Effects of platelet-derived growth factor-BB on the metabolic function and morphologic features of equine tendon in explant culture

Jennifer L. Haupt, BS; Brandon P. Donnelly, BS; Alan J. Nixon, BVSc, MS

Objective—To evaluate the effects of recombinant human platelet-derived growth factor-BB (rhPDGF-BB) on the metabolic function and morphologic features of equine superficial digital flexor tendon (SDFT) in explant culture.

Animals—6 euthanized horses (2 to 5 years old).

Methods—Forelimb SDFT explants were cultured for 6 days as untreated control specimens or treated with rhPDGF-BB (1, 10, 50, or 100 ng/mL of medium). Treatment effects on explant gene expression were evaluated via real-time PCR analysis of collagen type I, collagen type III, PDGF-A, and PDGF-B mRNA. Explants were assayed for total collagen, glycosaminoglycan, and DNA content; histologic changes were assessed via H&E staining and immunohistochemical localization of collagen types I and III.

Results—No morphologic or proliferative changes were detected in tendon explant sections. After high-dose rhPDGF-BB treatment, gene expression of collagen types I and III was increased and decreased, respectively. Expression of PDGF-A and PDGF-B mRNA was significantly increased at 24 hours, but later decreased to have few or negative autoinductive effects. Although PDGF gene expression waned after 48 hours of culture, collagen type I gene expression was significantly increased at 48 hours and reached peak value on day 6. Glycosaminoglycan and DNA content of explants were unchanged with rhPDGF-BB treatment.

Conclusions and Clinical Relevance—Results suggest that rhPDGF-BB use may be of benefit in the repair of equine tendon, particularly through induction of collagen type I mRNA. Positive autoinductive effects of PDGF-BB in equine SDFT explants were detected early following culture medium supplementation, but these diminished with time. (Am J Vet Res 2006;67:1595–1600)

Tendinitis and degenerative diseases of flexor tendons are common and debilitating musculoskeletal conditions in equine athletes.1-3 In horses with tendinitis, peptide, cell, or small-molecule therapies are used in attempts to decrease the high incidence of recurrence and bolster the limited intrinsic tendon repair capacity.1-3 Tendon healing is generally slow and involves a wide range of molecules in a complex but regulated mechanism. After injury, the repair process involves a sequence of events including hemorrhage, inflammation, fibroblast proliferation, collagen deposition, and tissue remodeling. Exogenous agents that positively influence any of these phases may be of major therapeutic value in tendon repair.

Anabolic growth factors have emerged as potentially useful recombinant products that improve healing of many musculoskeletal tissues. Their role in tendon healing—directing the proliferation and migration of tenocytes, stimulating angiogenesis, and driving matrix proliferation—is well documented.4 For tendon repair, IGF-I and TGF-β1 have proven benefits in vitro.5-7 Results of in vivo research8 have indicated that constitutive gene expression of both these anabolic peptides is depressed in the early phases of tendon healing, which suggests a therapeutic window for exogenous supplementation. Administration of IGF-I or TGF-β stimulates healing of tendinitis lesions through increases in cell proliferation and gene expression of collagen and other matrix components.8,9,10-12 Less information is available regarding the role of PDGF as an anabolic growth factor for tendon healing, but results of some investigations13,14 indicate that PDGF has considerable mitogenic and chemo-attractant activity for many cell types, including fibroblasts.

Platelet-derived growth factor is a dimeric polypeptide that exists in 3 isoforms. The PDGF-BB isoform has greater potential to stimulate cell proliferation than the AA or AB isoforms.15-17 In vivo, PDGF protein is stored within the α-granules of platelets and released following vessel damage and subsequent platelet activation. During activation, the α-granules release their contents (including adhesive proteins, plasma proteins, cellular mitogens [primarily growth factors], coagulation factors, and protease inhibitors) into the wound milieu.18

Recent interest has been directed toward PDGF as a potentially vital component of bone marrow aspirates or platelet-enriched plasma concentrates that have

ABBREVIATIONS

IGF-I Insulin-like growth factor-I
TGF-β1 Transforming growth factor-β1
PDGF Platelet-derived growth factor
EGF Epidermal growth factor
VEGF Vascular endothelial growth factor
rhPDGF-BB Recombinant human PDGF-BB
SDFT Superficial digital flexor tendon
GAG Glycosaminoglycan

Received October 23, 2003.
Accepted February 8, 2006.
From the Comparative Orthopaedics Laboratory, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. Ms. Haupt’s present address is College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824.
Supported in part by Luitpold Pharmaceuticals.
The authors thank Mary Lou Norman for processing and sectioning of histologic samples.
Address correspondence to Dr. Nixon.
been administered by direct injection to tendinitis lesions.\textsuperscript{19,20} Bone marrow aspirates are of clinical interest as a source of autogenous mesenchymal stem cells and often deliver growth factors to the tendinitis lesion.\textsuperscript{21,22} Platelet-enriched plasma concentrates have also emerged as useful agents, supplying an array of growth factors including PDGF, IGF-I, TGF-\(\beta\)-1, and, in smaller amounts, EGF, VEGF, hepatocyte growth factor, and basic fibroblastic growth factor.\textsuperscript{12,23,24} Despite the interest in PDGF as a therapeutic option for tendon healing, there is little compelling evidence to confirm that PDGF induces cell proliferation and matrix production in tendon samples.\textsuperscript{13,25,26}

The purpose of the study reported here was to evaluate the effects of rhPDGF-BB on the metabolic function and morphologic features of equine SDFT in explant culture. Additionally, the potential follow-on PDGFD expression associated with autocrine induction of PDGF action was investigated.

**Materials and Methods**

Assessment of the dose-dependent effects of PDGF-BB on equine SDFT explants—To establish tendon explant cultures, the superficial digital flexor tendon was aseptically excised from the midmetacarpal region of both forelimbs of 3 horses (aged 2 to 5 years); the horses were euthanized by barbiturate overdose as part of another study that was approved by the institutional animal care and use committee. Tendons were divided to be normal on the basis of findings via palpation, gross examination, and later dissection for explant culture. Following collection, the tendon segments were maintained in culture medium\textsuperscript{a} during the removal of the paratenon. The explants were then sectioned into 5 \(\times\) 5 \(\times\) 4 mm blocks and evenly distributed in 12-well plates. Explants were cultured for 6 days in culture medium\textsuperscript{a} (supplemented with penicillin [50 U/mL]; streptomycin [50 \(\mu\)g/mL]; ascorbic acid [100 \(\mu\)g/mL]; and 2\% fetal bovine serum) with 0, 1, 10, 50, or 100 ng of rhPDGF-BB/mL. Medium was exchanged every 48 hours, and tendon explants were collected on day 6. Each explant was divided, and a portion was snap-frozen in liquid nitrogen and pulverized for RNA isolation with a phenol-guanidine isothiocyanate reagent.\textsuperscript{c} A cross-sectional edge was fixed in 4\% paraformaldehyde, embedded in paraffin, and sectioned for H&E staining and immunohistochemical staining for collagen types I and III. The remainder of the explant was immersed in protease inhibitors and snap-frozen in liquid nitrogen for biochemical assays.

To evaluate gene expression, tendon explants were snap-frozen and powdered in a freezer-mill\textsuperscript{c} under liquid nitrogen prior to extraction of RNA. Following extraction, the RNA was purified over RNA-specific purification columns\superscript{27} and the quality assessed via UV spectrophotometry and electrophoresis. The RNA was then diluted to approximately 5 \(\mu\)g/mL and mixed with diluted primers and probes according to real-time PCR assay procedures.\textsuperscript{27} Gene expression for equine PDGF-A, PDGF-B, collagen type I, and collagen type III mRNAs was analyzed via real-time PCR procedures with a 1-step reverse transcription–PCR kit\textsuperscript{27} and laser excitation fluorescent real-time PCR detection system.\textsuperscript{28} Primers and probes for PCR were designed by use of proprietary software\textsuperscript{27} and known equine sequences that spanned exon-exon junction sites for greater binding specificity. The fluorescent-labeled probe had a reporter dye (6-FAM) at the 5'-end and a quenching dye ( TAMRA) at the 3'-end. Total copy number of mRNA was measured from a validated standard curve constructed from plasmid stocks. Each gene was normalized to 18S RNA expression.

For histologic evaluation, tendon explants were fixed in 4\% paraformaldehyde, processed, embedded in paraffin, and sectioned at 6 \(\mu\)m. Routine H&E staining was used to assess morphologic features of the explants. Patterns of collagen type I and III protein production and distribution were examined immunohistochemically. A custom rabbit anti-equine collagen type I primary antibody and mouse anti-human collagen type III antibody were used. Biotinylated goat anti-rabbit and goat anti-mouse antibodies were used as a secondary antibody, followed by color production from diaminobenzidine (catalyzed by peroxidase-conjugated streptavidin) for localization. The sections were counterstained with hematoxylin. Nonimmune serum from the same species as that used to develop the primary antibody was used as a negative-control treatment on serial sections, and positive-control specimens included equine late-term fetal costochondral junction tissue.

Biochemical analyses of DNA, GAG, and total collagen contents in tendon explants were performed. Explants were rinsed in protease inhibitors, snap-frozen, powdered in a freezer-mill in liquid nitrogen, and then lyophilized. Glicosaminoglycan content was quantified by use of a dimethylmethylene blue spectrophotometric assay.\textsuperscript{27} Dried tendon samples were digested in papain and then mixed with dimethylmethylene blue solution in a formate buffer. Optical density was measured at 525 nm. A standard curve was derived from mixed-isomer shark chondroitin sulfate. Papain-digested samples were also used to determine total DNA content by use of a fluorometric assay that was based on DNA-specific enhancement of dye fluorescence, compared with a standard curve derived from call thymus DNA.\textsuperscript{27} Lyophilized tendon samples were solubilized in pepsin and 0.5M acetic acid for total collagen quantitation by use of a Sirius red colorimetric assay.\textsuperscript{27} Data for the assays were normalized to the initial tendon dry weight.

Assessment of the temporal effects of PDGF on equine SDFT explants—The effects of rhPDGF-BB supplementation on the temporal expression of collagen type I and PDGF-A and -B mRNA were assessed in a subsequent experiment. Explants of SDFT were obtained from both forelimbs of 3 horses (aged 3 to 5 years) and treated as either control specimens or supplemented with 100 ng of rhPDGF-BB/mL (the PDGF dose that increased collagen type I and reduced collagen type III expression to the greatest extent in the dose-response portion of this study). Explants were collected for RNA isolation at 12, 24, 36, 48, 72, and 144 hours following the initiation of rhPDGF-BB treatment. Gene expression for equine PDGF-A, PDGF-B, and collagen type I was analyzed, and, for each, the total copy number of mRNA was measured from a validated standard curve constructed from plasmid stocks. Each gene was normalized to 18S RNA expression.

**Results**

Dose-dependent effects of rhPDGF-BB—The dose-dependent effects of rhPDGF-BB on the gene expression of equine SDFT explants were analyzed via real-time quantitative-PCR analysis of PDGF-A and PDGF-B mRNA, in addition to gene expression of collagen types I and III. Collagen type I and III message...
expression, which was measured after 6 days of culture exposure to increasing concentrations of rhPDGF-BB, did not differ significantly among doses (Table 1). At the high dose (100 ng/mL), rhPDGF-BB significantly decreased expression of both PDGF-A and PDGF-B mRNA, compared with untreated controls and lower PDGF doses (Figure 1). No significant differences in expression of PDGF-A and PDGF-B mRNA were detected among the lower dose concentrations of rhPDGF-BB.

The effects of rhPDGF-BB on cell and matrix morphologic features of the tendon explants were assessed via H&E staining. Among the control and rhPDGF-BB–treated explants, no morphologic differences were detected via microscopic examination (Figure 2). Immunohistochemical localization of collagen (types I and III) revealed no visible morphologic changes as a result of rhPDGF-BB supplementation. Both collagen types I and III appeared to be uniformly expressed throughout the matrix.

To determine the in situ consequences of PDGF-BB supplementation, total collagen, GAG, and DNA contents were measured in lyophilized tendon samples. Compared with the control explants, mean values for each variable in all rhPDGF-BB–treated explants were not significantly different (Table 2).

**Temporal effects of rhPDGF-BB on tendon explants**—The temporal effects of rhPDGF-BB on equine SDFT explants were assessed via real-time PCR analyses of matrix molecule and PDGF gene expression. The autoinductive effects of rhPDGF-BB on expression of both the A and B isoforms were apparent at 12 and 24 hours, respectively; mRNA concentrations were increased at those time points, compared with the control treatment (Figure 3). The increased PDGF gene expression decreased to levels similar to untreated control specimens by day 6. Despite the decrease in PDGF gene expression after 24 hours of culture, collagen type I gene expression significantly increased with rhPDGF-BB treatment during the 6-day culture period.

<table>
<thead>
<tr>
<th>PDGF-BB treatment (ng/mL)</th>
<th>Collagen type I</th>
<th>Collagen type III</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>5,397 ± 2,571</td>
<td>11,109 ± 5,467</td>
</tr>
<tr>
<td>1</td>
<td>4,085 ± 2,969</td>
<td>6,206 ± 2,663</td>
</tr>
<tr>
<td>10</td>
<td>4,420 ± 1,993</td>
<td>7,565 ± 2,810</td>
</tr>
<tr>
<td>50</td>
<td>9,075 ± 2,795</td>
<td>8,367 ± 2,359</td>
</tr>
<tr>
<td>100</td>
<td>8,629 ± 3,443</td>
<td>5,465 ± 1,967</td>
</tr>
</tbody>
</table>

Table 1—Expression of collagen type I and III mRNA in equine SDFT explants treated with various concentrations of rhPDGF-BB assessed via real-time quantitative PCR assay (expression determined as absolute copy number and samples normalized to 18S RNA).

<table>
<thead>
<tr>
<th>PDGF-BB treatment (ng/mL)</th>
<th>DNA (µg/mg)</th>
<th>GAG (µg/mg)</th>
<th>Total collagen (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>1.21 ± 0.07</td>
<td>27.47 ± 0.07</td>
<td>616.07 ± 4.33</td>
</tr>
<tr>
<td>1</td>
<td>1.19 ± 0.06</td>
<td>30.54 ± 0.07</td>
<td>631.42 ± 4.79</td>
</tr>
<tr>
<td>10</td>
<td>1.42 ± 0.19</td>
<td>27.39 ± 0.07</td>
<td>655.19 ± 4.92</td>
</tr>
<tr>
<td>50</td>
<td>1.27 ± 0.07</td>
<td>24.47 ± 0.07</td>
<td>619.70 ± 3.75</td>
</tr>
<tr>
<td>100</td>
<td>1.23 ± 0.07</td>
<td>27.30 ± 0.07</td>
<td>648.35 ± 8.89</td>
</tr>
</tbody>
</table>

Table 2—Tendon explant DNA, GAG, and total collagen content of equine SDFT specimens exposed to increasing concentrations of PDGF-BB (samples were normalized to tissue dry weight).
from all horses were considered as a group, no significant difference in total collagen content between the control and rhPDGF-BB–treated specimens was detected (580.03 ± 41.86 μg/mg and 588.10 ± 37.36 μg/mg, respectively).

Discussion

In the present study, exogenous PDGF-BB had a modest stimulatory effect on equine flexor tendon explants that could potentially enhance tendon healing in vivo. Explants were cultured for 6 days, and tendon matrix gene expression, cell proliferation, and GAG and collagen contents were determined. Additionally, the potential for autoinduction of PDGF isoforms in tendon explants, which might extend any positive effect of PDGF-BB exposure, was examined. Treated explants responded with increased collagen type I gene expression, and after extended culture periods at the highest dose (100 ng of rhPDGF-BB/mL), collagen type I expression was significantly greater than that of untreated control specimens. Platelet-derived growth factor BB has been shown to have potential for enhancing tendon healing through its mitogenic stimulation of fibroblasts.13,25 However, in the present explant study, DNA concentration remained constant, and contents of tendon matrix components such as GAG and total collagen were either unchanged or slightly increased (albeit not significantly). The initial steps in tendon healing involve hemorrhage and inflammation, wherein PDGF is released following activation of platelets and clot formation.1 Exposure to exogenous PDGF-BB duplicated some of this local inflammatory milieu of growth factors, but may have required coordinated exposure to IGF-I, TGFβ, VEGF, and other factors to generate a more robust response. These other growth factors, including IGF-I and TGF-B1, are present in tendon lesions during the early phase of the healing process and are thought to be important for tendon matrix synthesis.23–28 Significant stand-alone anabolic and mitogenic responses of SDFT to IGF-I were identified previously with this explant culture model.

The in vitro culture model of fresh normal tendon explants does not replicate in vivo tendinitis injury or healing conditions particularly well, given the lack of vascular access or mechanical forces. However, the explant model does allow the study of the impact of exogenous PDGF supplementation on tenocytes and subsequent matrix changes. Explant culture was chosen over monolayer conditions for the present study because in vitro monolayer systems do not represent the impact of recombinant supplementation within a tissue and fail to account for the impact of treatment on matrix content.

In the present study, treatment of tendon explants for 6 days with rhPDGF-BB failed to corroborate the reported25,29 mitogenic and proliferative effects of PDGF-BB. Quantitative DNA assay results were supported by similar cellular density and cell shape in histologic sections from PDGF-treated and untreated tendons. Other investigators have demonstrated mitogenic activity of PDGF in tendon explant culture,25,29 and culture conditions and the rhPDGF-BB doses used in our study seemed similar to those published previously. The effects of rhPDGF-BB on total collagen content in equine SDFT explants in the present study were limited. However, collagen typically is slow to change in dense connective tissue. Gene expression can be a more sensitive indicator of collagen metabolism than protein formation, and collagen type I mRNA content in rhPDGF-BB–treated explants on day 6 of the temporal study was increased, compared with untreated control explants. Conversely, collagen type III gene expression was suppressed. This suggested that rhPDGF-BB has positive matrix-healing and tendon-remodeling effects. Extended culture periods may have yielded collagen accumulations, but the short-term analyses performed in our study involving quantitative total collagen assay and
immunohistochemical staining suggested few profound anabolic changes.

The autocrine feedback aspects of exogenous PDGF supplementation were biphasic, which complicates the conclusions of the present study. Results of the escalating-dose–response experiment suggested that by culture day 6, 100 ng of PDGF-BB/mL of medium had suppressed PDGF-A gene expression and to some extent also PDGF-B expression. However, assessment of the temporal changes in PDGF gene expression in SDFT explants treated with 100 ng of rhPDGF/mL of medium suggested that there was an initial positive autocrine feedback at 24 hours, which diminished or reversed later. Similar responses have been detected in studies of the in vitro autocrine feedback effects of IGF-I and TGF-β in equine and human chondrocytes and periosteum-derived cells. In combination, the results of these studies suggest caution should be used in attempts to interpret autocrine effects, which vary substantially with time of assessment. Moreover, autoduction of gene expression and protein of both PDGF isoforms in bone cells for as long as 24 hours following PDGF-BB treatment has been described.

Analysis of the dose-related effects of rhPDGF-BB supplementation on tendon explants revealed potential benefit at higher doses. On the basis of those data, a second experiment was performed to analyze the early impact of rhPDGF-BB supplementation on collagen type I gene expression and the early autocrine changes in PDGF-A and PDGF-B expression. Over a period of 6 days, treatment with PDGF-BB significantly increased the expression of type I collagen, although this increase in gene expression failed to culminate in a measurable increase in total collagen content of the explants by the end of the 6-day experiment. Static total collagen content potentially reflects a reduction in collagen type III expression with PDGF treatment and warrants further study of long-term and in vivo effects of PDGF.

Overall, the findings of these 2 experiments verify the potential benefit of PDGF-BB supplementation on healing in tendons, given that supplementation resulted in an increase in collagen type I mRNA, despite a lack of predicted cell proliferation and increased DNA content in the tendon explants. Furthermore, it appears that the autoductive effects of PDGF-BB are present early following supplementation, and continued treatment results in limited feedback and eventually a negative autocrine-paracrine response for both isoforms. Results of recent studies indicate that there is a benefit to treatment of tendinitis lesions with bone marrow aspirates or platelet-rich plasma concentrates because of their high concentration of PDGF, which is hypothesized to stimulate tendon healing. Our data from equine SDFT explants suggest that rhPDGF-BB alone induces useful but moderate effects on tendon healing and most likely requires a combination of other growth factors that are present in platelet-rich plasma or bone marrow aspirates to more effectively contribute to tendon healing and maturation.

Figure 3—Temporal gene expression of PDGF-A (A), PDGF-B (B), and collagen type I (C) in equine SDFT explants following no treatment (control specimens; circles) or treatment with rhPDGF-BB (100 ng/mL; inverted triangles). Gene expression was normalized to 18S RNA. Data are presented as mean values ± SEM derived from 6 samples. a, b, c, d = Within each graph, data points for each treatment with different classification letters are significantly (P < 0.05) different. *= Within each graph, data points for each treatment at individual time points are significantly (P < 0.05) different.

a. M199 medium, Gibco-Life Technologies, Grand Island, NY.

b. Sigma-Aldrich Co, St Louis, Mo.

c. TRIzol reagent, Gibco-Life Technologies, Grand Island, NY.

d. 6750 Freezer/Mill, Spex Certiprep, Metuchen, NJ.

e. RNeasy column, Qiagen, Valencia, Calif.

f. TaqMan One-Step RT-PCR kit, Applied Biosystems, Foster City, Calif.

g. ABI PRISM 7900 HT sequence detection system, Applied Biosystems, Foster City, Calif.
References


