Platelet-rich plasma gel promotes differentiation and regeneration during equine wound healing

Charleata A. Carter, a,* David G. Jolly, a Charles E. Worden Sr., a Dennis G. Hendren, a and Cynthia J.M. Kane b

a Research Division, BeluMedX Equine Wound Healing and
b Department of Anatomy and Neurobiology, University of Arkansas for Medical Sciences, Little Rock, AR 72212, USA

Received 17 October 2002

Abstract

Nonhealing wounds of the lower equine limb represent a challenging model. The platelet is a natural source of a myriad of growth factors and cytokines that promote wound healing. This study evaluates the potential of platelet derived factors to enhance wound healing in the lower equine limb. Platelets were isolated from horse blood and activated with thrombin, a process known to induce growth factor release. This produced a platelet gel composed of platelet-rich plasma (PRP). To test this all-natural wound healant, 2.5-cm² full thickness cutaneous wounds were created below the knee and hock of a thoroughbred horse. Wounds were treated with PRP gel or left untreated. Sequential wound biopsies collected at Days 7, 36, and 79 postwounding permitted comparison of the temporal expression of differentiation markers and wound repair. To test the hypothesis that wounds treated with PRP gel exhibit more rapid epithelial differentiation and enhanced organization of dermal collagen compared to controls, tissues were stained for cytokeratin 10, a suprabasal differentiation marker, and the reestablishment of collagen was evaluated by trichrome staining. PRP gel-treated wounds at Day 7 expressed intense cytokeratin 10 staining near the wound junction in suprabasal epidermal layers, while staining in control tissues was less intense and restricted to apical epidermal layers distal to the wound junction. By Day 79, the staining was equal in both groups. However, PRP gel-treated wounds at Day 79 contained abundant, dense collagen bundles oriented parallel to each other and to the overlying epithelium, whereas control tissues contained fewer collagen fibers that were oriented randomly. Thus, treatment of wounds with PRP gel induced accelerated epithelial differentiation and produced tissue with organized, interlocking collagen bundles. This study reveals that this novel all-natural wound healant induced wound repair in injuries previously deemed untreatable.

© 2003 Elsevier Science (USA). All rights reserved.

Keywords: Growth factor therapy; TGF-β; PDGF; Epidermis; Collagen; Tissue repair

Introduction

Over the past 2 decades, extensive cellular and molecular details have been elucidated regarding the regulation of cutaneous wound healing (Chettibi and Ferguson, 1999; Gharaei-Kermani and Phan, 2001). Despite this knowledge, modulation of the intracellular and cell-to-cell communication systems that control wound healing and lead to effective repair has not been accomplished. In fact, costly treatment is often limited to wound maintenance rather than to the production of a successfully healed wound (Dyson, 1997). It is clear that no single exogenous agent can effectively mediate all aspects of a wound-healing response. Thus, successful cutaneous wound healing necessitates combination drug therapy. A rich source of the complex group of growth factors essential to natural wound repair is the platelet (Dugrillon and Kluter, 2002). Not only do platelets assist with clot formation and the subsequent cessation of local loss of blood and lymph, they are also rich in growth factors and cytokines that induce wound healing (Pierce et al., 1998b). Degranulation of platelets by proteins such as thrombin causes them to release these factors which
include transforming growth factor-β (TGF-β), fibrinogen, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-α (TGF-α), vascular endothelial growth factor (VEGF), platelet thromboplastin, thrombospondin, coagulation factors, calcium, serotonin, histamine, and hydrolytic enzymes (Harrison and Cramer, 1996).

The ability to repair cutaneous wounds following injury is critical for survival. Advanced age and diseases such as diabetes alter the effectiveness of the wound-healing process (Ashcroft et al., 1995; Singer and Clark, 1999). However, wound healing below the hock or knee in the horse is a problem even in the healthiest of animals (Bertone, 1989). Wounds below the hock or knee in the horse are resistant to healing. This may be due to the large distance between the trunk and lower limbs providing tissues of the lower limbs with a poor blood supply, thus lower oxygen, a lower temperature, and an imbalance of growth factors (Dyson, 1997; Theoret et al., 2001). In contrast, even extensive, deep wounds in the upper body heal very well with few complications. Interestingly, skin grafts taken from the neck region and placed on the lower limb induce faster wound contraction than skin grafted from the leg (Knotenbelt, 1997). Chronic nonhealing wounds of the lower equine limb have similarities to human leg ulcers and other wounds that resist healing (Cochrane, 1997). The cost of treating poorly healing human wounds in the United States has been estimated at one billion dollars annually (Cupp and Bloom, 2002). Therapies to efficiently treat nonhealing wounds, to speed wound healing, and to promote scar-free healing would be mutually beneficial to the equine and human.

We sought to solve this wound-healing problem by developing a platelet-derived wound healtant from horse blood. Concentrated platelets and their released growth factors are applied locally as a platelet gel whose function is to stimulate and coordinate the wound-healing process. Local application of growth factors has important therapeutic potential in the treatment of chronic wounds, although the exact mechanisms of synergistic action are not completely understood (Debus et al., 2000). Growth factors and their receptors regulate key aspects of soft and hard tissue repair (Bennett and Schultz, 1993). Several clinical studies demonstrate that growth factor treatment accelerates healing of normal tissues and promotes healing of impaired wounds (Bennett and Schultz, 1993). A previous study using topical application of diluted platelet-derived locally acting growth factors resulted in repair of previously nonhealing cutaneous wounds in humans (Knighton et al., 1990). In the treated group, 81% of the wounds achieved 100% epithelization in 8 weeks compared with 15% in the control group. There were no harmful side effects and there was no excessive scarring, malignant transformation, or excessive connective tissue growth (Knighton et al., 1990).

Growth factors are essential for regulating the cellular events involved in wound healing. Growth factors attract cells into the wound, stimulate their proliferation, and significantly influence extracellular matrix deposition (Declair, 1999). TGF-β is particularly important because once it is activated it affects most aspects of tissue repair, including its initiation and termination. TGF-β was predicted to be of therapeutic value in the treatment of chronic, nonhealing or slow to heal wounds over a decade ago (Ksander et al., 1990). PDGF improves dermal regeneration, acts to locally promote protein and collagen synthesis, causes endothelial migration or angiogenesis (Ross, 1987), and induces the expression of TGF-β (Pierce et al., 1989a). Because TGF-β and PDGF have been reported to be released at sites of tissue damage during degranulation of platelet α-granules (Assoian et al., 1983), we evaluated the concentration of TGF-β and PDGF in platelet-rich plasma (PRP) and in platelet-poor plasma (PPP). TGF-β and PDGF levels were elevated in PRP compared with PPP. Because TGF-β promotes differentiation and proliferation associated with wound healing (Choi and Fuchs, 1990), we hypothesized that wounds treated with PRP gel would exhibit enhanced wound repair compared to control tissues due to more rapid proliferation and differentiation. We further hypothesized that wounds treated with PRP gel would possess more organized collagen than control tissues, without excessive deposition of connective tissue or scar formation. The results were consistent with these hypotheses. Treatment of wounded horse tissue with PRP gel induced accelerated epithelial differentiation and restored a more organized collagen matrix in the dermis of the repaired tissues than controls in sequentially sampled biopsies.

**Materials and methods**

**Preparation of Platelet Rich Plasma (PRP)**

Equine platelet-rich plasma was prepared by collecting 1 liter of equine whole blood into a Viaflex bag containing acid citrate dextrose formula A anticoagulant (Haemovetics Corp., Braintree, MA). For enzyme-linked immunosorbent assay (ELISA) analysis, blood was collected from thoroughbred horses locally as well as from several horses in a donor herd in Pennsylvania. For the wound-healing study, blood was drawn from local thoroughbred horses. All blood was refrigerated until separation, which occurred within 24 h of collection, using a MCS+ blood cell separator (Haemovetics Corp.). A preset harvesting program for single donor platelets with concurrent plasma collection was used to collect a target yield of $1.4 \times 10^{11}$ platelets (PRP). Platelet yield averaged $4.9 \times 10^{11}$/liter. In addition, platelet-poor plasma (PPP) was collected simultaneously with a target volume of 250 ml. To prepare the PRP gel, the platelets were activated with 200 IU/ml of thrombin and 5 μM of ascorbic acid (vitamin C) (Fisher Scientific, Fair Lawn, NJ) was added.
ELISA analysis of PDGF and TGF-β1

After separation eight paired samples of platelet concentrate (PRP) and plasma (PPP) were aliquoted into 10-ml single-use vials and frozen at -30°C. For PDGF-AB analysis, samples were thawed, activated with 200 IU/ml of thrombin, and centrifuged to remove platelet membranes and the supernatant (releasate) was frozen. Samples for TGF-β1 analysis were processed identically except that they were not thrombin-activated. Commercial human PDGF and TGF-β1 ELISA kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer’s instructions. All samples for TGF-β1 analysis were acid-activated with 1 N hydrochloric acid. Equine TGF-β1 is 99% homologous to the human nucleotide sequence (Penha-Goncalves et al., 1997).

Study design

All procedures were approved by the Animal Care and Use Committee in compliance with the Guide for the Care and use of Agricultural Animals in Agricultural Research and Testing. A 16-year-old thoroughbred male horse in good general health weighing 1200 lb underwent a physical examination prior to the start of and daily throughout the study. The horse was anesthetized by intravenous administration of 5 cc of 100 mg/mL of xylazine (Akorn, Decatur, IL) and 10 cc of 100 mg/mL of ketamine (Ft. Dodge, IA) and isofluorane. The hair on selected areas, below the knee and hock, of lateral and medial areas of the front legs and lateral areas of the back legs was shaved and the sites were prepared for sterile surgery. Each 2.5-cm² full-thickness skin wound was created by excising the skin demarcated by a sterile plastic template and dissecting it free from the underlying subcutaneous tissue with a scalpel. Three full-thickness excisional skin wounds were created overlying the lateral aspect of the cannon bone and two full-thickness excisional skin wounds were created overlying the medial aspect of the cannon bone of each front leg. Two full-thickness excisional skin wounds were created overlying the lateral aspect of the cannon bone on each rear leg as described for the front legs. The wounds were placed in a vertical column and were separated from each other by a space of 3 cm. The wounds were bandaged with sterile nonadherent, semiocclusive gauze and covered with a Telfa or Duoderm bandage. After 2 days, the bandages were removed, the wounds were cleaned with sterile saline, and treatment was initiated. One of three treatments was randomly assigned to each wound: (1) no treatment other than wound cleaning and placement of a gauze pad, (2) application of saline to a gauze pad, and (3) application of PRP gel to a gauze pad. (Throughout the remainder of this article, untreated controls and saline controls are referred to as controls because there was no difference in the results obtained in the two controls.) Wounds were rebandaged with Telfa or Duoderm. Retreatment was done every 4 days, at which time each wound was cleaned, using sterile saline and gauze sponges. At Day 28, semiocclusive gauze containing the appropriate treatment was applied with Vet Wrap bandaging to permit wound aeration. This aeration limits the formation of excess granulation tissue and thus avoids surgical removal of excess granulation tissue. After Day 28, treatments were performed every 8 days.

For biopsy collection, the horse was sedated and the biopsy site cleaned with saline and gauze. Full-thickness specimens were collected using an 8-mm surgical biopsy punch (Miltex Instrument Co., Bethpage, NY) at Days 7, 36, and 79 after creation of the wounds. The biopsy was performed at the wound margins and included a 2- to 3-mm region of uninjured skin, migrating epithelium, and a 2- to 3-mm region of granulation tissue. Specimens were fixed in 10% neutral-buffered formalin for 24 h and transferred to 70% alcohol. Tissues were embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin using standard procedures for light microscopy.

Cytokeratin stain

Cytokeratin 10 was evaluated in sequential biopsies because it is a marker of suprabasal keratinocyte differentiation (Fuchs, 1995). Sections were deparaffinized and rehydrated prior to quenching endogenous peroxidases for 30 min with freshly made 0.3% H₂O₂ (Fisher Scientific, Springfield, NJ) diluted in methanol (Fisher Scientific). Following two rinses with phosphate-buffered saline (PBS), tissues were treated with 0.1% trypsin (Gibco Life Technologies, Grand Island, NY) in PBS for 20 min at room temperature. After rinsing in PBS, tissues were blocked with 3% horse serum (Sigma, St. Louis, MO) for 20 min. Sections were incubated in a 1:50 dilution of anticytokeratin 10 antibody (Novacastra Laboratories, Newcastle-upon-Tyne, UK) diluted in 3% horse serum in PBS for 1 h. Serial sections served as staining controls and received only 3% serum in PBS. Following rinses in PBS, tissues were incubated in diluted biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA) for 1 h. Following a PBS rinse, sections were incubated with Vectastain Elite ABC Reagent (Vector Laboratories) for 30 min. After a PBS rinse, sections were exposed to the chromagen NovoRed (Vector Laboratories) for 5 min. Following a water rinse, sections were counterstained with Gill’s I hematoxylin (Poly Scientific R and D Corp., Bay Shore, NY) for 45 s, rinsed gently with running tap water for 10 min, rehydrated, cleared in xylenes, and mounted in Permount (Fisher Scientific). Observation and photomicrography was with a Nikon Labophot microscope using Ektachrome tungsten film.

Masson’s trichrome stain

Staining was performed according to revised methods of Masson (1929; Prophet et al., 1992). Tissues were deparaffinized and rehydrated. Following treatment with Bouin’s
fixative (Sigma) for 1 h at 56°C, the slides were allowed to cool for 10 min. Sections were cleared in running water for 10 min and rinsed with distilled water. Following a stain in Weigert’s iron hematoxylin solution (Sigma) for 10 min, slides were washed in running water for 10 min and then rinsed in distilled water. Tissues were stained in Biebrich scarlet-acid fuchsin (Sigma) for 15 min, rinsed in distilled water, and exposed to phosphomolybdic–phosphotungstic acid solution (Sigma) for 10 min. Sections were counterstained in an aniline blue solution (Sigma) for 8 min and rinsed in distilled water. Following treatment in 1% acetic water for 4 min, sections were dehydrated, cleared, and mounted in PolySlip (Poly Scientific). Observation and photomicrography were performed as described above.

Results

Growth factor quantitation in PRP gel

Analysis of TGF-β1 and PDGF-AB protein concentrations in thrombin-activated PRP samples and PPP samples by ELISA revealed that growth factor levels were elevated in PRP samples. Thrombin-activated PRP contained 745 ± 120 pg/ml (n = 8) PDGF-AB and PPP contained 173 ± 68 pg/ml (n = 8) PDGF-AB (Fig. 1). The TGF-β1 concentration in PRP (7480.7 ± 1315 pg/ml; n = 10) was 4.6 times the level in PPP (1619.4 ± 227 pg/ml; n = 10) (Fig. 2).

PRP gel-treated wounds differentiate earlier than controls

Control tissues obtained 7 days after surgical wounding did not exhibit a differentiated morphology evidenced by the lack of keratohyalin granules and a persistence of undifferentiated cells in the suprabasal epithelial layers (Fig. 3A). There was a marked absence of melanin. A high rate of proliferation was suggested by multiple layers in the stratum basale in control tissues (Fig. 3A). At Day 36 postinjury, undifferentiated cells persisted in control tissues in the hyperplastic epithelia at the wound junction (Fig. 3C). Cells had differentiated in control tissues at Day 79 and keratohyalin granules were present, but melanin was not apparent (Fig. 3E). In contrast, PRP gel-treated wounds obtained 7 days after surgical wounding exhibited advanced reepithelialization evidenced by differentiated polyhedral keratinocytes in the stratum spinosum and keratohyalin granules in the stratum granulosum (Fig. 3B). Melanin was obvious in cuboidal cells in the stratum basale of PRP gel-treated wounds (Fig. 3B). PRP gel-treated wounds obtained at Day 36 postwound displayed differentiated cells in the hyperplastic epithelia at the wound junction (Fig. 3D) evidenced by keratohyalin granules in the stratum granulosum and polyhedral cells in the stratum spinosum. The presence of several dividing cells in the stratum spinosum (Fig. 3D) indicated that cell division was higher in this PRP gel-treated wound than in the paired control (Fig. 3C). A PRP gel-treated wound obtained at Day 79 postwound displayed keratinocytes in the upper epithelial layers with a differentiated phenotype, and melanin was apparent in the stratum basale (Fig. 3F).

PRP gel promotes differentiation evidenced by accelerated expression of cytokeratin 10

Because cytokeratin 10 is a suprabasal marker for keratinocyte differentiation in normal epidermis (Fuchs and Green, 1980; Fuchs, 1995), tissues were immunostained for

![Fig. 1. ELISA assay of PDGF-AB released upon activation with thrombin in eight samples of PRP (745.4 ± 120 pg/ml) and in eight samples of PPP (173.2 ± 68 pg/ml). PRP contains around 4 times more PDGF-AB than PPP.](image1)

![Fig. 2. ELISA assay of TGF-β1 released by 8 samples of PPP (1619.4 ± 227 pg/ml) and 8 samples of PRP (7480.7 ± 1314.6 pg/ml). PRP contained around 4.5 times more TGF-β1 than PPP.](image2)
this marker to assess progression of epithelial differentiation. Table 1 summarizes cytokeratin 10 staining in temporally sequential biopsies of the two treatment groups. Day 7 control tissues exhibited positive cytokeratin 10 staining in suprabasal layers but the staining was less intense in the stratum spinosum and stratum granulosum, and the stain was distant from the wound margin (Fig. 4A and C). The distance between the wound margin and the cytokeratin 10 positively stained epithelium in control tissues \((n = 3)\) was 1350 \(\mu\)m. On Day 36, tissues from control and PRP gel-treated wounds displayed staining near the wound margin (Fig. 4E and F). Suprabasal keratinocytes in control tissues were positive for cytokeratin 10 at Day 36 (Fig. 4E). However, tissues from Day 79 control wounds stained positively for cytokeratin 10 only in the apical epidermal layers, and the staining was often heterogeneous (Fig. 4G).

When PRP gel-treated wounds were stained for cytokeratin 10, Day 7 tissues exhibited intense staining in all epidermal layers except the stratum basale; this staining occurred near the wound margin (Fig. 4B and D). The distance from the wound margin to the positive cytokeratin 10 stain in PRP gel-treated wounds \((n = 3)\) was 900 \(\mu\)m. Thus, cytokeratin 10 positive epithelial staining was 1.5X further away from the wound margin in control tissues than in PRP gel-treated tissues. Intense positive cytokeratin staining in all suprabasal layers is obvious in a PRP gel-treated wound (Fig. 4D), while a paired control tissue exhibited less staining, which was located only in apical epithelial layers (Fig. 4C). Tissue from PRP gel-treated wounds obtained at Day 36 postwound was positive for cytokeratin 10 staining throughout the suprabasal epidermal layers, with an intense stain in the stratum corneum (Fig. 4F). Day 79 tissues treated with PRP gel that had been frozen and thawed prior to application (Fig. 4H) displayed similar cytokeratin 10 staining as the paired control (Fig. 4G) because heterogeneous cytokeratin 10 staining occurred in the apical epidermal keratinocytes. The tissues that received only secondary antibody and served as a staining control displayed no staining (data not shown).

The epithelial layer was thicker in control tissues (Fig. 4G) than in the PRP gel-treated tissues (Fig. 4H) at Day 79. Quantitation of epidermal thickness in 10 different areas of control and PRP gel-treated tissues revealed that the epithelial layers of control tissues \((n = 2)\) were thicker than PRP gel-treated tissues \((n = 2)\). The average thickness for control tissues was 338 \(\mu\)m, while the average thickness for PRP gel-treated tissues was 169 \(\mu\)m. Thus, the epithelial height in control tissues is twice that of PRP gel-treated tissues. This indicates that control tissues remain hyperplastic and less differentiated at this time point, while PRP gel-treated tissues have advanced to a normal, less hyperplastic phenotype.

**PRP gel promotes the formation of organized collagen**

In order to evaluate the structural organization of collagen in the dermis of the repaired wound, a trichrome stain was performed. Control tissues obtained at Day 79 post-

---

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 7</th>
<th>Day 36</th>
<th>Day 79</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>Staining of suprabasal epithelial layers. Staining is distal to wound margin</td>
<td>Intense staining of all suprabasal epithelial layers</td>
<td>Positive scattered staining in the stratum corneum and stratum granulosum</td>
</tr>
<tr>
<td>Saline control</td>
<td>Staining of suprabasal epithelial layers. Staining is distal to wound margin</td>
<td>Intense staining of all suprabasal epithelial layers</td>
<td>Positive scattered staining in the stratum corneum and stratum granulosum</td>
</tr>
<tr>
<td>Platelet-rich plasma (PRP)</td>
<td>Intense staining of all suprabasal epithelial layers. Staining is near the wound margin</td>
<td>Intense staining of all suprabasal epithelial layers. Stratum corneum has dark positive staining</td>
<td>Positive scattered staining in the stratum corneum and stratum granulosum</td>
</tr>
</tbody>
</table>

---

This page contains images and text discussing the use of platelet-rich plasma (PRP) in wound healing, specifically focusing on the expression and staining of cytokeratin 10 in different layers of the epidermis. It highlights differences between control and PRP-treated wounds, noting increased thickness and differentiation in PRP-treated tissues. The table summarizes staining patterns for cytokeratin 10 at different time points, with notable differences in intensity and distribution between untreated and PRP-treated samples. The text also mentions the use of hematoxylin and eosin staining to evaluate the structural organization of collagen in the dermis.
wound displayed disorganized collagen fibers at various angles to each other and to the apical epidermis (Fig. 5A and C). These disordered collagen fibers did not interconnect with each other to any large extent. In contrast, on Day 79, PRP gel-treated wounds displayed organized interconnecting collagen fibers running parallel to each other and to the epidermis (Fig. 5B and D). Several small vessels were also apparent in the PRP gel-treated tissues (Fig. 5D), whereas only a few vessels were present in control tissues (Fig. 5C). Because organized collagen fibers give tensile strength to the tissue, the arrangement of collagen observed in the PRP gel-treated tissues is consistent with tissues that possess good tensile strength and improved wound repair.

Discussion

We have developed a new wound healant composed of concentrated PRP, thrombin, and ascorbic acid that is delivered as a topical gel to cutaneous wounds. PRP gel accelerated epithelial differentiation in sequential biopsies from equine wounds. Additionally, collagen in the PRP-treated tissues was organized as dense, tightly packed fiber bundles parallel to the overlying epidermis, whereas control tissues exhibited fewer collagen fibers arranged randomly. An organized, dense collagen lattice suggests increased tensile strength in the repaired wound.

Cutaneous wound healing involves repair and regeneration. It is controlled by growth factors that regulate protein expression, enzyme production, cellular differentiation, proliferation, metabolism, and migration, as well as the synthesis and remodeling of extracellular matrix (ECM) proteins (Steed, 1998; Komarcevic, 2000). The ECM then coordinates cellular events and modulates cellular responsiveness to growth factors and cytokines. Several key factors in the enhanced PRP wound healing response likely include platelet-derived TGF-β1, thrombin, EGF, vitamin C, fibroblast growth factor (FGF), PDGF, VEGF, thrombospondin, and fibronectin. Platelets were activated with thrombin, a naturally occurring platelet activator that promotes wound healing. Others have shown that, even though thrombin is highly labile, on Day 7 compared to controls an encapsulated thrombin peptide decreased wound size by 60%, increased the fibroblast to macrophage ratio, and increased proliferating fibroblasts 150% (Strukova et al., 2001). Thrombin activation of platelets caused release of VEGF (Mohle et al., 1997), a key mediator of angiogenesis that stimulates endothelial cell proliferation (Kliche and Waltenberger, 2001). EGF is released by platelets and is chemotactic for fibroblasts (Adelmann-Grill et al., 1990), and topical application accelerated the rate of epidermal regeneration and increased wound tensile strength (Andersen and Ehlers, 1998). However, repetitive or sustained administration of EGF was necessary to maximize its actions, whereas basic FGF, PDGF, and TGF-β required only a single dose to induce their maximal effects (Hom and Maisel, 1992). Thrombospondin, the most abundant protein in platelet α-granules (Chung et al., 1997) is released by activated equine platelets (Lipscomb et al., 1997) and activates latent TGF-β in vivo (Crawford et al., 1998). PDGF is released by α-granules in activated platelets (Ross et al., 1986), which are the largest source of PDGF in the body (Pierce et al., 1991). PDGF induces fibroblast and smooth muscle cell migration and proliferation and is a chemotactic for neutrophils and monocytes (Hosgood, 1993). PDGF-AB is the dominant form of PDGF in human platelets (Hosgood, 1993). Thrombin-activated PRP contains 4 times more PDGF-AB than PPP (745 pg/ml in PRP and 173 pg/ml in PPP). Three milliliters of PRP gel was applied per wound containing 2.23 ng PDGF-AB. This low concentration of PDGF is in a range (2–5 ng/ml) reported to induce fibroblast mitosis (Ross, 1987). An increased number of fibroblasts in our PRP-treated wounds may help explain the increased collagen and repair in the dermis following treatment.

Growth factors are thought to be imbalanced or insufficient in the lower equine limb, leading to a major impairment of ECM and delayed wound healing (Stashack, 1991; Knottenbelt, 1997; Theoret et al., 2002). Concentration and activation of platelets into a PRP and topical application of this PRP as a gel is shown in this study to be an effective method for replacing essential growth factors, especially TGF-β and PDGF-AB, at increased concentrations in physiologic balance, thereby creating an environment conducive

Fig. 4. Cytokeratin 10 staining in tissues obtained at Days 7, 36, and 79 after wounding. (A) Control tissue displays intense staining for cytokeratin 10 in the stratum corneum, while a less intense stain is seen in the stratum granulosum and stratum spinosum. Note that the region of positive cytokeratin staining is far from the wound margin (arrow). (B) Tissue from a wound treated with PRP gel displays intense staining for cytokeratin 10 in all suprabasal epidermal layers with a very intense stain in the stratum corneum. Note that the cytokeratin 10 staining is close to the wound margin (arrow). Bar for A and B = 500 μm. (C) Tissue from a control wound exhibits cytokeratin 10 staining only in the stratum corneum and stratum granulosum. Positive cytokeratin 10 staining is heterogeneous in the epidermis near the wound margin. (D) Intense cytokeratin 10 staining is obvious in tissue from a wound treated with PRP gel. Staining occurs in all suprabasal epidermal layers. This image is a higher magnification of B and shows the close proximity of the cytokeratin stain to the wound margin (arrow). Bar for C and D = 200 μm. (E) Tissue obtained at Day 36 after wounding from a control wound displays positive cytokeratin 10 staining near the wound junction in all suprabasal epidermal layers. (F) Tissue obtained at Day 36 after wounding from a PRP gel-treated wound displays positive staining for cytokeratin 10 in the advancing epithelial tissue. All suprabasal epidermal layers are positive for cytokeratin 10 with an intense stain in the stratum corneum. Bar for E and F = 500 μm. (G) Control wound tissue obtained at Day 79 after wounding shows positive staining for cytokeratin 10 arranged in a heterogeneous pattern in the stratum corneum and stratum granulosum. Note that the epithelial keratinocytes in control wounds at this time point are more hyperplastic than paired tissues from PRP gel-treated wounds. (H) Tissue obtained at Day 79 after wounding from a wound treated with PRP gel displays heterogeneous cytokeratin 10 staining similar to tissues from control wounds. Bar for G and H = 200 μm.
to wound healing. TGF-β1 is produced by a variety of cells normally recruited to an injury site. TGF-β1 regulates cellular differentiation, proliferation, chemotaxis, and synthesis of many ECM components (Roberts et al., 1988; Kane et al., 1991). The release of H$_2$O$_2$ by macrophages is suppressed by TGF-β (Tsunawaki et al., 1988) so that growth factors and cytokines secreted by macrophages exert their effects, but the ability of the cells to induce cell death by oxidative stress is alleviated (Roberts and Sporn, 1990). In vivo studies have confirmed that exogenous TGF-β1 increases granulation tissue, collagen formation, and wound tensile strength when applied locally in animal models (Ashcroft et al., 1999). Because TGF-β plays a role in differentiation and has multiple roles in wound healing (Roberts and Sporn, 1990; Kane et al., 1991), we analyzed the amount of TGF-β by ELISA in PRP and PPP. Even in non-thrombin activated PRP, the concentration of TGF-β was significantly increased compared to PPP (7481 pg/ml in PRP compared to 1619 pg/ml in PPP). TGF-β released from blood platelets at sites of tissue damage is physiologically targeted to intervene in wound healing (Assioan et al., 1983). Topical application of TGF-β1 to cutaneous wounds in pig and rat promotes healing (Mustoe et al., 1987; Pierce et al., 1989b). A single dose of 1 μg/wound of TGF-β1 significantly enhanced wound healing in rats; this effect was most prominent when TGF-β was applied topically as a polyoxamer gel (Puolakkainen, 1995). Topical application of TGF-β1 in our PRP gel at a 45-fold lower dose (0.022 μg/wound) produced regenerative healing in the epidermis and dermis without fibrosis or scar formation.

Differentiation can be induced by natural compounds (Carter and Parham, 1997; Carter, 2000; Carter and Madden, 2000), including growth factors. TGF-β decreases basal keratinocyte proliferation and induces suprabasal cell differentiation to stimulate epidermal regeneration associated with cutaneous wound healing (Choi and Fuchs, 1990; Kane et al., 1990, 1991). Ascorbic acid is a free radical scavenger that promotes keratinocyte differentiation and accelerates wound healing (Komarcevic, 2000; Savini et al., 2002). PRP gel containing PDGF-AB, TGF-β1, and ascorbic acid induced accelerated differentiation of the suprabasal epidermis evidenced by accelerated expression of keratohyalin granules and cytokeratin 10 in suprabasal keratinocytes following injury. Cytokeratin 10 is located only in differentiating keratinocytes in the spinous and granular layers (Kane et al., 1991; Fuchs, 1995). PRP gel-treated wounds exhibited intense cytokeratin 10 suprabasal staining near the wound margin at Days 7 and 36. In contrast, paired control wounds exhibited a less intense suprabasal stain distant from the wound.

PRP gel-treated wounds possess mature granulation tissue characterized by dense parallel dermal collagen bundles. Dense, tightly packed, organized collagen fiber bundles are characteristic of mature granulation tissue; whereas thin, randomly organized collagen bundles containing many fibroblasts characterize the early stages of granulation tissue deposition (Moyer et al., 2002). Collagen synthesis occurs in the expanded population of fibroblasts in the wound. Ascorbic acid is a known stimulator of collagen production (Nowak et al., 2000). Treatment of fibroblasts with TGF-β1 significantly increases cellular synthesis of collagen, fibronectin, and glycosaminoglycans and promotes matrix formation (Hsuan, 1989; Ignotz and Massagué, 1986). In skin incisions in rabbits, TGF-β1 triggered synthesis and rapid maturation of collagen in early wounds (Pierce et al., 1991). When PDGF was used in diabetic rats, collagen deposition in experimental wounds increased to the level of control, nondiabetic animals (Grotendorst et al., 1985). PDGF and TGF-β1 in combination results in higher collagen deposition than in rats treated with TGF-β1 alone (Lawrence et al., 1986). PDGF, TGF-β1, and ascorbic acid are important components of PRP gel that promote collagen formation associated with optimal wound healing.

Collagen deposition is normally elevated for a specific and limited time during wound healing. However, if a chronic inflammatory state is initiated, enhanced fibroblast production of collagen may lead to a pathologic lesion and excess scarring. Even without the use of antibiotics, catastrophic equine wounds treated with PRP gel do not exhibit prolonged inflammation, which commonly leads to scarring, or develop bacterial infections (unpublished results). Interestingly, lack of acute inflammation is a consistent feature of fetal wound healing (Armstrong and Ferguson, 1995; Cowin et al., 1998). Fetal wounds also contain low levels of growth factors (Whitby and Ferguson, 1991). These features are thought to underlie the absence of scar formation in fetal wounds (McCallion and Ferguson, 1995; Whitby and Ferguson, 1991; Longaker et al., 1990). In contrast, adult wounds usually undergo acute inflammation leading to excess collagen deposition and scar formation (Cowin et al., 1998). Using PRP gel and an aerated wound environment, growth factors in the wounds and subsequent cell-mediated events were regulated to create an environment with accelerated epidermal differentiation and an organized, dense collagen matrix in the absence of a prolonged inflammatory response.

Not only does the lower limb of the horse represent an ideal model of hard-to-heal acute equine wounds, it is also representative of chronic nonhealing mammalian wounds. The use of growth factors derived from activated platelets to stimulate wound repair is a unique approach to improving wound healing compared with conventional wound management, including debridement and antibiotic treatment (Komarcevic, 2000). Moreover, the costs of wound management worldwide are staggering. An accelerated and improved quality of healing would be advantageous for horses and humans. The use of PRP gel as an improved therapy for poorly vascularized or non-healing wounds in the lower equine limb and in immunocompromised, diabetic, or elderly individuals could provide quality healing of acute wounds leading to limb...
salvage that would be of significant personal, economic, and social advantage.

Acknowledgments

We are grateful to Mr. Robert Skinner and Ms. Francis Swain for histological expertise, to Ms. Erika Lowe for image processing, and to the veterinary assistants who assisted with biopsy collection.

References


Fig. 5. Tissues obtained at Day 79 after wounding when healing appeared nearly complete by gross observation. Tissues were stained with Masson’s trichrome stain in order to analyze collagen amount and arrangement. (A) Tissue from a control wound shows an intact epidermis overlying randomly oriented collagen bundles in the dermis. Blue-staining collagen bundles are not prominent because of their immature structure, but red-staining fibroblasts are obvious. Bar = 200 μm. (B) Tissue, from a PRP gel-treated wound, displays well-organized collagen bundles in the papillary dermis near an intact epithelia. Blue-staining collagen bundles are prominent in this tissue and the collagen is oriented parallel to the overlying epithelia. Note also the fibroblasts lying perpendicular to the epidermis. Bar = 200 μm. (C) Higher magnification of an area in A showing the papillary dermis adjacent to the overlying epithelia demonstrates that collagen bundles are randomly oriented and do not form organized close associations with each other, a pattern characteristic of immature granulation tissue. Note the numerous fibroblasts interspersed among the collagen. Bar = 100 μm. (D) Higher magnification of an area in B showing the papillary dermis with abundant, dense, tightly packed collagen bundles oriented parallel to the overlying epithelia. These blue-staining collagen bundles are prominent throughout the dermis and are characteristic of mature granulation tissue. Bar = 50 μm.