Effect of Various Platelet Preparations on Retinal Müller Cells

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Purpose. Peeling of the internal limiting membrane is the treatment of choice for macular holes. Fresh platelet suspension (PS) is used to support wound healing in persistent macular holes. The concentration of growth factors in fresh, frozen, and thrombin-activated PSs were compared, to optimize their trophic potential and examine their capacity to support proliferation, migration, and contraction of human retinal Müller cells.

Methods. The concentration of various growth factors in frozen PS, thrombin-activated PS, and plasma were evaluated by ELISA. The effect of these preparations on proliferation, migration, and contraction of human Müller cells were evaluated with an ATP-assay, a colony-dispersion assay, and a detached collagen gel contraction assay respectively. Plasma was tested as a control.

Results. Frozen and thrombin-activated PSs contained significantly more EGF, TGF-β1, and PDGF than did plasma. The highest concentrations of EGF and FGF were found in frozen PS. All platelet preparations and plasma supported cell growth significantly better than the control, which was serum-free culture medium. Müller cells migrated better when incubated with thrombin-activated PS than with any other test solution. Contraction was extremely strong after incubation with fresh PS compared with plasma or thrombin-activated or frozen PSs.

Conclusions. Frozen and thrombin-activated PSs may be suitable alternatives to fresh PS for persisting macular holes, due to their superior effect on Müller cell migration. (*Invest Ophthalmol Vis Sci.* 2009;50:4881–4886) DOI:10.1167/iovs.08-3057

Since the initial publication by Kelly and Wendel[1] in 1991, the anatomic success of surgery for macular hole has steadily increased from 58% to over 95%. This success is certainly the result of improved surgical techniques, especially peeling of the internal limiting membrane (ILM).[2] The supposed pathomechanism of macular hole closure is the stimulation of glial cell proliferation (i.e., of Müller cells and astrocytes) by surgical decapitation of their basal membrane, ultimately leading to closure of the macular hole.[3,4] However, for patients with a persistent macular hole despite ILM peeling, no successful treatment alternative has been established so far.[5–8] To promote the healing process, adjuncts like growth factors, autologous serum, plasma, or concentrated platelet suspension (PS) have been investigated.[3] Particularly, PS has been shown to achieve a high anatomic success rate and good visual results, even in recurrent macular holes.[9–15]

These positive effects may be explained by the content of various growth factors in concentrated platelet solutions, which support tissue repair by regulation of cell migration, mitosis, and differentiation shown in various models of normal and impaired wound healing.[16,17] Müller cells, which are the predominant glial cells in the retina (90%) and the stabilizers of the complex retinal architecture, are affected by growth factors and play an important role in the healing process of macular holes.[18–23] Histologically, closure of a macular hole has been associated with glial proliferation,[3] which is also observed after various forms of retinal injury.[4] Müller cells express growth factors, neurotransmitter transporters, antioxidant agents and— under hypoxic conditions—facilitate neovascularization.[24] Furthermore, they have essential functions for the normal activity of the retina, including glutamate uptake to reduce its extracellular concentration below neurotoxic level, K+-homeostasis, nutrient supply, pH regulation, and retinoid metabolism.[18,19]

Currently, fresh suspensions of autologous platelets are used in the clinical set up of macular hole surgery. They are often produced from and for every patient individually. During surgery, the PS is applied to the macular hole, where it is thought to release its growth factor content and support healing of the retinal defect. The remaining platelet membranes, however, are also known for their proapoptotic effects.[25] Alternatively highly concentrated solutions of platelet derived growth factors can also be produced ex vivo by activating platelets with thrombin or by freeze–thaw cycles. To optimize the trophic potential of the PS product, we compared the effect of various production protocols on the concentration of growth factors and its capacity to support aspects of retinal wound healing, such as proliferation, migration, and contraction of human Müller cells.

Materials and Methods

Preparation of Test Solutions

Platelet suspension (PS) were prepared from six single-donor aphereses of healthy volunteers for proliferation (mean age, 36 ± 9.6 years) with a blood cell separator (Amicus; Baxter, Deerfield, IL) used according standard procedures. Acid citrate dextrose was used as an anticoagulant. The apheresis products, containing approximately 3×10^{11} platelets, were washed three times with 0.9% NaCl at 800*g* for 10 minutes before careful resuspension in serum-free medium (DMEM; Invitrogen-Gibco, Grand Island, NY) with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) to a concentration of 1×10^{10} platelets per milliliter. Platelet counts were obtained with an automated blood count analyzer (GenS; Beckman Coulter Inc., Fullerton, CA).

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The PS were divided in three equal-volume samples from which the following test-solutions were prepared:

1. Fresh PS (as is currently used in the clinical setup).
2. Frozen PS, stored for 1 hour at −70°C for growth factor release.
3. PS stimulated with human thrombin (Tissucol Duo S, diluted in 40 mM $CaCl_2$; Baxter Deutschland GmbH, Unterschleissheim, Germany) at a concentration of 1 U/mL for 20 minutes, followed by centrifugation at 3500*g* for 15 minutes to obtain a cell-free, growth-factor rich–supernatant.

As the control, plasma was obtained from the same group of volunteers, to test its effect on proliferation ($n = 6$) and contraction ($n = 3$). Briefly, volunteers' whole blood was taken by venipuncture and immediately mixed with the anticoagulant citrate phosphate dextrose adenine (CPDA), before it was centrifuged at 3500 U/min (800*g*) for 2 minutes to obtain the plasma supernatant. Aliquots of all samples were stored at −70°C until the day of analysis.

Growth Factor Quantification

The concentrations of PDGF-AB, basic FGF, and EGF in plasma, frozen, and thrombin-activated samples were measured in duplicate with ELISA kits (R&D Systems, Minneapolis, MN). For the quantitative determination of TGF-β1, an ELISA kit from BenderMedSystems (Vienna, Austria) was used, according to the manufacturer's instructions.

Cell Culture

Spontaneously immortalized human Müller cells (MIO-M1 Müller cell line) were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37°C and 5% CO_2 atmosphere.[19]

Proliferation

Proliferation was determined with a luminescence-based luciferin-luciferase ATP-assay.[26] Briefly, 5000 cells were seeded per well in 96 well-culture plates (Falcon, Plymouth, UK) and cultured for 24 hours. Before exposure to the test substances, the culture medium was changed to a non–growth-supporting medium for 24 hours. Defined DMEM contained 1% BSA and 1% penicillin-streptomycin, but no growth factors, serum, or tissue extracts. The cells were then washed twice with phosphate-buffered saline (PBS) and exposed to the test solutions (plasma and fresh, frozen, and thrombin-activated PSs). On each culture plate, the cells were also exposed in separate wells to serum-free medium (DMEM) containing 1% BSA, as a positive control, or to 1% benzalkonium chloride (BAC; Haltermann Ltd., Workington, UK), a maximum inhibitor of cell proliferation, as a negative control. As a control thrombin (1 U/mL) was also tested. All test solutions were diluted in serum-free medium (DMEM) containing 1% BSA from 100% (1×10^{10} platelets/mL) to 50%, 20%, 10%, 1%, and 0.1%, and triplicate cell cultures were exposed to these solutions for 24 hours. After incubation, the test substances were removed, and all wells were washed with PBS three times before cellular ATP was extracted by adding 100 μL PBS and 50 μL cell extraction reagent to each well with a multichannel pipette. The cells were left for at least 5 minutes at room temperature before 25 μL of culture extract was mixed with 25 μL luciferin-luciferase reagent, previously equilibrated to room temperature, into the wells of a white 96-well assay half-area plate (Dynex, Chantilly, VA).[27,28] The resultant luminescence was read after 15 minutes with a luminometer (FLUOstar Optima; BMG Labtech GmbH, Offenburg, Germany). The ATP assay reagents, including extraction buffer and luciferin-luciferase, were obtained from PerkinElmer Life Sciences (Boston, MA). The luminescence intensity is proportional to the amount of cellular ATP, which is a marker of cell viability, present in all metabolically active cells and can therefore be used as a marker for cell growth. The percentage of cell growth (CG) for each substance was calculated according to the formula:

$$[(\text{Test} - \text{MI})/(\text{MO} - \text{MI})] \cdot 100 = \%\text{CG}$$

where MO is the mean count for positive control cultures, MI is the mean count for maximum inhibition control cultures, and Test is the mean count for triplicate test solutions.

Migration

Six-well culture plates (9.6 cm^2/well; Nunclon; Thermo Fisher Scientific, Roskilde, Denmark) were coated with 0.01% collagen type 1 (Sigma-Aldrich) per well, sterilized, and dried overnight under UV light. Three silicone cylinders (flexiPerm micro 12; Vivascience; Sartorius AG, Göttingen, Germany) were placed in each well and human Müller cells were seeded within the cloning rings at a density of 30,000 per cylinder in 150 μL of cell culture medium. After the cells were confluent, proliferation was stopped with 100 μM hydroxyurea in culture medium for 24 hours.[29] For a starvation period, the cells were then washed with PBS and incubated with serum-free medium (DMEM + 1% BSA without any growth factors) for 24 hours. Then, the silicone cylinders were removed, and the cells were washed twice with PBS, before the test solutions—fresh, frozen, and thrombin-activated PSs, in a dilution of 50% (5×10^9 platelets/mL, diluent: serum-free medium)—were added in triplicate. At 48, 96, and 144 hours the cells were thoroughly washed with PBS and fixed with 90% methanol for 10 minutes. The cell cultures were then stained with hematoxylin for 30 minutes, washed with PBS, and photographed in standard conditions with a digital camera (Sony Corp., Tokyo, Japan). The size of each colony was measured with Image Tool software (UTHSCSA image tool, version 2α, http://ddsdx.uthscsa.edu/dig/ provided in the public domain by University of Texas Health Sciences Center, San Antonio, TX).

Contraction

First, concentrated medium was prepared by mixing 15 mL 10 × DMEM (Sigma-Aldrich) with 35 mL sterile distilled H_2O, 1.5 mL 2 mM glutamine, 1.5 mL penicillin/streptomycin (10,000/mL), and 4 mL 7.5% sodium bicarbonate. Then a gel mixture was prepared by adding 160 μL concentrated medium in a 50-mL tube to 830 μL type 1 collagen (First Link, Birmingham, UK). The pH was raised to 7.4 by drop-wise addition of sterile 0.1 M NaOH, before 500,000 cells, diluted in 150 μL serum-free medium, were added gently. All substances, except the cell suspension, were used cold to delay the precipitation of the collagen. Quickly, but very carefully, to avoid air bubbles, 150 μL of the collagen/cell mixture was cast into the wells of a 48-well plate (Thermo Fischer Scientific). The plates were then incubated at 37°C for 15 minutes, before addition of 75 μL test solutions (plasma and fresh, frozen, and thrombin-activated PSs) per well. Dilutions of 100%, 50%, and 20% (diluent: serum-free medium) were tested in duplicate. After another 2 hours in the incubator, the gels were detached gently from the plate walls with a yellow pipette tip. At 24, 48, and 96 hours, the gels were photographed under standard conditions with a digital camera (Sony Corp., Tokyo, Japan). The size of each gel area was measured with the Image Tool software.

Statistical Methods

Statistical analysis was performed with paired Friedmann, paired Wilcoxon, and one-sided *t*-tests (SPSS for Windows, ver. 15; SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Content of Growth Factors

The mean concentrations ± standard deviations of epitheliotrophic factors in blood preparations are given in Table 1. Frozen- and thrombin-activated PS contained significantly more EGF, TGF-β1, and PDGF than plasma. The highest concentrations of EGF and FGF were found in frozen PS. The differences between the three groups were statistically significant with $P < 0.002$.

TABLE 1. Content of Growth Factors in Undiluted Platelet Preparations and Undiluted Plasma

	Frozen PS	Thrombin-Activated PS	Plasma
EGF	4.99 ± 2.82*†	1.40 ± 0.73†	0.14 ± 0.08
TGF-β1	356.39 ± 123.5†	433.39 ± 132.47†	56.72 ± 26.10
PDGF	307.11 ± 75.52†	240.13 ± 115.97†	8.41 ± 4.35
FGF	0.47 ± 0.43*†	0.07 ± 0.11	0.04 ± 0.01

Data are expressed as nanograms per milliliter.

* Significant difference between frozen and thrombin-activated PS ($P < 0.001$).

† Significant difference between frozen or thrombin-activated PS and plasma ($P < 0.002$).

Proliferation

ATP bioluminescence is interpreted as relative cell growth, and its mean coefficient of variation was calculated as the SD. For cells cultured with the positive control, this was 15%, which represents the normal variability of a cell culture–based proliferation assay.[30] Figure 1 shows relative ATP bioluminescence in cells after incubation for 24 hours with the test solutions (plasma and fresh, frozen, and thrombin-activated PSs) at concentrations between 0.1% (1×10^7 platelets/mL) and 100% (1×10^{10} platelets/mL). All test solutions were able to support proliferation significantly better than the control culture with serum-free medium, which in the graphs in Figure 1 is represented by the 100% cell growth rate ($P < 0.035$). Fresh PS achieved an approximate 50% increase in cellular ATP level compared with serum-free medium at a 10% dilution ($P < 0.03$). Frozen PS–stimulated cell growth was best at a concentration of 100%, whereas fresh, and thrombin-activated PSs achieved the highest ATP-levels when diluted to 10%. Plasma stimulated cell growth best when diluted to 50%.

Migration

Support of cell migration was investigated for 50% fresh, frozen, and thrombin-activated PSs and the control culture serum-free medium over 144 hours. Müller cells migrated better when incubated with thrombin-activated PS than with fresh PS ($P < 0.04$) or serum-free medium ($P < 0.01$; Fig. 2). Frozen PS also supported cell migration, but the effect was inferior compared with thrombin-activated PS at 96 hours ($P < 0.04$). Fresh PS did not support significant migration. The effect of plasma on migration was not tested, since previous experiments showed that it is not capable of sustaining cell viability and migration beyond 48 hours.[31] Thrombin alone did not significantly effect proliferation at 12 and 48 hours (data not shown).

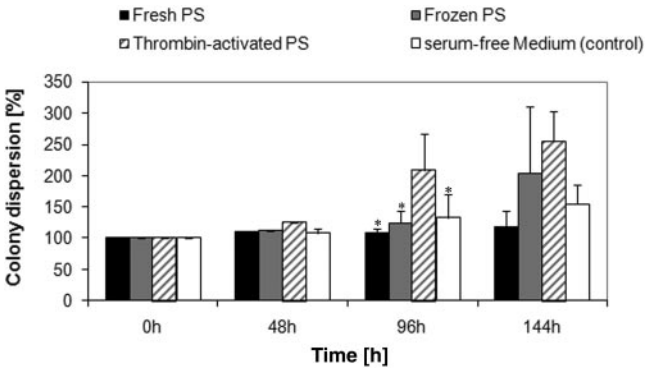


FIGURE 2. Colony-dispersion assay of Müller cells incubated for up to 144 hours with 50% (5×10^9 platelets/mL) test solutions. Cells incubated with thrombin-activated PS migrated faster than with the others test substances.

Contraction

Induction of cell contraction was investigated for the plasma and frozen, fresh, and thrombin-activated PSs in dilutions of 100%, 50%, and 20% for up to 96 hours in time– and dose–response-experiments (Figs. 3B, 3C). Cell contraction was strongest when the cells were incubated with fresh PS. Whereas fresh and frozen PS showed maximum gel contraction at a concentration of 20%, thrombin-activated PS and plasma had its maximum at 100%. The control culture serum-free medium induced no cell contraction. However, the differences were not significant.

DISCUSSION

Our intent was to assess various protocols for the preparation of platelet-based suspensions for their concentration of growth factors and their wound-healing properties on human retinal Müller cells. All cellular activities assessed (proliferation, migration, and contraction) can contribute to macular hole closure, a disorder for which fresh concentrated PSs have been

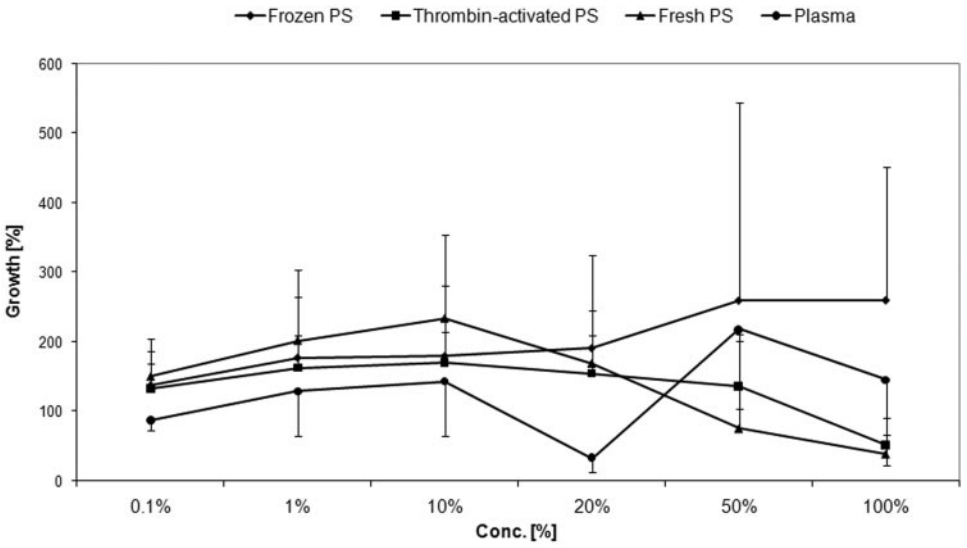
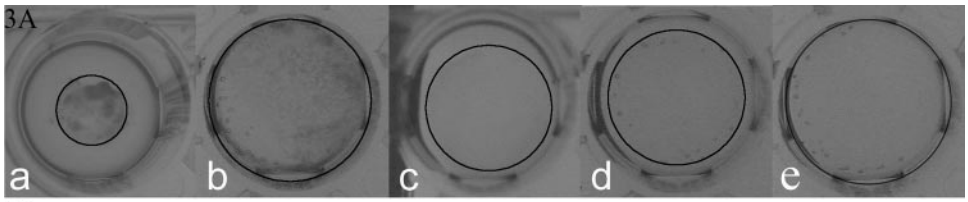
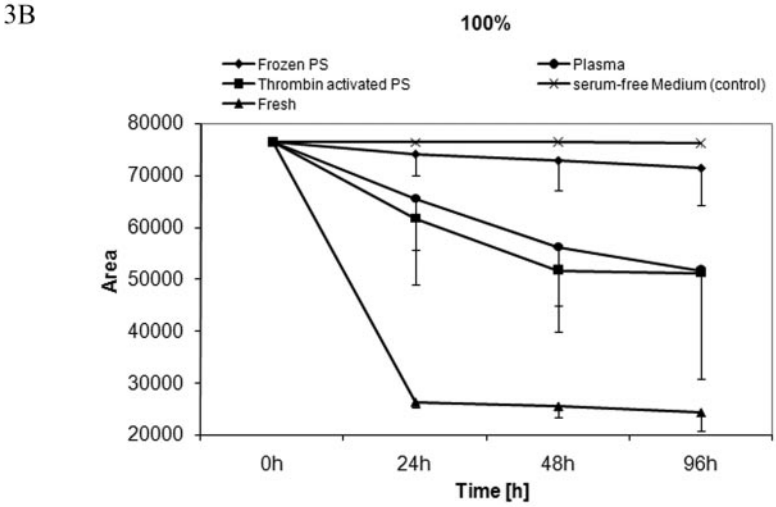


FIGURE 1. Dose–response of mean ± SE of cellular ATP levels in Müller cells incubated for 24 hours with various blood preparations. Cell growth was best supported with 100% (1×10^{10} platelets/mL) frozen PS, 10% fresh PS, 10% thrombin-activated PS, and 50% plasma. Support of proliferation was significantly better than with the serum-free medium culture control ($P < 0.035$).





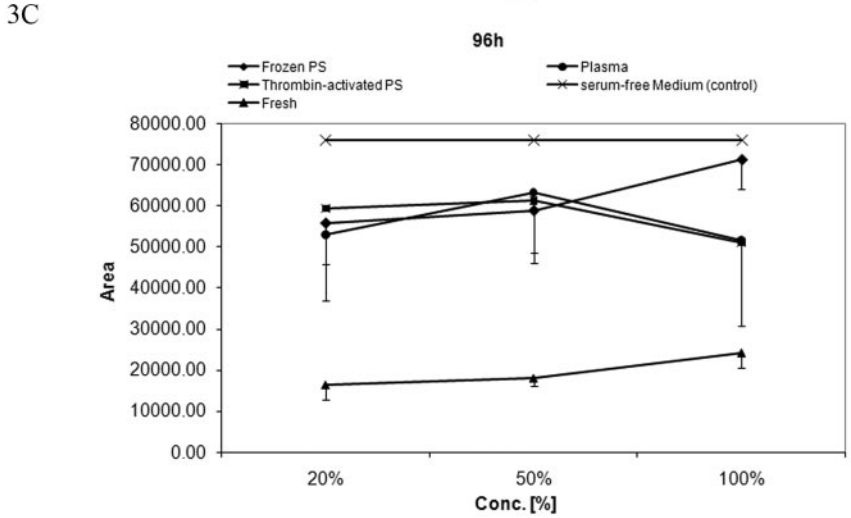


FIGURE 3. (**A**) Cell contraction at 24 hours with 100% (1×10^{10} platelets/mL) concentration. (**Aa**) Fresh PS, (**Ab**) frozen PS, (**Ac**) thrombin-activated PS, (**Ad**) plasma, and (**Ae**) serum-free culture medium. (**B**) Time–response of cell-contraction assay of Müller cells incubated with 100% (1×10^{10} platelets/mL) of various blood preparations for 24, 48, and 96 hours (*x*-axis). The mean area of gels ± SE is given on the *y*-axis. Cell contraction was strongest after incubation with fresh PS, which induced maximum gel contraction within 24 hours. The control culture serum-free medium showed no cell contraction. (**C**) Dose–response-curve of the contraction assay of Müller cells incubated for 96 hours with various blood preparations in a dilution of 100 (1×10^{10} platelets/mL), 50% and 20%. Thrombin-activated PS and plasma induced the highest level of contraction at a concentration of 100%, whereas this maximum was found for frozen and fresh PS after dilution to 20%. In comparison, cell contraction was strongest after incubation with fresh PS.

used as a wound-healing supportive adjunct during vitreoretinal surgery. In addition to fresh platelet suspension, we also investigated the effects of freezing and thrombin activation of platelets. In contrast to plasma, which was included as a control, platelets contain high concentrations of platelet-derived growth factors and can be cryopreserved for months, providing logistic liberty.

Our experiments showed that the production protocol had a significant impact on the composition (i.e., the growth factor content, as well as the induction of wound healing activities by human retinal Müller Cells), with none of the protocols supporting proliferation, migration, and contraction equally well. Table 2 summarizes the findings. All test solutions supported the growth of human Müller cells—as measured with a luminescence-based ATP-assay—significantly better than serum-free cell culture medium.[26] Cell migration was clearly better when the cells were incubated with thrombin-activated PS. This effect was reduced if the PS was activated by freezing. Fresh PS had no effect on cell migration at all. In contrast cell–matrix contraction was extremely high after incubation with fresh PS when compared with thrombin-activated or frozen PS and plasma.

These results suggest that fresh PS used clinically at 1×10^{10} may achieve macular hole closure predominantly by contraction of Müller cells. At a lower platelet concentration, proliferation is also supported. The results confirm previously

TABLE 2. Summary of Wound-Healing Parameters Assessed in Human Retinal Müller Cells

	Fresh PS	Frozen PS	Thrombin-Activated PS	Plasma
Proliferation at				
10%	++	+	+	+
100%	−	++	−	+
Migration	0	+	++	Not done
Contraction	++	0	+	+

100% = 1×10^{10} platelets/mL. −, inhibition; 0, no effect; +, support; ++, maximum support.

published data by Castelnovo et al.,[32] who also showed that fresh PS supports cell growth better at concentrations lower (2.6×10^7 platelets/mL) than that currently used during surgery. This finding may be explained by the proapoptotic effect of platelet membrane aggregates, which remain in the solution after platelet activation.[25] The supporting effects of a concentrated platelet suspension is usually explained by its high concentration of various growth factors, which are known to be involved in the biological activity of many cells and in the wound-healing processes of several tissues.[16,17] EGF, FGF, TGF-β1, and PDGF are known to modulate Müller cell proliferation, migration, and contraction.[20–23]

Thrombin-activated PS could constitute an alternative to fresh PS, which has already been shown to support proliferation of human corneal epithelial cells.[33] In our experiments, it also had a statistically significant better effect on Müller cell migration. This may not only be explained by the absence of cell membrane remnants but also by the known promigratory effect of thrombin itself.[34] In addition the thrombin used for coagulation is known to support proliferation as well as contraction of retinal glial cells and fibroblasts, although cell proliferation in our experiments was not positively affected by thrombin alone at 48 hours.[35,36] A note of caution: Remnants of active thrombin could induce thrombosis in intraocular vessels if a thrombin-activated PS is used in vitreoretinal surgery. Growth factor released by freeze-thaw cycles would avoid this potential risk. In contrast to fresh PS, frozen PS also showed cell-migration–supporting activities, which might be explained by higher concentrations of EGF, FGF, and TGF-β.

In our experiments, plasma was found to support the wound-healing activities of human retinal Müller cells much less than platelet-based suspensions, perhaps due to the significantly lower concentration of various growth factors, including EGF and FGF, in plasma, which by definition is obtained before activation and release of platelet-derived growth factors. However, plasma is not completely free of growth-supporting substances. It contains substantial amounts of TGF-β1 and other cytokines, from sources other than platelets. Also, other factors not examined in this study, such as fibronectin and vitamins, which also modulate migration and proliferation, may be present in plasma and are likely to be involved in modulation of the wound-healing of Müller cells.[21,32,37–40]

Cell culture models have been used successfully in the past to optimize the production protocols of other blood products, such as the serum eye drops currently used to promote healing of corneal epithelial defects.[28,33] Certainly, the complex physical and molecular interactions in the retina and macular holes cannot be replicated by cell culture models in vitro. However, we used spontaneously immortalized human Müller cells, which retain the characteristics of primary isolated cells in culture.[19] Also since Müller cells in the retina have a nutrient supply from the retinal vasculature, vitreous, and choroid, all test substances were diluted in a non–growth-factor-supplemented culture medium to provide some basic nutrients during long-term incubation. To differentiate the effect of the test substance from serum-free medium itself the serum-free medium was used as a control in every assay. However, in vivo, other factors such as the method of application and vitreous turnover are likely to influence the final concentration of active growth factors in the vicinity of the macular hole and the efficacy of trophic wound-healing effects of any platelet-based suspension.

While the peeling of the ILM has increased the success of macular hole surgery to more than 95%, some holes persist or recur.[1–3,13–15,41,42] Factors that affect such closure rates include the pathogenesis, size, and chronicity of the macular hole. Small and recent holes may close regardless of the type of intervention, even without ILM peeling. However, persistent and recurrent macular holes may require a different approach. Clinicopathologic correlation suggests that Müller cells and astrocytes are involved in macular hole closure. Therefore, adjuvants such as platelets which stimulate Müller cell–based wound healing continue to be used in patients with large, chronic, persistent, or recurrent macular holes. The production protocol for platelet suspensions should therefore be optimized to improve the wound-healing capacity of this adjuvant. A potentially serious risk of the use of every blood-derived product, especially if it is pooled from more than one donor, is transmission of disease, such as HIV or hepatitis. However, the risk of transmission is reduced by combined testing with PCR and serology to probably less than $1{:}4 \times 10^5$ for hepatitis V, less than $1{:}4 \times 10^6$ for HIV, and $<1{:}10^7$ for HCV.[43,44] A prestorage pooling system for whole-blood–derived platelets has just been licensed in the United States.[45]

In conclusion, our results demonstrate that the blood products plasma and fresh, frozen, and thrombin-activated PSs enhance proliferation, migration, and contraction of human retinal Müller cells in culture. Both, growth factor concentrations and trophic capacity of blood-derived products depend on the production protocol. Our experiments indicate that a lower concentration of platelets may be beneficial to what is currently used in the clinical setting. Also frozen PS or thrombin-activated PS may be suitable as an alternative adjunct for recurrent or persistent macular holes with a superior effect on migration. These experimental findings should be verified in well-controlled and randomized clinical studies.

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