as per manufacturer's instructions.

Enzyme-Linked Immuno-Sorbant Assay (ELISA)

Human PDGF-BB, TGF- β and VEGF-A contents from PRFM conditioned media were measured at specified times using commercially available assay kits as per manufacturer's recommendation (R&D Systems Inc., MN, USA).

Human endothelial cell proliferation assay

Human microvascular endothelial cells (HMEC-1) cells were cultured under standard culture conditions (5% CO_2 at 37°C) in MCDB-131 growth medium (GIBCO-BRL, Invitrogen) supplemented with 10% FBS, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 2 mol/L L-glutamine (10). Cells were counted using a Coulter Z1 particle counter (Beckman Coulter Inc., Miami, FL). For proliferation assay, cells were seeded (5,000 cells per well) in 96-well plates. After 24 hours of seeding, the media was changed to serum free media. Cells in serum free media were treated with conditioned media from PRFM as indicated in figure legends. The proliferation of cells following 48h of PRFM conditioned media treatment was determined using CyQUANT™ cell proliferation assay kit (Molecular Probes, Invitrogen).

SiRNA transfection

HMEC cells were seeded in antibiotic-free medium 24h prior to transfection. Dharma FECT™ 1 transfection reagent (Dharmacon RNA technologies, Lafayette, CO) was used to transfect cells with 100nM of siRNA pool for ERK1/2 (Dharmacon RNA technologies, Lafayette, CO) for 72h as described previously (11). For controls, si Control non-targeting siRNA pool (mixture of 4 siRNA, designed to have 4 mismatches with the corresponding gene) was used. After transfection with SiRNA, HMEC cells were seeded in 96-well plates and followed by treatment with conditioned media from PRFM as described for the cell proliferation assay. For quantification of protein using Western blot, samples were collected after 72h of siRNA transfection. Protein extraction was done and immunoblot analysis for ERK1/2 was performed.

Immunoblot analyses

For ERK, phospho-ERK immunoblots, HMEC cellular protein extracts were separated on a 10% SDS-polyacrylamide gel under reducing conditions, transferred to nitrocellulose and probed with anti-ERK (1: 1000, Biosource Invitrogen) and phospho-ERK (1:1000, Biosource, Invitrogen) antibody. Immunoblotting was performed using standard procedures described previously (12).

Porcine ischemic excisional wound model and PRFM treatment

All experiments were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee (ILACUC). Pigs (70–80lb) were anesthetized using Telazol® followed by isoflurane. The dorsal region was shaved. The skin was surgically prepared with alternating Betadine™ and alcohol scrubs. Under such aseptic conditions, four full-thickness bipedicle skin flaps measuring 15 x 5 cm were developed on each animal by means of parallel incisions as described previously (8). Ischemia of the flap tissue was verified by laser Doppler imaging of blood flow (8). Full thickness excisional wounds were developed in the middle of each flap with an 8mm disposable biopsy punch. Six more wounds were developed similarly on non-ischemic skin to serve as controls. Blood from the wounds was blotted and the PRFM were placed in the treatment wounds such that the bulk of it was within the wound with a small margin around the wound edge. One thoracic and one lumbar wound were treated for both the ischemic and control wound groups (Fig 6A). The incisions were dressed with VAC Drape (KCI Inc, TX). The dressing was changed every three days and any accumulating wound fluid was drained. All wounds were digitally imaged. On day 14 post wounding, the entire wound tissue (1.5 x 1.5 cm with the 8mm wound at the center) was harvested for tissue analyses (RNA, protein and histological analyses). The pigs were euthanized after the completion of experiments.

Histology

Formalin-fixed paraffin-embedded or OCT-embedded frozen wound-edge specimens were sectioned. The paraffin sections were deparaffinized and stained with hematoxylin& eosin (H&E), Masson's Trichrome and picro-sirius red staining using standard procedures. Immunohistochemical staining of paraffin- or frozen- sections was performed as described earlier(8) using the following primary antibodies: anti-vWF (1:400; BD Pharmingen, San Diego, CA); anti-Ki67 (1:200, Neomarkers, Fremont, CA) after heat induced epitope retrieval when necessary. Secondary antibody detection and counterstaining were performed as described previously (13).

Image quantification

The mosaic images of whole wounds were collected under 20X magnification guided by MosaiX software (Zeiss) and a motorized stage. Each mosaic image was generated by

combining minimum of ~100 images. Between 7–10 high-powered representative areas from mosaic image were quantified for each data point from each animal. Quantification was performed employing Automeasure software (Zeiss).

Laser Doppler blood flow imaging

The Moor LDITM-Mark 2 laser Doppler blood perfusion imager (resolution: 256 x 256 pixels in the region of interest; each pixel being an actual measurement) employs a visible red laser beam (633 nm) to map tissue blood flow and enable quantification (Fig. 12)(8).

Laser microdissection pressure catapulting (LMPC)

Tissue sectioning & fixation—Frozen tissue blocks were cut into 8 μ m sections. The sections (2–3) were mounted on each RNAP-treated thermoplastic (polyethylene naphthalate)-covered glass slide (PALM Technologies, Bernreid, Germany) and kept at -80°C until use. Sections were fixed and treated as described (14).

Granulation tissue staining—After rinsing slides in diethyl pyrocarbonate (DEPC)-treated water for 2 min, the sections were stained using a quick hematoxylin QS for granulation tissue identification(15). The sections were rinsed in DEPC phosphate buffer (PBS) for 2 min, and sequentially dehydrated as described (14). Sections were stained using a quick hematoxylin QS protocol (15). The granulation tissue was identified as hypercellular region (Figure 10A–C). 1×10^6 sq micron granulation tissue was captured in Trizol for mRNA extractions.

LMPC—LMPC was performed using the laser microdissection system from PALM Technologies (Bernreid, Germany) containing a PALM MicroBeam and RoboStage for high-throughput sample collection and a PALM RoboMover (PALM RoboSoftware version 4.0) (14, 15). Typical settings used for laser cutting were UV-Energy of 75–85 and UV-Focus of 52. The granulation tissue was cut and captured under a 10x ocular lens.

mRNA extraction and CD31 quantification was performed as described before (14).

Statistics

Data are reported as mean $\pm$ SD of at least three experiments. Difference between means was tested using Students *t* test or ANOVA as appropriate. A value of $p < 0.05$ was considered statistically significant.

RESULTS

In this study PRFM were prepared as described (5), from 20 human donors. The PRFM consists of a cross-linked fibrin matrix containing platelets. The system of preparation employed here separates a platelet rich plasma (PRP) fraction from whole blood by centrifugation (1100xg) for six minutes in a Vacutainer[®] tube (Figure 1A). The heavier red blood and white blood cells reside in the lower layer of the tube and a thixotropic separator gel separates this layer from the upper fraction containing the platelets. The transfer of the PRP fraction to a second ‘membrane vial’, containing calcium chloride, is facilitated by a transfer device. The presence of calcium ions initiates the thrombin-catalyzed cleavage of fibrinogen to fibrin the process is further accelerated by a further centrifugation of the second tube at a higher g force (4500xg) for 25 minutes. This results in a PRFM disc or membrane, which adopts the contour of the base of the vial. The conditions of preparation are such that the membrane contains a concentrated platelet population, which are intact. In the wound care application described above, this membrane, which can be readily removed from the Vacutainer[®] tube, is applied directly to the wound (Figure 1B).

The recovery of platelets in PRP using the FIBRINET[®] system varied in subjects between 35–95% with mean recovery was 63% (Figure 1C). The freshly prepared PRFM were incubated in cell culture media under standard culture conditions and the viability of platelets in PRFM was determined using lactate dehydrogenase (LDH) system. Under these conditions, a gradual loss of viability of platelets in PRFM was observed (Figure 1D). The growth factor levels in media overlying PRFM (conditioned media) were determined using ELISA (Figure 2). An increase in the levels of PDGF-BB, TGF β 1 and VEGF-A was noted in culture media indicating a gradual release of growth factors by platelets embedded in PRFM (Figure 2).

To determine whether the growth factors released by PRFM are biologically active, the PRFM conditioned media was tested in a human micro-vascular endothelial cells (HMEC) proliferation assay. The HMEC were cultured in 96-well plates followed by serum starvation to restrict the proliferating HMEC in G1 phase. PRFM conditioned media was collected day 7 after culture. The HMECs were either treated with varying percentage (v/v) of day 7 PRFM conditioned media and serum free HMEC culture media or 10% (v/v) fetal bovine serum (FBS) with HMEC culture media. As anticipated, serum starvation resulted in growth suppression compared to cells cultured using standard 10% FBS (Figure 3A). Increasing the concentration of day 7 PRFM conditioned media in serum free HMEC culture media resulted in progressive increase in HMEC proliferation (Figure 3A). Treatment of serum starved HMEC with days 1, 3 or 7 PRFM conditioned media (25% v/v) resulted in significant but similar increase in cell proliferation (Figure 3B). Treatment of serum starved HMEC with conditioned media from platelet poor plasma (PPP) gel did not change the rate of in cell proliferation suggesting that the factors released by platelets is required for endothelial cell proliferation (Figure 3C). The growth factor levels (especially PDGF and TGF β) in conditioned media increased gradually (Figure 2), whereas, the extent of cell proliferation induced by the media collected on these days was comparable. This data shows that the cell proliferation is not correlated to PDGF or TGF β levels in PRFM conditioned media (Figure 3B). Treating PRFM conditioned medium with neutralizing antibody against PDGF and VEGF resulted in depletion (>90%) of the levels of these growth factors from the media (data not shown). Depletion of PDGF from conditioned media did not affect HMEC proliferation. A small but significant decrease in HMEC proliferation was noted in cells treated with VEGF depleted PRFM conditioned media (Figure 4A) suggesting VEGF released by PRFM is at least in part involved in HMEC proliferation.

VEGF is a strong activator of ERKs (extracellular-signal-regulated protein kinases) 1 and 2 via its major receptor, kinase-insert-domain-containing receptor (KDR) resulting in endothelial cell mitogenesis. The treatment of HMEC with day 7 PRFM conditioned media resulted in an increase in ERK 1/2 phosphorylation while the total levels of ERK in the cells remained unchanged (Figure 4B–C). A pharmacological inhibitor of ERK UO126 significantly inhibited day 7 PRFM conditioned media-induced endothelial cell proliferation (Fig 4D). To determine a specific role of ERK 1/2 in PRFM conditioned media induced endothelial cell proliferation, we used the siRNA approach. Transfection of HMEC with ERK1 or 2 siRNA either alone or in combination resulted in knockdown of the respective proteins (Figure 5A–C). ERK 1/2 knockdown using siRNA significantly inhibited day 7 PRFM conditioned media-induced endothelial cell proliferation (Fig 5D).

To determine effects of PRFM on wound healing we used a porcine ischemic wound model. These ischemic wounds show impairment in angiogenesis and subsequent closure (16). PRFM was applied to ischemic excisional wounds and the quality of the regenerated tissue was determined using the Masson-Trichrome and Hematoxylin-Eosin (H&E) staining of the wound tissue on day 14 post-wounding. Histological evaluation of the wounds sections shows that PRFM-treated ischemic wounds at day 14 show presence of mature collagen

fibers in the treated wounds compared to untreated wounds (Figure 7). Von Willebrand factor (vWF) is a large multimeric glycoprotein produced constitutively in the endothelium. On day 14 post-wounding, PRFM treated ischemic wounds displayed increased number of vWF+ cellular structures indicative of vascular formation (Figure 8).

Based on the data derived from effect of PRFM on endothelial cell proliferation under cell culture conditions, we asked whether the increased number of blood vessels in wound treated with PRFM is attributable to increased proliferation of the cells. Cell proliferation in wounds was detected using antibody against Ki67 antigen. Ki-67 is found in growing, dividing cells but is absent in the resting phase of cell growth. PRFM treated ischemic wounds displayed greater numbers of Ki67+ compared to non- treated control ischemic wounds (Figure 9). Colocalization studies show that many of these proliferating Ki67 + cells were also positive for vWF(Figure 10). To quantitatively assess that PRFM treatment to ischemic wounds increases endothelial cells numbers in the granulation of the healing wounds we utilized the laser capture micro dissection (LCM) approach. Granulation tissues were captured using LCM near (within 500 μ m) the epithelial margins, CD31 gene expression was measured in the granulation tissue as a marker for endothelial cells (Figure 11). Laser Doppler imaging is a useful technique for measuring microvascular perfusion in wounds because it involves no contact and produces a color image representing flow distribution over an area of tissue (17). Finally, to show that the more CD31 and vWF positive endothelial cells were actually functional vessels, we performed laser Doppler blood flow imaging (Figure 12). Taken together, the LCM, laser Doppler data and immunohistochemical data indicate presence of functional vessels and angiogenesis in PRFM treated wounds.

DISCUSSION

The study characterized recovery and viability of as well as growth factor release from platelets in a novel preparation of platelet gel and fibrin matrix, namely, platelet rich fibrin matrix (PRFM). The growth factors released by platelets in PRFM induced endothelial cell proliferation and promoted wound angiogenesis in porcine ischemic wounds. Platelet-rich plasma (PRP) has been utilized and studied since the 1970s (18). PRP is used clinically in humans for its healing properties attributed to the increased concentrations of autologous growth factors and secretory proteins that may enhance the healing process on a cellular level (18). Conventional PRP has been defined as plasma with a platelet concentration above the “normal” physiologic levels found in whole blood (19). Currently, several methods and systems are available for the preparation of PRP, most producing a liquid end product. Platelet gel and fibrin sealant derived from PRP mixed with thrombin and calcium chloride have been exogenously applied to tissues to promote wound healing, bone growth, hemostasis and tissue sealing (20). Platelet-Rich Fibrin Matrix (PRFM) prepared as a membrane based preparation of PRP that is without the use of exogenous thrombin (5). Ultrastructural analysis of PRFM with confocal scanning fluorescence microscopy and SEM revealed a dense highly cross-linked fibrin matrix with the intact platelets localized on one side of the membrane (21).

A large number of *in vitro* studies have been performed characterizing PRP and products derived from PRP (20, 22). This study reports, for the first time, the viability of platelets within PRFM. Determination of viability of platelets in PRFM is critical to understanding the dynamics of the growth factor release by these cells while in fibrin matrix. Viability assay shows that under *in vitro* conditions, platelets gradually lose viability over a period of time. It is also important to note that the platelets produced with this system immediately following production are intact (5). The loss of cell viability is expected to be more rapid in the harsh environment of wounds where high levels of proteases are known to be present

(23). Studies are required to understand the fate of platelets viability in a wound environment. A significant difference between the platelet number in the whole blood samples and their experimental PRP has been reported (22). In the majority of the studies reported, the data presented are difficult to interpret because of lack of reference value of the whole blood platelet numbers (24, 25). The recovery of platelets in this study was of the order of 65% with some inter-donor variation. The platelet yields and recoveries (~60%) are comparable to studies reported previously with other commercially available systems (26).

The alpha granules are the storage site for a plethora of factors notably amongst which are growth factors (27). The level of PDGF and VEGF released in conditioned media from platelets embedded in PRFM is comparable to the levels of PDGF & VEGF released from platelet gels (22, 26). A steady release of all the three growth factors over a period of seven days suggest that the release kinetics is distinct compared to the PRP gel preparations where use of thrombin causes an immediate release of growth factors as a result of degranulation (26, 28). Endogenous release of these mediators by local tissue at the site of delivery is known to result in a slower release of growth factors and chemical mediators (28). Earlier studies using PRFM and “washed-out” protocol demonstrated that the conditioned media from PRFM cultures produced high levels PDGF, TGF β 1, VEGF, bFGF, and EGF supports the finding of present study on the release of growth factors over a period of 7 days from PRFM cultures in vitro.

The supernatants from PRP gels stimulated with thrombin and calcium chloride has been shown to be mitogenic for endothelial cells (26, 29, 30). The current study provides evidence that conditioned media derived from PRFM cultures for 7 days show potent mitogenic activity. Proliferation of endothelial cells, treated with day 1,3 or 7 conditioned media, were comparable. The growth factor levels in the conditioned media increased in period from 1–7 days suggesting that the increasing concentration of growth factors did not further stimulate the endothelial cell proliferation. The effect of individual growth factor on endothelial cell proliferation was investigated using neutralizing antibodies against growth factors. The sequestering of PDGF using anti-PDGF antibody did not affect endothelial cell proliferation induced by PRFM conditioned media. Anti-VEGF antibody, however, significantly inhibited the proliferative response. VEGF, a well known endothelial cell-specific mitogen, promotes many of the events necessary for angiogenesis (31, 32). PRP supernatants have been shown to be highly mitogenic for endothelial cells at least in part, to the presence of epidermal growth factor (EGF) in media (33). Thus, a number of factors present in PRFM conditioned medium may be responsible for endothelial cell proliferation. We therefore investigated the net response of the conditioned medium on key signaling pathways that are involved in endothelial cell proliferation. MAPK/ERK activity has been shown important to the induction of endothelial cell proliferation by VEGF via VEGFR2 (KDR/Flk-1)(34). This study provides maiden evidence that PRFM derived medium potently activates ERK phosphorylation and that knock down of this signaling mediator inhibits the conditioned media-induced endothelial cell proliferation.

Finally, to correlate the in vitro findings of endothelial cell proliferation in an experimental model of delayed wound angiogenesis in pigs was studied. Vascular complications commonly associated with problematic wounds are primarily responsible for wound ischemia. Ischemia limits the supply of blood-borne products, including nutrients, oxygen, and circulating cells to the wound site, thereby severely impairing the healing response (35). Thus, clinically presented ischemic wounds do not readily lend themselves to the study of biological mechanisms because the collection of tissue biopsies at multiple time points from the same wound poses ethical challenges. The need for preclinical models of ischemic wounds is therefore compelling (16). We recently characterized a porcine ischemic wound model. Wound closure in the ischemic pig skin tissue was severely impaired, resulting in

wounds that persisted for 30 days or longer. Impaired angiogenesis was reported in these experimental wounds (16). Angiogenesis, or the formation of new blood vessels from existing ones, is a critical step in the regeneration of hard and soft tissues (9). Increased wound angiogenesis in ischemic wounds was comparable to a recently reported study where a slow and sustained release of growth factors from PRP via hydrogel approach increased neovascularization in a murine model of hind limb ischemia (36). These observations suggest that a slow sustained release of growth factor in this preparation because of absence of thrombin-induced stimulation may be one of the critical factors in promoting wound angiogenesis in PRFM treated ischemic wounds. This preparation has showed effectiveness in the treatment of hard to heal wounds (5). Other clinical applications that have been reported using PRFM include rotator-cuff repair (37); bony repair in the oral cavity (38) and aesthetics (39).

In summary, the study for the first-time shows the kinetics of the viability of platelets embedded in fibrin matrix. A slow and steady release of growth factors from PRFM was observed because of the use of non thrombin activation approach. The VEGF released from PRFM was primarily responsible for endothelial mitogenic response via ERK activation pathway. Finally, the preparation effectively induced endothelial cell proliferation in wounds and improved wound angiogenesis in ischemic wounds indicating potential mechanisms of action of PRFM in healing of chronic ulcers.

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Figure 1. Preparation of PRFM, quantification of platelet recovery and viability

A. An outline of the PRFM preparation protocol. **B.** Red arrow indicates a ready PRFM membrane that was used for *in vitro* and *in vivo* wound studies. **C.** Platelet recovery determined in the first 20 subjects enrolled. Individual % recovery data as well as average (error bar) recovery is shown. Average data shown are mean $\pm$ SD (n=20). **D.** Changes in platelet viability in PRFM over time. LDH release from cells was used as measure of loss of platelet viability. Data shown are % change compared to 0h or baseline sample (100% viable). Mean $\pm$ SD (n=10); **, p<0.001 compared to baseline.

Figure 2. Release of growth factors from PRFM in culture

RFM were prepared and placed in 6-well culture plates with RPMI 1640 media. Growth factors (PDGF-BB, TGF-β1, and VEGF-A) released in media was measured using ELISA. Data shown are actual growth factor levels (ng/ml) in media bathing PRFM. Data are mean $\pm$ SD (n=10 for PDGF and n=5 for VEGF and TGFβ1); **, $p < 0.01$ compared to 0h (baseline) sample.

Figure 3. Conditioned media from PRFM induced human endothelial cell proliferation

Human microvascular endothelial cells (HMEC) were cultured in 96-well plates. The cells were serum starved and simultaneously treated with conditioned media from PRFM. The cells were cultured with 10% fetal bovine serum (FBS) in culture media served as positive control. **A**, Cell proliferation in the presence of PRFM conditioned media (dilution = % conditioned media of the total culture media). Data are mean $\pm$ SD (n=5); †, $p < 0.01$ compared to positive control; *, $p < 0.05$ & **, $p < 0.01$ compared to conditioned media untreated group. **B**, HMEC cells cultured in serum free conditions were treated with 25% (i.e. 1 part condition media added to 3 parts of cell culture media) PRFM conditioned media collected on days 1, 3 or 7 post PRFM formation. Data are mean $\pm$ SD (n=4). †, $p < 0.01$ compared to positive control; *, $p < 0.05$ & **, $p < 0.01$ compared to conditioned media untreated group; **C**, Cell proliferation in the presence of platelet poor plasma-gel conditioned media (PPP-M, dilution = % conditioned media of the total culture media). Data are mean $\pm$ SD (n=5); †, $p < 0.01$ compared to positive control.

Figure 4. Role of PDGF-BB and ERK activation in HMEC proliferation induced by PRFM-conditioned media

A. PRFM conditioned media was treated or not with anti-PDGF or anti-VEGF (20 μ g/ml) blocking antibody to sequester PDGF-BB or VEGF in PRFM conditioned media. Cells cultured in serum free conditions were treated with 25% PRFM for 48h. Data are mean $\pm$ SD (n=4). *, $p < 0.05$ compared to PRFM treated & blocking antibody untreated group. **B.** PRFM-conditioned media (25%) induced rapid (30 min) ERK phosphorylation (p-ERK) in HMEC. **C.** Quantification of the immunoblot data (B) using densitometry. Ratio of p-ERK/ERK is shown. * $p < 0.05$ compared to PRFM untreated group. **D.** ERK inhibition attenuated HMEC proliferation induced by PRFM (25%). ERK inhibition was achieved by pretreatment of cells with the ERK inhibitor UO126 (10 μ M) 5 min prior to PRFM exposure. Data are mean $\pm$ SD (n=5). *, $p < 0.05$ compared to PRFM treated and inhibitor untreated group.

Figure 5. ERK knock-down inhibited PRFM-conditioned media induced proliferation of HMEC
 Cells were transfected with siRNA to knockdown ERK 1 and 2. Control cells were transfected with scrambled (control) siRNA. **A**. ERK immunoblot showing effective knock down of ERK following siRNA tranfection. **B–C**. Quantification of the immunoblot densitometry data of ERK1 (**B**) and ERK2 (**C**) shown in A. Ratio of ERK/GAPDH is shown. GAPDH was used as a housekeeping protein standardization of sample loading. Data are mean $\pm$ SD (n=3); * p<0.05 compared to control siRNA treated group. **D**. ERK knockdown attenuated PRFM (25%) induced proliferation of HMEC. HMEC were transfected with ERK 1 & 2 siRNA to knockdown ERK levels in cells. Control cells were transected with scrambled (control) siRNA. ERK siRNA and control siRNA transfected cells were activated with 25% PRFM as described earlier. Results are mean $\pm$ S.D. *, p<0.05 compared to scrambled (control) siRNA treated group.

Figure 6. Treatment of porcine ischemic wound with PRFM

A. Four Ischemic wounds (solid circles) were developed on the back of pigs as described in *Methods*. Six additional non-ischemic wounds were developed (open circles). Arrows indicate direction of blood flow in flaps containing ischemic wounds. The upper left and lower right ischemic wounds were treated with PRFM at the time of wounding. **B.** Representative digital images of excisional wounds treated or not with PRFM on days 0 and 4 post-wounding.

Figure 7. Ischemic wound histology following treatment with PRFM membranes
Wound-edge tissues were collected on 14 d post-wounding. Formalin-fixed paraffin-embedded biopsy tissues were sectioned (5 μ m) and stained using **A.** Masson's Trichrome staining. This staining results in blue-black nuclei; blue collagen and cytoplasm. Epidermal cells appear reddish. The arrows indicate the start and the end of wound. Scale bar = 500 μ m. Right panels are the zoom of boxed area of images shown in left panel. Scale bar = 50 μ m. **B.** Picro-sirius red staining. The red staining is the birefringence of collagen fibers, which is largely due to co-aligned molecules of Type I collagen. Bar graph show quantitation of the collagen fibers in PRFM-treated or untreated wounds. Scale bar = 50 μ m. Data are mean $\pm$ SD (n=3); * p<0.05 compared to untreated wounds.

Figure 8. Increased vascularization of PRFM-treated ischemic wounds

Wound-edge tissues were collected 14d post-wounding. Formalin fixed paraffin embedded biopsy tissues were sectioned (5 μ m) and stained using von Willebrand Factor (brown) and counterstained with hematoxylin (blue). Compared to non-treated ischemic wounds, the treated wounds showed increased vascular endothelial cell staining. Arrows indicate vWF positive vessels. *Right panel.* Zoom of the boxed area shown in corresponding images to the left. Scale bar = 50 μ m. Bar graph show quantitation of the blood vessels in PRFM-treated or untreated wounds. Data are mean $\pm$ SD (n=3); * $p < 0.05$ compared to untreated wounds.

Figure 9. Increased number of Ki67 positive cells in PRFM treated porcine wounds

Wound-edge tissues were collected 14 d postwounding. Formalin fixed paraffin embedded biopsy tissues were sectioned (5 μ m) and stained using Ki67 (brown), a marker of proliferating cells. The sections were counterstained with hematoxylin (blue). Compared to non-treated ischemic wounds, the treated wounds showed increased number of proliferating cells. Arrows indicate the Ki67 positive proliferating cells. *Lower panel.* Scale bar = 50 μ m. Bar graph show quantitation of the Ki67 positive cells in PRFM treated or untreated wounds. Data are mean $\pm$ SD (n=3); * $p < 0.05$ compared to untreated wounds.

Figure 10. Co-localization of Ki67 and vWF positive cells in PRFM treated porcine wounds
Wound-edge tissues were collected 14 d post wounding. Formalin fixed paraffin embedded biopsy tissues were sectioned (5 μm) and stained using Ki67 (red) and vWF (green). Compared to non-treated ischemic wounds, the treated wounds showed increased number of VWF positive proliferating cells. Arrows indicate Ki67 and vWF positive blood vessels. *Top two panels.* Scale bar = 200 μm . *Bottom panel.* Scale bar = 20 μm .

Figure 11. Increased CD31 expression in LCM captured granulation tissue from PFRM treated wounds

Wound-edge tissues were collected 14 d post wounding. LCM capture was performed on H&E stained OCT embedded tissue **A**, visualization of section; **B**, demarcation (in blue) of region of interest; **C**, post-dissection and catapulting. Scale bar, 150 μ m; **D**, Real-time PCR was performed on LCM captured granulation tissue from wounds treated or not with PFRM to quantify CD31 expression normalized against PPIA levels. Data are mean $\pm$ SD (n=4); *, p<0.05.

Figure 12. Increased blood flow in PFRM treated wounds

Doppler images at 14 d post wounding of the back of swine with ischemic flaps treated or not with PFRM. Bar graph presents the quantitative data from the Doppler images. Data are mean $\pm$ SD (n=3); *, $p < 0.05$.