Assessment of platelet growth factors in supernatants from rehydrated freeze-dried equine platelets and their effects on fibroblasts in vitro

Fern Tablin, VMD, PhD; Naomi J. Walker, BA; Sara E. Hogle, DVM; Suzanne M. Pratt, DVM; Jeffrey W. Norris, PhD

Objective—To determine whether platelet growth factors are preserved in supernatants obtained from rehydrated trehalose-stabilized, freeze-dried (lyophilized) equine platelets and whether those growth factors stimulate fibroblast proliferation and migration and enhance fibroblast-associated contraction in a collagen gel assay.

Animals—6 clinically normal adult horses.

Procedures—Blood samples were obtained from 6 horses, and washed platelets were prepared via differential centrifugation. Washed platelets were freeze-dried in a physiologic buffer with a mixture of trehalose and polyethylene glycol 4000. Rehydrated platelet supernatants and releasates prepared from fresh washed platelets stimulated with thrombin or platelet-activating factor were evaluated for transforming growth factor β1 and platelet-derived growth factor BB by use of ELISAs. Effects of rehydrated freeze-dried platelet supernatants on fibroblast proliferation, migration, and collagen gel contraction were compared with effects of 1%, 2.5%, or 10% fetal bovine serum (FBS).

Results—Supernatants from freeze-dried platelets contained similar amounts of growth factors as thrombin- and platelet-activating factor–stimulated platelet releasates. The supernatants significantly enhanced fibroblast proliferation and migration in a scratch assay, compared with FBS-free control or low (1%) FBS conditions. Additionally, supernatants from freeze-dried platelets enhanced contraction of fibroblast-seeded collagen gels, compared with the effect of 1% FBS.

Conclusions and Clinical Relevance—The preparation technique preserved platelet growth factors, enhanced fibroblast proliferation and migration, and improved fibroblast-seeded collagen gel contraction under conditions of low FBS concentration; these platelet supernatant preparations may prove useful as an aid to conventional wound management. (Am J Vet Res 2008;69:1512–1519)

Wounds on the distal portion of limbs of horses are a therapeutic challenge, both with regard to the time required for complete healing as well as long-term expense to the horse owners. These wounds frequently heal by second intention; compared with other species, these wounds typically have a slower rate of healing and have a propensity to develop exuberant granulation tissue that results in subsequent delays in epithelialization and contraction.

Because of the limited vasculature and excessive skin tension in the distal portion of equine limbs, these lesions often become chronic nonhealing wounds, which have been equated with chronic nonhealing wounds in humans with diabetes mellitus. Numerous studies have been conducted to evaluate different treatment methods for limb wounds in horses, including topical administration of growth factors, cytokines, and other biological agents, yet healing times remain prolonged.

Typically, wound healing is initiated by the traumatic event that results in tissue injury. The vascular response to the injury is hemostasis; the coagulation cascade is initiated and then thrombin is produced, which results in the conversion of fibrinogen to fibrin.

Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
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<tr>
<td>PAF</td>
<td>Platelet-activating factor</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>TGF</td>
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Fibrin forms a provisional matrix for incoming inflammatory cells and fibroblasts as well as the presentation of growth factors to endothelial cells and fibroblasts. Platelet α granules contain a wide variety of growth factors and matrix proteins, which participate in all phases of wound healing. During hemostasis, platelets are activated; platelet granular contents are subsequently released into the wound and mediate the initial wound healing events. Platelet mediators, largely present within dense granules, have a role in vascular reactions as well as roles in leukocyte chemotaxis and stimulation. Growth factors in platelet α granules include (but are not limited to) PDGF, TGF-β, basic fibroblast growth factor, and epidermal growth factor. These granules also contain extracellular matrix molecules, including fibronectin, thrombospondin, and fibrinogen.13

The role of platelet-derived mediators in inflammation and tissue repair is well characterized, and an adequate inflammatory response is a prerequisite for rapid and effective healing.3 It has been suggested that horses have an initially weak inflammatory response, compared with that of ponies, and that this contributes to a prolonged inflammatory state.1,12-14

The use of autologous platelet gels for improvement of wound healing in humans has recently received considerable attention.13,16 Findings of a study17 of wound healing in horses suggested that treatment with a fresh autologous platelet gel accelerated epithelial differentiation and resulted in development of tissue with greater collagen organization (reflective of more mature granulation tissue). We have previously developed methods to store intact cells in a dry state by use of various substances, including the sugar trehalose.18

The purpose of the study reported here was to determine whether platelet growth factors are preserved in supernatants obtained after rehydration of washed, trehalose-stabilized, freeze-dried (lyophilized) equine platelets and whether those growth factors are able to support both fibroblast proliferation and migration in vitro as well as enhance fibroblast-associated collagen gel contraction.

Materials and Methods

Sample preparation—Blood (60 mL) was collected in acid citrate dextrose from each of 6 healthy horses, according to approved institutional protocols. Plateletrich plasma was prepared via differential centrifugation; platelets were washed twice in a Tyrode's HEPES buffer (pH, 7.2)19 and resuspended in an iso-osmotic mixture (pH, 7.2) of Tyrode's HEPES, trehalose, and polyethylene glycol 4000 with apyrase (0.1 U/mL) to a final concentration of 3 to 4 × 10⁸ platelets/mL.

Freeze-drying—Platelets (3 to 4 × 10⁹/mL) were not treated with agonist and were freeze-dried by use of a commercial freeze-drier.5 After freeze-drying, vials containing the platelets were flushed with dry nitrogen gas and stored in the dark prior to use. Samples were sealed in a sterile manner with gas permeable membranes5 prior to lyophilization (freeze-drying). For use in the assays, lyophilized platelets were rehydrated with 1 mL of sterile water and pelleted; the supernatants were then frozen. Supernatants from lyophilized platelets, which contained released and preserved granule contents, were used in all experiments.

Growth factor measurements—Transforming growth factor-β1 and PDGF-BB were evaluated by use of 2 ELISAs, according to the manufacturer's instructions. Both of these ELISAs have previously been validated for the measurement of equine growth factors.20,21 All samples for TGF-β1 analysis were acid-activated with 1N hydrochloric acid. Briefly, washed fresh platelets (3 to 4 × 10⁶/mL; same concentration as the freeze-dried platelets) were used without activation or were activated either with thrombin (1 U/mL) or PAF (1 × 10⁻⁹M). Washed platelets were pelleted, and the releasates were frozen at –80°C. Equine serum was prepared by allowing whole blood samples (10 mL) to clot in a glass tube in the absence of anticoagulants. Serum served as positive controls for ELISAs. Lyophilized platelets were rehydrated, as described, and the supernatants were used in the assay. For all conditions evaluated, 50 µL of supernatant was used in both types of ELISAs. Growth factors were measured in duplicate for each sample. Samples were evaluated for all 6 horses under all conditions.

Cell culture—For all in vitro cell culture studies, equine dermal fibroblasts (CCL-57) were grown in MEM with Earle's salts (115 mM NaCl, 0.5 mM KCl, 2 mM NaHCO₃, 0.3 mM NaH₂PO₄, 0.2 mM CaCl₂, and 0.8 mM MgSO₄) and supplemented 1 mM sodium pyruvate and 2 mM L-glutamine in 10% FBS. Cell cultures were split at confluence. Cells were used for assays through passage 30.

Cell proliferation assay—Ninety-six–well plates were prepared, in which each well contained 100 µL of medium with 1%, 2.5%, or 10% FBS and 10 µL of each rehydrated freeze-dried platelet supernatant or rehydrated freeze-dried buffer (negative control condition) that had been filtered through a 0.22-µm-pore filter to remove cell membrane fragments. Two initial cell populations were evaluated: 500 cells/well or 5,000 cells/well, and cell proliferation was monitored for 4 days. Proliferation was evaluated by use of a cell proliferation assay3; the assay relies on the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonylphenyl)-2H-tetrazolium, which results in the formation of formazan that is detected colorimetrically as absorbance on a microtiter-plate reader. Data were collected at 48, 72, and 96 hours after plating. Each assay was run in quadruplicate with platelets from each of 4 of the 6 study horses. Specimens from 2 of the 6 horses included in the cell migration and collagen gel contraction experiments were not available for this portion of the study. The effects of increasing concentrations of FBS were evaluated in quadruplicate.

Collagen gel contraction assays—Collagen gels were produced in medium and seeded with fibroblasts in 24-well plates.22 Briefly, type I rat tail collagen (0.75%) and 3 × 10⁶ fibroblasts/mL were cultured in MEM with either 1% or 10% FBS. Five hundred microliters of gel (0.75% collagen) was plated in each well, allowed to polymerize for 1 hour, and then released from the sides and bottom of the wells via rimming with a
sterile spatula. Each individual experimental culture received 500 µL of 2X MEM with 2% FBS and 300 µL of rehydrated freeze-dried platelet supernatants (final volume, 1 mL; n = 9), such that the final concentration in each experimental culture was 1% FBS. Individual control cultures received a total volume of 1 mL of medium and either 1% or 10% FBS (n = 6 and 5, respectively). Collagen gels were measured initially (day 0) and then incubated at 37°C for 15 days and photographed daily for analysis. The percentage shrinkage of gels relative to the initial diameter and both control diameters were determined.

Cell migration assay—Fibroblasts were plated at a concentration of 2 × 10^4/mL in 60-mm dishes (5 mL/ dish), and confluent cultures were established prior to wound simulation (day 1). Plates were rinsed with PBS solution and disrupted by dragging the rounded end of a sterile disposable spatula across the plate, creating a streak approximately 1 mm wide. Plates were rinsed twice in PBS solution, and 3 mL of medium with 1% or 2.5% FBS was added to each dish. Three experimental conditions were evaluated: the addition of 2 mL of medium, the addition of freeze-drying buffer in a final volume of 2 mL of medium, and the addition of lyophilized platelet supernatants in 2 mL of medium. All conditions had a total of 5 mL of medium. Phase-contrast photomicrographs of all dishes were obtained daily; cellular migration in the lesioned area was evaluated during a 4-day period (ie, on days 1, 2, 3, and 4).

Statistical analysis—Data were analyzed by use of either t tests or ANOVA. A value of P ≤ 0.05 was considered significant. Results are presented as mean ± SEM.

Results

Assessment of growth factors in lyophilized trehalose-polyethylene glycol–stabilized platelet supernatants—For each assay, samples of rehydrated freeze-dried platelet supernatants were compared with releasates from fresh platelets activated by thrombin or PAF and equine serum. On the basis of ELISA results, resting fresh platelet supernatants (negative control samples) had no discernable amounts of TGF-β1 or PDGF-BB, suggesting that no α granule contents were released in the starting populations (data not shown).

Platelet releasates and supernatants were analyzed for TGF-β1. The mean ± SEM concentration of TGF-β1 in supernatants from rehydrated freeze-dried platelets was 1,719.0 ± 227.0 pg of TGF-β1/mL, which was slightly more than the value in thrombin-treated fresh platelet releasates (1,514.2 ± 149.2 pg/mL) and slightly less than the value in PAF-treated fresh platelet releasates (2,075.1 ± 132.6 pg/mL). The TGF-β1 concentrations in the agonist-activated platelet releasates did not differ significantly, and neither value differed significantly from the concentration in the rehydrated freeze-dried platelet supernatants. The concentration of TGF-β1 in equine serum (2,973.8 ± 269.9 pg/mL) was significantly (P ≤ 0.05) greater than the value in any of the other experimental conditions.

Evaluation of PDGF-BB concentration in the experimental groups revealed a similar degree of α granule preservation. However, the rehydrated freeze-dried platelet supernatants contained significantly (P < 0.001) less PDGF-BB than the releasates of thrombin-treated fresh platelets (868.8 ± 69.6 pg/mL vs 1,851.5 ± 121.8 pg/mL). There was no significant difference in concentration of PDGF-BB between the rehydrated freeze-dried platelet supernatants and PAF-treated fresh platelet releasates (948.7 ± 99.9 pg/mL). Similarly, there was no significant difference in concentration of PDGF-BB between the rehydrated freeze-dried platelet supernatants and equine serum (1,324.4 ± 210.1 pg/mL).

Effects of rehydrated freeze-dried platelet supernatants on cellular proliferation—The effects of rehydrated freeze-dried platelet supernatants on cultured fibroblast proliferation were evaluated. At 48 hours, rehydrated freeze-dried platelet supernatants from 3 of the 4 horses significantly (P < 0.01) increased fibroblast proliferation, compared with the effects of the negative control conditions (rehydrated freeze-dried buffer included instead of supernatant), in cultures grown in medium containing 1% FBS. By 72 hours, all rehydrated freeze-dried platelet supernatants had significantly (P < 0.01) increased proliferation, compared with the effects of the negative control conditions, in cultures grown in medium supplemented with 1%, 2%, 5%, or 10% FBS. The same was true at 96 hours after plating, which indicated that the growth factors preserved by the freeze-drying process had a significant impact on the growth rate of the cultured fibroblasts, compared with the effects of the negative control conditions, at each concentration of FBS (Figure 1).

Effects of rehydrated freeze-dried platelet supernatants on fibroblast contraction of collagen gels—The role of rehydrated freeze-dried platelet supernatants in fibroblast migration and alteration of the fibroblast phenotype was investigated by use of contractile collagen gels, in which fibroblasts were seeded in collagen prior to treatment with either FBS alone (1% or 10%) or 1% FBS supplemented with rehydrated freeze-dried platelet supernatants. All conditions were also compared with fibroblasts that were seeded in collagen with supplemental 10% FBS (positive control conditions). Culture in 1% FBS (n = 11) resulted in limited (not significant) collagen contraction over a 15-day period; however, there was a progressive contraction of the collagen gel when the cultures were treated with 1% FBS and rehydrated freeze-dried platelet supernatants (18; Figure 2). By day 7, the fibroblast-seeded collagen gels that had been treated with freeze-dried rehydrated platelet supernatants had significantly (P = 0.02) greater contraction, compared with contraction in gels cultured in medium with 1% FBS alone. This difference was apparent at days 8 (P < 0.001), 9 (P < 0.001), 10 (P < 0.001), and 15 (P < 0.001). This progressive contraction of the collagen gel was similar to but not as considerable as the pattern observed with 10% FBS positive control conditions.

Effects of rehydrated freeze-dried platelet supernatants on migration of fibroblasts—The effects of rehydrated freeze-dried platelet supernatants on fibroblast migration were evaluated by use of a scratch assay in which a defined lesion was made through the center...
of a confluent fibroblast culture. Rehydrated freeze-dried platelet supernatants were added to medium containing 1% or 2.5% FBS, and effects on migration were compared with findings in comparable cultures containing 1% or 2.5% FBS in the absence of supernatant. Under both conditions, the presence of rehydrated freeze-dried platelet supernatants enhanced alignment of fibroblasts perpendicular to the lesion and enhanced migration across the lesion, compared with changes among cells cultured in medium containing 1% or 2.5% FBS alone. Fibroblasts cultured with both 2.5% FBS and rehydrated freeze-dried platelet supernatants had a distinctive spindle morphology; the number of cells that had migrated into the simulated wound area was greater than the number of cells that had migrated into the control (2.5% FBS) lesion. Most cells were aligned perpendicular to the simulated wound (Figure 3). Although there were fewer cells in the 1% FBS that migrated into the lesioned area, the addition of freeze-dried platelet supernatant to 1% FBS cultures also resulted in a greater number of cells with spindle morphology in the area of the simulated wound (Figure 4).
The use of platelet-rich plasma gels and platelet releasates has recently received a great deal of interest as an aid to wound healing. This interest is attributable, in large measure, to the growth factors in platelet α granules that can be locally released into tissue to enhance wound healing. Clearly, the development of a freeze-dried platelet product that could be used by field veterinarians for wound healing would be an important expansion of current treatments.

During initial phases of inflammation, platelet growth factors and thrombin generated by the enzymatic reactions of the coagulation common pathway work together to bring neutrophils, monocytes, and T lymphocytes to the site of injury. This is followed by production of granulation tissue, migration of a wide variety of cell types, synthesis of extracellular matrix and associated growth factors, angiogenesis, and eventually reepithelialization. In the present study, we developed a method for stabilizing platelet growth factors in a dry state such that, under conditions of low serum concentration in vitro, they stimulated fibroblasts during 3 key stages of wound healing, namely proliferation, migration, and contraction.

Although the concentration of platelet growth factors in platelet-rich plasma should, in theory, be similar to that of serum, the experiments of the present study were performed with platelets that had been washed free of plasma proteins and most contaminating leukocytes. The role of leukocytes in enhancing platelet activation through the tissue factor pathway is well characterized. Furthermore, results of recent studies provide evidence that there is differential packing and release of platelet α granule proteins subsequent to agonist stimulation. Compared with platelets in either platelet-rich plasma or whole blood, preparations of washed platelets may therefore react differently to agonists and release different concentrations of growth factors.

Proliferation of fibroblasts, an early stage in wound healing, is stimulated by PDGFs, in particular, TGF-β. In the present study, we simulated wounds areas in the cultures were examined on days 2 (A, D, G), 3 (B, E, H), and 4 (C, F, I); day of examination is indicated as D2, D3, and D4, respectively. Notice that fibroblast orientation, proliferation, and migration were substantially enhanced by the addition of rehydrated freeze-dried platelet supernatants in the presence of 2.5% FBS. Bar = 200 µm (applies to all panels).
However, increasing concentrations of TGF-β1 are associated with excessive granulation tissue; thus, the effects of this potent mitogen must be mitigated by other growth factors such as PDGF-BB through cellular cross-talk. The rehydrated freeze-dried platelet supernatants generated in our study, which contained both TGF-β1 and PDGF-BB, stimulated fibroblast proliferation in cultures grown in medium containing 1%, 2.5%, 5%, and 10% FBS. These growth factors enhanced cellular proliferation in medium containing 10% FBS, indicative of the additional mitogenic effect of rehydrated freeze-dried platelet supernatants in the presence of growth factor–rich medium.

By use of a scratch assay, the rehydrated freeze-dried platelet supernatants stimulated fibroblast migration across a simulated wound area, compared with fibroblast migration in cultures incubated in medium containing 1% FBS alone. The stimulated migrating fibroblasts had a distinct motile spindle phenotype and a polar perpendicular orientation to the wound site, which are typical features of fibroblast migration during wound healing. In preliminary experiments that we performed with medium containing higher serum concentrations (5% and 10%), the scratch lesion was almost completely filled with fibroblasts within 36 hours following the addition of the rehydrated freeze-dried platelet supernatants (data not shown). As detected at lower serum concentrations, those fibroblasts cultured in either 5% or 10% FBS had a spindle morphology and polar orientation to the wound site. Platelet α-granule components PDGF, TGF-β1, and fibronectin increase fibroblast chemotaxis to wound sites. Furthermore, PDGF from human platelet releasates acts in conjunction with collagen matrices to increase the expression of fibroblast α2, α3, and α5 integrins, which are required for enhanced migration in vitro. The concentrations of PDGF required for this increased integrin expression were comparable with those generated in the rehydrated freeze-dried platelet supernatant preparations of the present study.

Figure 4—Representative phase-contrast photomicrographs illustrating the effects of rehydrated freeze-dried platelet supernatants on fibroblast migration as determined by use of a scratch assay. After wound simulation in confluent cultures (day 1), 3 experimental conditions were established: addition of 2 mL of medium containing 1% FBS (A–C), addition of freeze-drying buffer in a final volume of 2 mL of medium containing 1% FBS (D–F), and addition of rehydrated freeze-dried platelet supernatants in 2 mL of medium containing 1% FBS (G–I). Simulated wounds areas in the cultures were examined on days 2 (A, D, G), 3 (B, E, H), and 4 (C, F, I); day of examination is indicated as D2, D3, and D4, respectively. Compared with findings in conditions of 2.5% FBS, fewer fibroblasts are present in the simulated wound area. However, in cultures treated with rehydrated freeze-dried platelet supernatants in the presence of 1% FBS, fibroblasts were more elongated in shape than either those in cultures treated with 1% FBS alone or with 1% FBS and freeze-drying buffer and were oriented perpendicular to the simulated wound area. Bar = 200 µm (applies to all panels).
Collagen gel contraction occurs when fibroblasts differentiate and secrete collagenase, which degrades the collagen matrix and facilitates contraction. Fibroblast contraction, differentiation, and collagenase secretion are critical events during contraction, which is an important stage of wound healing in vivo. Platelet-derived growth factors and TGF-β1 promote changes in the fibroblast contractile phenotype that are characterized by increased expression of smooth muscle actin and smooth muscle myosin as well as by the downregulation of expression of fibroblast-specific proteins. These growth factors directly stimulate the production of collagen and thus play a key role in collagen reorganization in vitro. In our study, rehydrated freeze-dried platelet supernatants enhanced collagen gel contraction under conditions of low serum concentration, consistent with the fibroblast contraction and secretion of collagenase.

The abilities of supernatants of rehydrated freeze-dried equine platelets to enhance fibroblast proliferation, migration, and collagen gel contraction in vitro, under conditions of low serum concentration, suggest that this preparation may be a useful adjunct to enhance healing. In particular, this preparation may be useful in the treatment of limb wounds in horses, in which limited vasculature and skin tension may result in low serum concentrations locally and an initially weak inflammatory response. Future studies of the effects of rehydrated freeze-dried platelet supernatants on angiogenesis and epithelialization in vitro as well as their effects in wounds in vivo should be undertaken to provide more thorough understanding of the potential therapeutic use of freeze-dried platelet supernatants.

References


