Corneal Epitheliotrophic Capacity of Three Different Blood-Derived Preparations

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Purpose. Serum eye drops have been successfully used in the treatment of severe ocular surface disorders. Fresh frozen plasma (FFP) and platelet concentrates have not yet been tested for use as eye drops, although they are easily available as quality-controlled products from blood banks and are routinely used for transfusion. To test whether FFP or platelet-derived growth factor solutions could be used for ocular surface diseases, we compared the epitheliotrophic capacity of platelet releasate and FFP with that of serum in cell culture models.

Methods. The concentrations of EGF, TGF-β1, PDGF-AB, fibronectin, vitamin A and vitamin E in serum, FFP, and platelet releasate were evaluated with ELISA and HPLC. Corneal epithelial cells were incubated with the various preparations and cell proliferation, migration, and differentiation were evaluated by means of a luminescence-based adenosine triphosphate (ATP) assay, a colony dispersion assay, and scanning electron microscopy.

Results. Growth factor concentrations were significantly higher in platelet releasate than in serum and were lowest in FFP. Fibronectin and vitamins were found in higher concentrations in serum than in FFP and were lowest in platelet releasate. Cell proliferation was best supported by platelet releasate followed by serum and FFP; however, cell migration and differentiation were better supported by serum than by platelet releasate and FFP. The reduced nutrient capacity of FFP was in part found to be due to an antiproliferative effect of citrate used as an anticoagulant in the production process.

Conclusions. Platelet releasate but not FFP may offer additional potential for the treatment of severe ocular surface disease. Platelet releasate may be suitable as a novel treatment option for ocular surface disease with a superior effect on cell growth. (*Invest Ophthalmol Vis Sci.* 2006;47:2438–2444) DOI:10.1167/iovs.05-0876

A persistent epithelial defect (PED) of the cornea is a serious disorder that can result in a severe impairment of vision and may ultimately lead to corneal blindness. PEDs can be due to toxic and mechanical injuries or infections of the ocular surface, but are more common in patients with severe aqueous tear deficiency (e.g., Sjögren syndrome or lagophthalmus).[1,2] Chronic ulcerations of the cornea are also often caused by neurotrophic disorders seen in patients with diabetes, injuries of the trigeminal nerve,[3] or a variety of autoimmune diseases.[4]

For conventional treatment of a PED, especially in patients with dry eye, the topical application of artificial tear substitutes is most widely used. However, natural tears also offer microbicidal activity and support epithelial proliferation, migration, and differentiation due to their content of proteins, vitamins, and lipids, which are not present in this complexity in pharmaceutical tear substitutes.[5] Another important drawback of artificial tears is that, to ensure a long shelf life, they often contain preservatives, stabilizers, and other additives that potentially induce toxic or allergic reactions.[6,7]

In the past few years, autologous serum has been advocated frequently for topical therapy in patients with ocular surface disorders, and several studies have reported an improvement in healing PEDs.[8–11] Serum is the clear liquid part of full blood that remains after cellular components and clotting proteins have been removed. Eye drops made from autologous serum are thought to be superior to artificial tears in several aspects. First, their pH, osmolality and biomechanical properties are similar to natural tears. Second, they contain essential ocular surface nutrients, such as growth factors, vitamins, and bacteriostatic components such as IgG, lysozyme, and complement. Third, they are free of preservatives.[2,5,12] As mentioned, fresh frozen plasma (FFP) is a colorless, acellular fluid; however, it is distinguished from serum in that FFP still contains clotting proteins of full blood such as fibrin. Platelet concentrates contain platelets at a concentration of choice resuspended in donors' FFP or buffer solution. They contain a variety of growth factors involved in the wound-healing process and are therefore used as a topical agent to accelerate healing of wounds in several tissues.[13,14] If required, the platelet content of growth factors can be released by thrombin stimulation. After removal of the platelet membranes by centrifugation, the cell-free supernatant (platelet releasate) can serve as a preparation for wound-healing therapy.[15–17]

In contrast to serum, FFP, or platelet concentrates are readily available from blood banks as quality-controlled products and are therefore theoretically attractive for topical use in ophthalmology. We hypothesized that FFP or platelets releasate have wound-healing–supporting effects on corneal epithelial cells that may be similar to or better than autologous serum, and we therefore compared these three preparations in a culture model of corneal epithelial cells. Important parts of the wound-healing process are migration and proliferation of corneal epithelial cells to cover the wound and differentiation of these cells into a stable surface epithelium.[18] We measured the concentration of factors considered to be important in corneal epithelial wound healing and tears in the three different blood-derived products and determined the effects of these preparations on growth, migration, and differentiation of immortalized human corneal epithelial cells.[19]

Methods

Preparation of Blood-Derived Products

All blood donations were obtained according to the guidelines of the Scientific Council of the Bundesärztekammer and Paul-Ehrlich Institute

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for blood donation and use of blood products, and the study adhered to the provisions of the Declaration of Helsinki. All individuals were free of disease and were not taking any medication. Serum ($n = 8$), FFP ($n = 5$), and platelet releasate ($n = 8$) were obtained from independent donors to reduce the total sample size per individual to an acceptable volume. The number of FFP samples was limited to five because this blood product reproducibly was found to have inferior epitheliotrophic potential.

Serum. Samples of 200 mL of full blood were obtained from eight healthy volunteers (mean age, 50 ± 4 years) by venipuncture, allowed to clot at room temperature (18–25°C) for 120 minutes, and centrifuged at 3000*g* for 15 minutes. The serum was carefully recovered in a sterile manner and aliquotted.

Fresh Frozen Platelets. Whole blood was obtained from five healthy volunteers (mean age, 59 ± 5 years) and was immediately mixed with the anticoagulant CPDA to a concentration of 10% before it was centrifuged at 3000*g* for 15 minutes. The final CPDA concentration in the undiluted FFP obtained after centrifugation was approximately 18% but obviously depended on the hematocrit. The FFP was withdrawn and aliquotted.

Platelet Releasate. The platelet releasate was prepared from single-donor apheresis platelet concentrates containing approximately 3×10^{11} platelets. Platelet pheresis was performed on eight healthy volunteer blood donors (mean age, 45 ± 9 years) at the blood donation unit of the Institute of Immunology and Transfusion Medicine at the University of Lübeck, using a cell separator (Amicus; Baxter, Deerfield, IL) according to the standard manufacturer's directions with acid citrate dextrose as anticoagulant. On the day of apheresis, the fresh platelet concentrates were washed three times with a washing buffer containing 50 mM HEPES, 10 mM NaCl, 6 mM KCl, 3 mM glucose, and 0.35% human serum albumin. Centrifugation was performed at 800*g* for 15 minutes. After this, the platelets were carefully resuspended in phosphate-buffered saline to a concentration of 4×10^9/mL. Platelet counts were obtained with an automated blood count analyzer (GenS; Beckman-Coulter; Fullerton, CA). Growth factor release was mediated by stimulation of platelets with human thrombin (Sigma-Aldrich) at a concentration of 1 U/mL. After 20 minutes, the solution was centrifuged at 3500*g* for 15 minutes to remove all platelet remnants.

All aliquots were stored at −70°C until the day of analysis or experiment, when they were thawed and diluted with isotonic saline to 50%, 25%, 12%, 6%, or 3% concentration.

Quantification of Epitheliotrophic Factors

Epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), platelet-derived growth factor-AB (PDGF-AB), and fibronectin were quantified in undiluted serum, FFP, and platelet releasates by means of enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. ELISA kits for human EGF and PDGF-AB were from R&D Systems Inc. (Minneapolis, MN), for human TGF-β1 from Bender MedSystems Diagnostics GmbH (Vienna, Austria), and for human fibronectin from Chemicon International Inc. (Temecula, CA). The concentrations of vitamin A and E in the samples and controls (Chromsystems, Instruments & Chemicals GmbH, Munich, Germany) were quantified by means of reversed-phase high performance liquid chromatography (HPLC) with detection by UV absorbance. Calcium concentrations of $n = 4$ samples of platelet releasate, serum and FFP each were obtained using the photometric method from Abbott (Wiesbaden, Germany) performed on the clinical chemistry analyzer (Aeroset; Abbott).

Cell Culture Models

Human SV-40 Immortalized Corneal Epithelial Cells. The cells (RCB1384, HCE-T; Riken Cell Bank, Ibaraki, Japan) were cultured at 5% CO_2 at 37°C in an equal mixture of Ham's F12 (Biochrom AG, Berlin, Germany) and MEM (Invitrogen-Gibco, Grand Island, NY) supplemented with 25 mM HEPES buffer, 5% fetal bovine serum (FBS), 10 ng/mL EGF, 5 μg/mL insulin, 0.1 μg/mL cholera toxin, 100 IU/mL penicillin, and 100 μg/mL streptomycin. After confluence, cells were passaged with 5 mg/mL trypsin-EDTA (Biochrom AG) and 5 mg/mL soybean trypsin inhibitor. All supplements were purchased from Sigma-Aldrich except HEPES buffer and FBS, which were from Invitrogen-Gibco.

Primary Rabbit Corneal Epithelial (RCE) Cells. One male adult New Zealand rabbit was provided by the animal center of the University of Lübeck. The animal was handled according to the guidelines described in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbit was killed by intravenous injection of pentobarbital sodium solution (100 mg/kg), and the globes were enucleated. Both corneas were excised including the corneal rim and washed three times with 100 μg/mL streptomycin and 100 IU/mL penicillin. Each cornea was cut into four sections and incubated at 37°C for 2 hours in 2 mg/mL Dispase II (Roche, Mannheim, Germany) dissolved in DMEM containing 10% serum. The epithelium was stripped off with gentle scraping from the limbus to the center into the well of a six-well-plate containing 3 mL PBS. The solution was pipetted to disperse cells and centrifuged at 100*g* for 5 minutes. PBS was carefully removed, and the cells were suspended in 5 mL keratinocyte-serum-free medium (KSFM; Invitrogen-Gibco) supplemented with 5 ng/mL EGF, 50 μg/mL BPE, 100 IU/mL penicillin, and 100 μg/mL streptomycin and seeded in a 25-cm^2 cell-culture flask. The cells were cultured at 37°C in 5% CO_2 until confluent and expanded by using routine cell culture techniques.

Endpoint Assays

The effect of the three blood preparations on cell proliferation, migration and differentiation were tested in dose- and time-response experiments. Proliferation was determined with a luminescence-based luciferin-luciferase ATP-assay,[20] migration with a colony dispersion assay,[21] and differentiation with scanning electron microscopy. All assays were performed in triplicate.

The dose–response ATP assay was performed after incubation of HCE-T cells with serum, FFP, and platelet releasate diluted from 100% to 50%, 25%, 12%, 6%, and 3% over 24 hours. In addition, primary rabbit corneal epithelial cells were exposed to platelet releasate from two donors for 24 hours, and their relative cell growth was compared. Because FFP did not support cellular ATP levels in the dose–response experiments, an ATP time–response assay was performed only for serum and platelet releasate at a concentration of 20% over 2, 12, 24, 48, 72 and 96 hours. This concentration is commonly used in clinical practice and, from the dose–response experiment, was found to be near the maximum of cell growth support. For all ATP-assays, 3000 cells were seeded per well in 96-well culture plates (Falcon, Plymouth, UK) and cultured until approximately 30% confluent. Before exposure to the test substances, the culture medium was changed to a non–growth-supporting medium (defined [D]KSFM containing 1% serum albumin, 100 IU/mL penicillin, and 100 μg/mL streptomycin for 24 hours, but no growth factors, serum, or tissue extracts). The cells were then washed twice with PBS and exposed to 200 μL test substances. On each culture plate cells were also exposed in separate wells to DKSFM with growth factor supplement as a positive control for maximum proliferation or to 1% benzalkonium chloride (BAC; Haltermann Ltd., Workington, UK) as a negative control (no growth support). After incubation, the test substances were removed, and all wells washed with PBS once before cellular ATP was extracted by adding 200 μL PBS and 50 μL cell extraction reagent to each well with a multichannel pipette. The cells were left at least 20 minutes at room temperature before 25 μL of culture extract was transferred and 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). The resultant luminescence was read immediately using a luminometer (FLUOstar Optima; BMG Labtech GmbH, Offenburg, Germany). The ATP assay reagents, including extraction buffer and luciferin-luciferase, were obtained from DCS Innovative Diagnostic-System (Hamburg, Germany). The luminescence intensity is propor-

tional to the amount of ATP of cells. ATP is a marker of cell viability and presents in all metabolically active cells. It correlates with cell proliferation and can therefore be used as a marker for cell growth. The percentage of cell growth (CG) for each drug and test situation was calculated.

$$\frac{\text{Test}-\text{MI}}{\text{MO}-\text{MI}} \times 100 = \%\text{CG},$$

where MO is mean counts for no inhibition control cultures, MI is mean counts for maximum inhibition control cultures, and Test is mean counts for triplicate test situations. To investigate the influence of the anticoagulant CPDA present in the FFP samples on cell growth, HCE-T cells were incubated with the DKSFM medium with increasing concentrations (0.5%, 1%, 2%, 4%, 9%, and 18%) of the anticoagulant ($n = 3$). As controls, the same medium with equivalent concentrations of distilled water instead of CPDA was used. After 24, 48, and 72 hours, ATP content was determined by the ATP assay as just described.

For the colony-dispersion assay, HCE-T cells were seeded and cultured in cloning rings (flexiPERM micro 12, Vivascience; Sartorius AG, Göttingen, Germany) to confluence with 1:1 Ham's F12-MEM on 0.01% acid-extracted rat tail collagen I–coated plates (Sigma-Aldrich). The cells were cultured for a further 24 hours in the presence of 200 μM hydroxyurea (Sigma-Aldrich) to induce growth arrest[22] and then were starved for 24 hours with the non–growth-supporting medium. After removal of the rings, the cells were thoroughly washed with PBS and incubated with 25% serum diluted with saline, 65% platelet releasate diluted with DKSFM without growth factors, or 25% FFP in saline for 24, 48, 96, or 144 hours. These concentrations were chosen based on the results of the proliferation assay, because cell growth was best supported with serum or FFP at around 12% to 25%, but platelet releasate as an undiluted solution. Because the cells were cultured for up to 144 hours, the platelets were diluted with serum-free medium to provide basic nutrient factors. The cells were washed with PBS three times, fixed with 90% (vol/vol) methanol and stained with Mayer's hematoxylin. The colony dispersion areas were photographed with a digital camera (Sony Corp., Tokyo, Japan) under standardized conditions and the areas were measured in pixels with image-analysis software (Uthesca Image Tool, Version 2.00, The University of Texas Health Science Center, San Antonio, TX; http://ddsdx.uthscsa.edu/dig/download.html). The areas of the cell colony after test exposure were compared to areas at time 0 hour.

For scanning electron microscopy (SEM), 10^4 cells were seeded on plastic cell culture inserts (Thermanox; Nalge Nunc Intenational, Rochester, NY) and incubated for 48 hours with the three different blood preparations at the approximate concentration found to yield maximum cell growth (20% serum, 20% FFP, 100% platelet releasate). After washing with PBS, specimens were fixed in Monti-Graziadei fixative (2.5% glutaraldehyde, 0.5% paraformaldehyde, 0.1 M Na-cacodylate buffer [pH 7.4]), dehydrated through ascending alcohol concentrations, critical point dried, mounted, and sputter-coated with platin-palladium before examination with a scanning electron microscope (SEM 505; Phillips Inc., Eindhoven, The Netherlands). Images were then generated (APX 100 film; Agfa-Gevaert AG, Leverkusen, Germany). The surface morphology of the cells was evaluated by two independent examiners.

Data Evaluation and Statistical Methods

Statistical analysis was performed with unpaired two-sided *t*-tests and the analysis of variance (ANOVA) on computer (SPSS for Windows, ver. 1.0.1; SPSS, Chicago, IL). $P \leq 0.05$ was considered statistically significant.

Results

Quantification of Epitheliotrophic Factors

The mean ± SD concentrations of epitheliotrophic factors in the three blood preparations are given in Table 1. Platelet releasate contained significantly more EGF, TGF-β1, and PDGF than serum, which contained significantly more than FFP ($P < 0.0001$). However, the highest concentrations of fibronectin, vitamin E, and vitamin A were found in the serum ($P < 0.0001$). Calcium concentrations of the three blood products ($n = 4$) were 2.27 ± 0.12 mM (SD) for serum, 1.87 ± 0.04 mM for FFP, and 0.43 ± 0.19 mM for platelet releasate. The differences between the three groups were statistically significant ($P < 0.05$).

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 6% ± 4%. Figure 1A shows the results of relative ATP bioluminescence in cells after incubation for 24 hours with serum or platelet releasate with different dilutions. Cell growth was best supported with serum diluted to 12%. A more linear positive correlation of ATP and test substance concentration resulted when the cells were incubated with platelet releasate, which supported cell growth best at 100%. Undiluted platelet releasate supported cell proliferation better than serum at any concentration. Primary rabbit corneal epithelial cells and HCE-T cells showed similar dose–response pattern after exposure to platelet releasate. FFP also stimulated cell growth best when diluted to 12%, but the supporting effect was much lower than serum and testing was therefore limited to five donor samples. In the time-response assay platelet releasate was found to support ATP-levels significantly ($P < 0.05$) better than serum for up to 12 hours (Fig. 1B). The incubation for up to 72 hours with different dilutions of CPDA in culture medium reproducibly showed toxic effects of the anticoagulant at concentrations of more than 2% of CPDA (Fig. 1C). The negative effects on cell growth were detectable at 24 hours' incubation and became stronger with longer incubation times. Control cultures incubated with culture medium containing 18% of distilled water instead of an equal volume of CPDA showed only a slight decrease of cell growth after 72 hours. This is likely to be due to the dilution of the culture medium with water (Fig. 1D).

Table 1. Concentration of Epitheliotrophic Factors in Different Blood Preparations

Blood Preparations	EGF (ng/mL)	TGF-β1 (ng/mL)	PDGF (ng/mL)	Fibronectin (ng/mL)	Vitamin A (nmol/mL)	Vitamin E (nmol/mL)
Serum	0.82 ± 0.46*	45.84 ± 3.24*	15.58 ± 5.98*	455.09 ± 65.64*†	3.52 ± 1.02*†	35.58 ± 8.18†
FFP	0.01 ± 0.01	6.26 ± 1.89	1.37 ± 1.27	128.06 ± 66.64	2.35 ± 0.39	29.22 ± 3.23
Platelet releasate	1.55 ± 0.3†	287.6 ± 132.36†	86.04 ± 22.6†	6.32 ± 1.83	0.06 ± 0.04	0.46 ± 0.18

The higher values are marked for significance.

* Significant difference between serum and FFP ($P < 0.05$).

† Significant difference between serum and platelet releasate ($P < 0.05$).

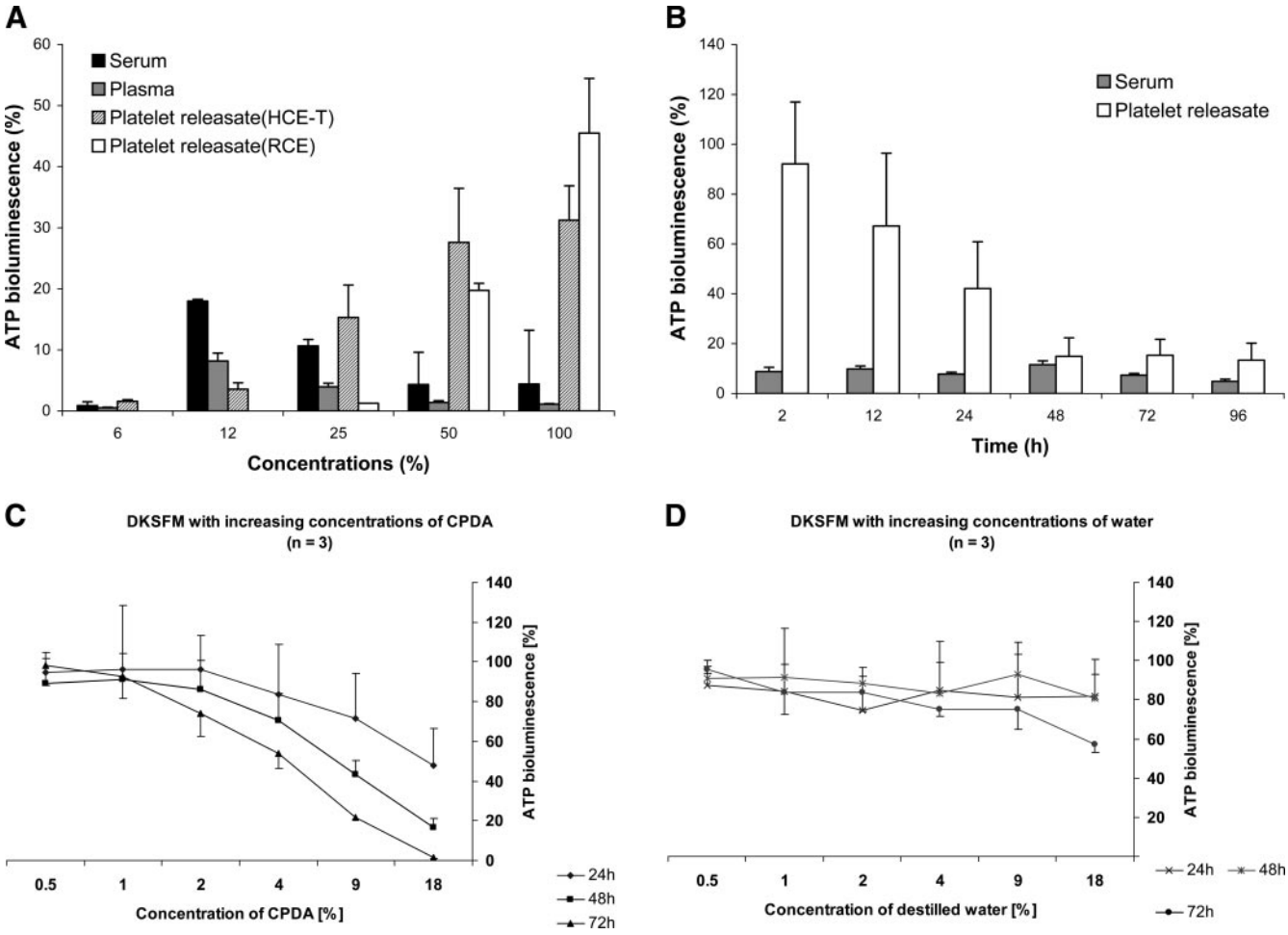


FIGURE 1. (**A**–**D**) Dose–response of ATP content of corneal epithelial cells incubated 24 hours with various blood preparations. Error bars, SE. (**A**) Cell growth was better supported with undiluted platelet releasate than with serum at any concentration when tested on HCE-T cells. The proliferative response of primary rabbit corneal epithelial cells (RCE) was stronger than that of immortalized human corneal epithelial cell line cells (HCE-T) when incubated with platelet releasate. (**B**) Time response of mean relative ATP content with SE of cells incubated with 20% of serum or platelet releasate. Cell growth was significantly better supported for up to 12 hours with platelet releasate than with serum. (**C**) Incubation of HCE-T cells with medium containing increasing concentrations of the anticoagulant CPDA used for generation of FFP indicated toxic effects after 24 hours and concentrations of >2% of the anticoagulant. (**D**) Medium diluted with distilled water served as control. A slight growth inhibition was detectable but only at concentrations of 18%, which is likely to be due to the dilution of the medium.

Migration

Support of cell migration was investigated for 25% serum and 25% FFP, because these products were found to support cell growth best when diluted. With platelet releasate support of cellular ATP-levels was best if the blood product was not diluted. However, because migration was tested in a long-term assay (144 hours), we decided that additional basic nutrient factors were required. We therefore diluted platelet releasate with serum-free culture medium to a concentration of 65%. HCE-T cells migrated clearly better when incubated with serum than with FFP. Cells incubated with 25% FFP did not migrate within the first 48 hours of incubation, and became completely detached from the surface of the culture plate as a sign of cell death at 96 and 144 hours (Fig. 2). The 65% platelet releasate also supported cell migration, but its effect was slightly inferior compared to serum at the later time-points of the experiments although this was not statistically significant.

Differentiation

After incubation of HCE-T cells with various blood preparations for 48 hours, cellular differentiation patterns were assessed by SEM (Fig. 3). Cells incubated with 20% serum formed a coherent monolayer of flattened and polygonal shaped cells ("fried-egg" appearance) equipped with densely and homogeneously distributed microvilli, reflecting typical features of fully differentiated corneal epithelial cells (Fig. 3A). Treatment with 20% FFP led to a similar morphologic picture. However, the amount of microvilli was slightly decreased, and the cellular monolayer was less coherent, leaving small gaps between adjacent cells (Fig. 3B). In contrast, exposure of the cell culture to platelet releasate did not result in a homogeneous monolayer. Most cells changed from a polygonal to an ovoid shape and formed numerous cytoplasmic processes bare of microvilli which radiated from the cell bodies to contact adjacent cells (Fig. 3C). Generally, their degree of differentiation was inferior to cells treated either with serum or FFP.

DISCUSSION

We investigated the effect of three blood preparations—serum, FFP, and platelet releasate—as potential therapeutic agents for topical use in patients with ocular surface disease such as PED

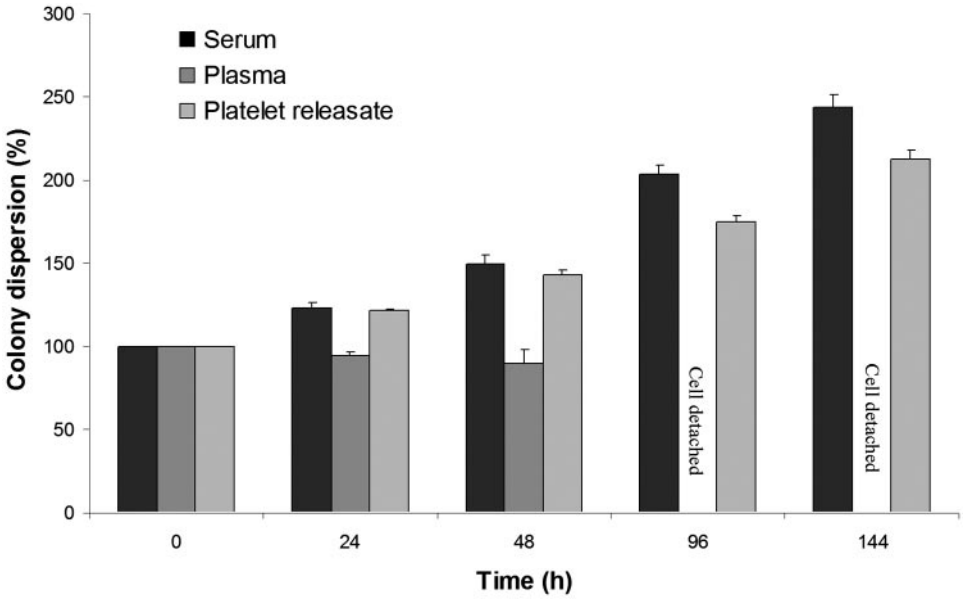


FIGURE 2. Colony-dispersion-assay of HCE-T cells incubated with 25% serum, 25% FFP, or 65% platelet releasate. Exposure time is given on the *x*-axis and the relative area of colony on the *y*-axis. Error bars, SE. Cells incubated with serum migrate faster than with platelet releasate or FFP.

or severe dry eye. Clinically, only serum is currently used. However, it needs to be prepared in a time- and labor-intensive way from an autologous blood donation for each patient individually.[8–11] Heterologous FFP and platelet concentrates have the advantage that they can be obtained as quality-controlled routine blood products from blood banks where a supply is kept for intravenous transfusion.[23] Platelets are a source of a variety of growth factors with important functions in the wound-healing process of many tissues.[24–28] Therefore, they may also be used locally for improvement of wound healing. Because for ocular surface disorders eye drops have to be applied daily usually for weeks, and while platelet concentrates cannot be stored for more than a few days, we used a preparation in which the growth factors stored in platelets are released into the supernatant by stimulation with thrombin. The resulting aggregates of platelet membranes, which are thought to induce apoptotic cell death, are removed by centrifugation.[29] The supernatant termed "platelet releasate" contains the various platelet-derived growth factors. It is a clear, transparent liquid suitable for use as eye drops and can be cryopreserved for months.

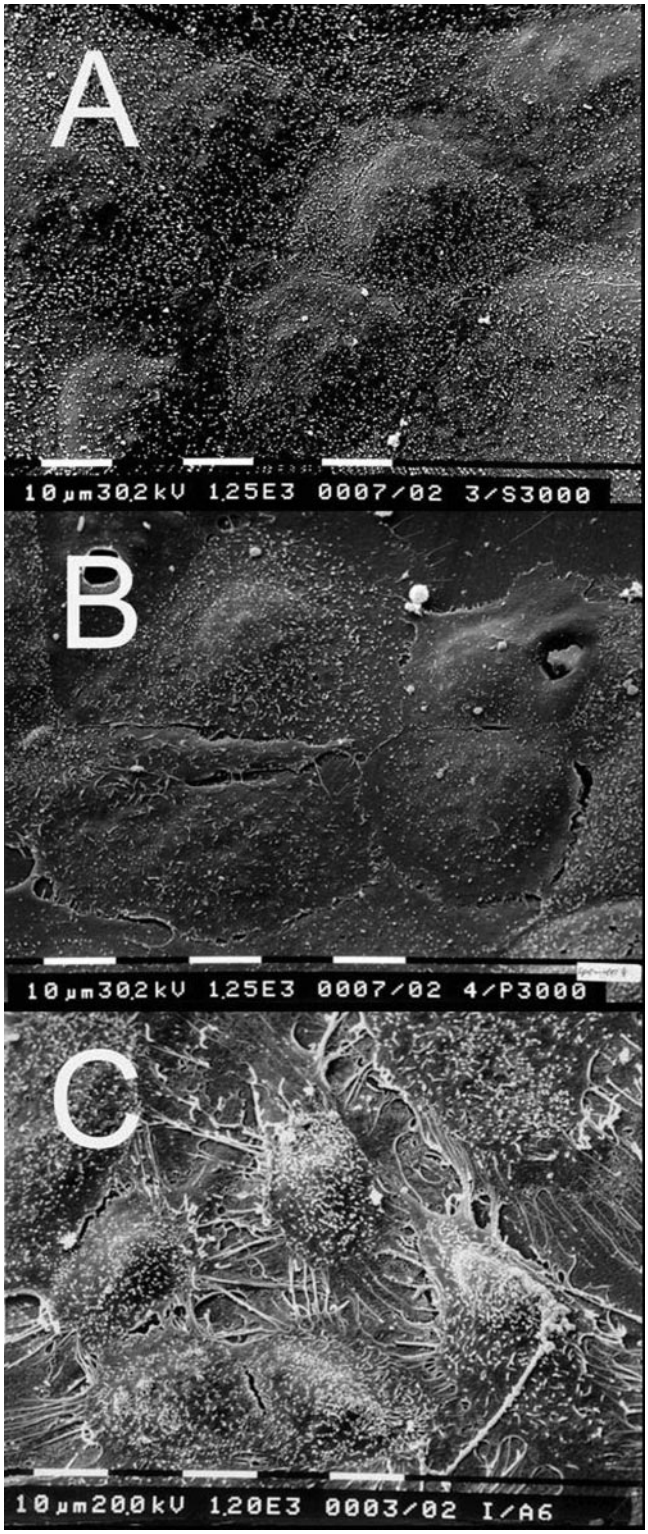
To determine the epitheliotrophic capacity of serum, FFP, or platelet releasate on corneal epithelium, a culture model of primary rabbit and immortalized human corneal epithelial (HCE-T) cells was used to investigate the influence of these blood products on cell growth, migration, and differentiation.[5,19] The type and concentration of the blood products tested was predominantly guided by clinical considerations as to which product would be easily available in the routine clinical setting.

Platelet releasate was clearly better than serum or FFP in stimulating the metabolic activity of immortalized human corneal epithelial cells, which is thought to represent a parameter of cell proliferation.[30] The results of the serum experiments confirmed previously published data.[31] Platelet releasate was tested for the first time. Repeat experiments using primary rabbit epithelial cells as a culture model, however, confirmed the pattern of response. Cell migration and differentiation were slightly better supported with serum, whereas FFP reduced cell viability and migration.

The concentrations of a variety of cytokines, fibronectin, and vitamins, which are important factors that influence corneal wound healing, were found to be distinctively different among the three blood preparations and the different effects of the blood products in the in vitro system may be, at least partially, explained by these biochemical differences. The substantially higher concentrations of EGF, PDGF, and FGF in platelet releasate may be the reason for its superior effect on the growth of corneal epithelial cells compared with serum and FFP. In contrast, FFP, which is obtained from anticoagulated whole blood, where intact platelets are removed from the liquid phase before they can release any growth factor, showed little stimulation of cell growth. The ATP bioluminescence showed a linear increase with the concentration of platelet releasate for 24 hours' incubation, but decreased during a long-term experiment. The latter observation is likely to be due to a loss of activity of growth factors over a 96-hour incubation at 37°C. At 24 hours, serum and FFP best supported metabolic cellular activity at 12%. The decrease of cell growth with increasing serum concentration may be due to serum derived inhibitory factors, which are removed from platelet releasate during the repeated washes with PBS; but this is an untested hypothesis.

For cell migration, however, serum was superior to platelet releasate, although the first was diluted with saline and the latter with basic culture medium. From the ATP assay results, an undiluted platelet releasate would preferably have been tested in the migration assay. However, the rational for diluting platelet releasate with a non–growth-factor–supplemented culture medium was to provide some basic nutrients during long-term incubation. This may direct reduce comparability for platelet releasate with FFP and serum diluted with saline to a lower concentration but was the best way to allow assessment of migration over 144 hours with near optimum concentrations of the blood products. The dimension of the difference observed in this assay between serum and platelet releasate indicates that this is not a result of comparing a 25% with a 65% solution, but that despite an increased concentration of platelet releasate this was unable to support cell viability in the long term. The superior effect of serum may be attributed to the significantly higher concentration of fibronectin in serum, which was almost completely absent in platelet releasate. Fibronectin is a glycoprotein that supports cell adhesion and is an important mediator of cell migration.[32] FFP showed no migration within the first 48 hours of the experiments. After 96 hours, all cells were necrotic and detached from the surface of the culture plate. This fatal effect of FFP on corneal epithelial cells after an extended incubation time could be due to toxic

substances potentially only present in the FFP, such as the anticoagulant CPDA. Indeed, subsequently performed experiments investigating the effect of CPDA clearly indicated a toxic effect even at concentrations found in diluted FFP. The toxicity of CPDA may be due to the generation of toxic metabolites from citrate by the cells. In addition, the reason for the cell death after incubation with FFP could be the lack of growth factors in this blood product, which may be essential for long term survival of corneal epithelial cells.



Morphologic criteria for cell differentiation, such as a coherent monolayer of flattened and tightly connected epithelial cells equipped with microvilli, were assessed by SEM after long-term incubation with the three different blood products. In contrast to serum and FFP, exposure to platelet releasate was judged to result in a lesser degree of differentiation. Platelet releasate induced a heterogeneous cellular surface morphology characterized by ramifying dendritic processes and a loss of continuous intercellular contacts. These findings most likely reflect a state of enhanced proliferation with a higher cellular dynamic and turnover and are in accordance with the results of the ATP assay where the highest proliferation rate was found after treatment with platelet releasate. The calcium concentration of platelet releasate was approximately five times lower than in serum or FFP. As calcium is an important factor for cell differentiation, the low calcium content of this blood product may substantially contribute to its inferior capacity to support cell differentiation. In addition, vitamin A and vitamin E are thought to be essential factors for cell differentiation and survival.[33,34] Both vitamins are virtually absent in platelet releasate.

The slightly better support of cell differentiation induced by serum than by FFP may be due to its higher content of vitamins and slightly higher calcium concentrations, factors that are thought to promote the differentiation of corneal epithelial cells.[1,35] In addition, as mentioned earlier, the anticoagulant CPDA in FFP had toxic effects on the cells.

The more differentiated character of epithelial cells after serum incubation seems to be contradictory to the finding that this blood product induces a relatively high migratory activity. However, FFP was shown to have toxic effects on the HCE-T cells, and platelet releasate contained almost no fibronectin, an important factor in cell migration. This may explain why serum was the best of the three blood products to support migration.

Certainly, the complex physical and molecular interactions of the tear film and ocular surface in vivo cannot be replicated by cell culture models in vitro. Cell culture models may be more susceptible to toxicity because, for example, they cannot provide neural pathways that are essential for long-term epithelial integrity in vivo. However, one can also argue that the indications for the use of serum are ocular surface diseases, which certainly equally increases the susceptibility of epithelial cells in vivo. Well-characterized human cell lines that retain the characteristic features of the original tissue can be used for reproducible and comprehensive toxicity testing.[36,37] The HCE-T cell line was immortalized using SV-40-adenovirus vector and shows the properties of normal corneal epithelial cells. It exhibits well-developed desmosomes and abundant microvilli and expresses cornea-specific cytokeratin[19] and this phenotype was judged to be sufficient stable to allow reproducible toxicity testing.[38] HCE-T cells also showed a similar proliferative response to platelet releasate as primary rabbit corneal epithelial cells, and we previously could show that human corneal epithelial cell line cells (ATCC11515) respond

FIGURE 3. Scanning electron microscopy of HCE-T cells incubated for 48 hours with 20% serum (**A**), 20% FFP (**B**) or undiluted platelet releasate (**C**). (**A**) Cells incubated with 20% serum formed a coherent monolayer of flattened, polygonal shaped cells with upright microvilli homogeneously and densely distributed at the cellular surfaces. (**B**) Cells exposed to 20% FFP also formed microvilli and microcristae on their surfaces, however, of reduced density in comparison to the serum treated group. The cellular monolayer was slightly less confluent and exhibited small clefts between adjacent cells. (**C**) Cells incubated with platelet releasate showed a less distinctive degree of differentiation. Although they were also equipped with microvilli, their surface morphology was unhomogeneous. Instead of a flattened coherent monolayer, most cells formed multiple cytoplasmic processes bare of microvilli which extended from the cell body and interdigitated with those from adjacent cells. Original magnification, ×1250.

equally sensitive in the ATP-assay as primary human corneal epithelial cells.[5] Therefore, we believe that the culture model used here allows for some extrapolation to the clinical situation.

In conclusion, platelet releasate but not FFP may be a promising additional treatment modality for severe ocular surface disease, especially if stimulation of cell proliferation is required. This possibility should be evaluated in a controlled clinical trial.

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