Nucleotide structure of equine platelet-derived growth factor-A and -B and expression in horses with induced acute tendinitis

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Objective—To characterize the nucleotide sequence of equine platelet-derived growth factor (PDGF)-A and -B and analyze temporal expression of these genes in equine tendon after induced tendinitis injury.

Animals—18 mature horses.

Procedures—Genes for equine PDGF-A and -B were reverse transcribed and sequenced from synovial tissue mRNA obtained from a 3-year-old horse. Collagenase-induced lesions were created in the tendinotendinous region of the superficial digital flexor tendon in 14 horses; 3 horses served as uninjured control animals. Tendons were harvested and total RNA was isolated from experimental horses 1, 2, 4, 8, and 24 weeks after collagenase injection. Temporal gene expression for PDGF-A and -B was determined by use of quantitative PCR analysis.

Results—Equine PDGF-A shared 83.8% sequence and 87.5% peptide homology with human PDGF-A, with a discrepancy of 70 bp from the human sequence. Equine PDGF-B was similar in length to the human gene, sharing 90.3% and 91.7% nucleotide and peptide identity, respectively. Expression of PDGF-A mRNA in collagenase-induced tendinitis lesions was unchanged, compared with expression for normal control tendon, and remained steady throughout the 24-week study. Expression of PDGF-B mRNA decreased over time, and the expression at 24 weeks was significantly reduced, compared with expression in normal and acutely injured tendon.

Conclusions and Clinical Relevance—Injured tendon mounts a minimal constitutive PDGF-A or -B mRNA response. Serial exogenous treatment with either PDGF isoform within the first 2 to 4 weeks after tendon injury may bolster the meager PDGF paracrine-autocrine intrinsic response to injury. (Am J Vet Res 2006;67:1218–1225)

Injury of flexor tendons is common in recreational and competitive horses. Some injuries are a result of an extreme overload on the tendon and cause a gross tear. More often, however, injuries develop over a longer period and are the consequence of accumulated microtears in the tendon.1-3 These cyclical injuries induce a degradative enzyme flux that exacerbates fiber disruption and can propagate to a gross lesion that rarely reforms normal elastic architecture.4 In most cases, injured tendons heal slowly, and the resultant scar tissue is weaker and less elastic than normal tissue and therefore prone to further injury during return to work.5

The role of many growth factors in the healing process for tendons and ligaments has been documented.6 Growth factors are polypeptides that interact with cell receptors and direct many cellular responses, including cell migration, proliferation, and metabolic regulation, all of which are important during healing.5 In particular, PDGF is a potent mitogen found in α-granules of platelets and activated macrophages that induces chemotaxis and fibroblast proliferation. During tendon healing, it is hypothesized that PDGF influences collagen and proteoglycan extracellular matrix concentrations through 2 secondary messengers, IGF-I and TGF-β1.7,4

Platelet-derived growth factor is a dimeric protein formed from A and B chains that are coded by 2 genes. Through a disulfide linkage, the 2 chains form 1 of 3 dimers (PDGF-AA, PDGF-AB, or PDGF-BB), all of which are biologically active and found in mammalian tissues.8-10 The dimers bind to 2 tyrosine kinase receptors (α and β) in the cell membrane. The α receptor can bind both chain varieties and therefore binds all 3 dimers. However, the β receptor binds only the B chain and therefore has specificity for the PDGF-BB homodimer. Because of the differential binding of the protein, expression of PDGF receptors on various cells enables the isoforms to promote differing cellular responses and intensities. For example, addition of the 3 human PDGF isoforms to human wound fibroblast cultures results in varying effects on cell proliferation.11 Although all 3 isoforms of PDGF can have a beneficial effect on general wound healing, the temporal expression of PDGF during tendon healing is not fully understood or characterized. Knowledge of when PDGF has the greatest effect on tendon healing would enable use of this growth factor as a potential therapeutic agent for patients with tendinitis.

The purpose of the study reported here was to determine the temporal mRNA expression of PDGF-A

ABBREVIATIONS

PDGF Platelet-derived growth factor
IGF-I Insulinlike growth factor-I
TGF-β1 Transforming growth factor-β1
SDFT Superficial digital flexor tendon

1 Transforming growth factor-β
and -B in normal tendons and tendons with tendinitis caused by injection of collagenase. At the time the study was conducted, the PDGF-A and -B genes were not characterized for the equine species; therefore, both of these genes were cloned. Published human sequences were used to design primers, and clones were developed and subsequently sequenced. Probes were then generated for real-time PCR analyses to determine the gene expression of PDGF-A and -B in healing tendons.

Materials and Methods

Cloning and sequence determination of equine PDGF-A and -B—Samples of synovial tissue were aseptically harvested from a 3-year-old horse. The tissue was then pulverized by use of a freezer mill and liquid nitrogen. The RNA was extracted by use of a phenol and guanidine isothiocyanate solution and chloroform, and further purified by use of RNA affinity columns. Quantification of RNA was accomplished by use of UV spectrophotometry at 260 nm, and RNA quality was assessed from the ratio of absorbance at 260 and 280 nm and evidence of ribosomal RNA on a 1% agarose gel. RNA was subsequently stored at –80°C.

Reverse transcriptase was used to reverse transcribe RNA to complementary DNA (cDNA). The cDNA was primed by use of (dT) oligonucleotides to hybridize the 3’ poly (A) tails of mRNA. The cDNA was amplified and then treated with RNase H to remove the RNA template. The reverse-transcribed cDNA template was used to clone the coding sequences of PDGF-A and -B genes by use of PCR procedures. Published sequences of human PDGF-A (GenBank X03795) and -B (GenBank BC029822) were used to design primers (Appendix 1). Regions of high nucleotide homology among human, bovine, ovine, and murine sequences were generally better annotated and representative for use in primer design. The PCR mixture was added to thin-walled PCR tubes and placed in a thermocycler. The PCR conditions included 95°C to activate the DNA polymerase and 30 cycles of 1 minute at 95°C, followed by 55°C for 30 seconds and 72°C for 1 minute. The resulting cloned products were evaluated on 1% agarose gels for comparison of anticipated nucleotide products.

Transformation of clones was accomplished by use of a cloning vector. The cloning reaction, which consisted of 1 μL of cloning vector and 4 μL of PCR cloned product, was added to a vial of chemically competent Escherichia coli and incubated on ice for 5 to 30 minutes for transformation. Cells were incubated at 37°C for 1 hour, and aliquots were then transferred to plates containing Luria-Bertani medium with 50 μg of ampicillin/mL. Plates were coated with 40 μL of 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/mL and 100 mM isopropyl-β-D-thiogalactopyranoside and then incubated overnight at 37°C.

Positively transformed colonies were identified as white or light-blue colonies. Positively transformed colonies were transferred to agar plates containing ampicillin-selective Luria-Bertani medium and then incubated overnight at 37°C. Colonies from these plates were used to perform colony PCR analyses and subjected to gel electrophoresis (1% agarose gels) to confirm that the transformed cells contained the correct fragment size. Conditions for the PCR procedure were the same as described previously, except that E. coli colonies were used as the template. Colonies were transferred by use of a toothpick and placed in PCR tubes, which were then incubated for 5 minutes. The PCR assay was conducted by use of a thermocycler, beginning with incubation for 10 minutes at 95°C. This was followed by 30 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C. The PCR procedure was completed by incubation for 7 minutes at 95°C. Plasmid DNA from transformed colonies was then purified by use of a commercially available kit. An EcoRI restriction enzyme was used to excise DNA inserts, and plasmid DNA was evaluated for size by use of gel electrophoresis (1% gels). Ultraviolet spectrophotometry (260 nm) was also performed on purified DNA for quantification.

The PDGF-A and -B clones of correct size and highest DNA concentrations were sequenced. Two DNA samples from each colony were sequenced in forward and reverse directions to generate a consensus sequence. Sequencing was performed by use of an automated DNA analyzer with universal primers.

Sequence data from each clone were aligned against each other by use of commercial alignment software, and a consensus sequence was generated. The consensus sequence was further compared with sequences of other species. The predicted protein sequence was determined by use of commercial computer software and compared with PDGF protein data for other species. The percentage of homology was determined by use of commercial alignment software.

Temporal expression of PDGF-A and -B during tendon healing—Seventeen clinically normal mature (2- to 6-year-old) horses (7 males and 10 females) without a history of tendinitis were used for the study. These horses were used for other experiments, the results of which have been reported elsewhere. Experimental horses were administered nonsteroidal anti-inflammatory drugs to prevent inflammation and pain. Tendinitis lesions were induced in the tensile region of the SDFT of both forelimbs of 14 experimental horses by injection of filter-sterilized bacterial collagenase type I diluted in sterile water. Time of injection was designated as week 0. Horses were sedated, hair was clipped, and the skin aseptically prepared. Regional nerve blocks were used for management of pain. A total of 2,097 units of collagenase (in a volume of 0.30 mL) was administered at 2 sites that were 1 cm apart and centered in the tensile region of the SDFT to create 1 continuous lesion. Horses were confined to stalls for the duration of the study, with walking exercise initiated at 4 weeks after collagenase injection. Forelimbs were bandaged for 4 weeks after injection to control swelling. Horses were allocated into 4 groups of 3 horses and 1 group of 2 horses. Horses were euthanized by barbiturate overdose at 1, 2, 4, 8, or 24 weeks after collagenase injection (3 horses for weeks 1, 2, 4, and 8 and 2 horses for week 24). For control samples, SDFT tissue was harvested at week 0 from both forelimbs of 3 healthy control horses that were not treated with collagenase.

After the experimental horses were euthanized, the SDFT from the carpus to the distal portion of the metacarpus was harvested for analysis by use of RNase-free conditions and stored on ice for immediate dissection. Tissue samples for RNA isolation were collected from grossly damaged areas. Tendon samples were snap frozen in liquid nitrogen for analysis of gene expression and stored at –80°C until the RNA was isolated by use of the procedures described previously. Samples for histologic examination were dissected and included lesions and a portion of the surrounding normal tendon; these samples were fixed in 4% paraformaldehyde and stored at 4°C until processed. Parafomaldehyde-fixed sections were dehydrated, cleared in xylene, embedded in paraffin, sectioned, and mounted on microscope slides. Morphologic characteristics were examined by use of H&E stain.

The RNA was isolated from tendon specimens by use of procedures described previously. Gene expression was quantitated by use of real-time PCR procedures (a 1-step real-time PCR technique and DNA polymerase). Primers and fluoros...
Figure 1—Complete coding sequence for the equine PDGF-A prepropeptide and the human, mouse, and rat PDGF-A sequences. The 581-nucleotide coding sequence shared 83.8%, 85.6%, and 86.0% homology with human, rat, and mouse sequences, respectively. Differences in nucleotide sequence between species are indicated. Overlined codon represents the stop codon for translation end point in human and rat sequences. Numbers on the right side indicate nucleotide number.

Figure 2—Peptide structures of equine PDGF-A (deduced from the primary nucleotide sequence) and human PDGF-A. The 192-amino acid sequence of equine PDGF-A shared 87.5% homology with the amino acid sequence for human long-form PDGF-A protein. Homology of the amino acid sequence between equine and human is indicated by boxed regions.
cent probes were designed from the determined equine sequence by use of primer software. Total copy number of mRNA was obtained for the 2 genes of interest from validated standard curves adjusted on the basis of 18S RNA expression. Gene expression data for each horse were recorded, and differences over time were compared to detect statistical differences.

Statistical analysis—Differences in mRNA expression among weeks were analyzed by use of an ANOVA with a Tukey honest significance difference test. Expression of PDGF-A and the logarithm of PDGF-B expression were used to determine significant differences. Commercially available software was used for the statistical analysis. Significance was defined at values of $P < 0.05$.

Results

Characterization of nucleotide sequence—Nucleotide sequence for the 581-bp equine PDGF-A complete coding sequence was compared with human, mouse, and rat sequences (Figure 1). Nucleotide identity between the horse and human sequences was 83.8%. Homology of the horse sequence with rat and

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<td>Rat</td>
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Figure 3—Complete coding sequence for the equine PDGF-A prepropeptide and the human, mouse, and rat PDGFA sequences. The 726-nucleotide coding sequence shared 90.3%, 89.5%, and 85.1% homology with human, sheep, and rat sequences, respectively. See Figure 1 for remainder of key.
mouse sequences was 85.6% and 86.0%, respectively. However, there was a 70-bp segment that was not detected in the horse and mouse sequences that was evident in the human (long-form variant) and rat sequences. Additionally, a 12-bp deletion was unique to the equine nucleotide sequence, compared with all other species. Homology between the horse and human PDGF-A short-form variant sequences was also analyzed. The human short-form variant lacked a 69-bp segment, compared with the human long-form variant, in the same location as for the equine sequence.

The amino acid sequence for equine PDGF-A was derived from the nucleotide sequence and shared 87.5% homology with the human sequence (Figure 2). For the equine PDGF-A sequence, the signal peptide spanned bases 1 to 60; the 66 amino acid propeptide extension region followed, being encoded by bases 61 to 258; and the 106 amino acid mature peptide was encoded by bases 259 to 576. The mature peptide in humans contains 125 amino acid residues, compared with 106 for the sequence in horses (despite an 82-bp nucleotide difference), attributable to an alternative active stop codon included only in the nucleotide sequence for human and rat sequences.

Sequence data for the 726-bp coding sequence of equine PDGF-B were determined and aligned with human, sheep, and rat sequences (Figure 3). Sequence homology with other species was 90.3% for human, 89.5% for sheep, and 85.1% for rat. Homology of the amino acid sequence for PDGF-B between horse and human sequences was 91.7% (Figure 4). The 13-amino acid signal peptide (nucleotides 1 to 45) and 161-amino acid mature peptide (nucleotides 244 to 726) were also similar to that of humans, except for an additional codon in horses that provided a sequence for an additional amino acid, compared with the 160-amino acid composition of human PDGF-B.

The nucleotide and amino acid sequences for equine PDGF-A and -B were aligned and compared. This comparison revealed homology of 50.4% for the nucleotide sequences, and the amino acid sequences had homology of 41.7%.

Temporal expression of PDGF-A and -B mRNA in horses with tendinitis—Gene expression for PDGF-A and -B were determined from RNA isolated from healing equine tendons after injection of collagenase to induce lesions. Histologic characterization of tendinitis lesions at each time period has been described elsewhere. Tissue amounts of PDGF-A mRNA were determined (Figure 5). Although mRNA expression for PDGF-A did not have a discernable pattern during the 24-week period, copy number of PDGF-A mRNA expression at weeks 1 and 4 were significantly lower, compared with values at week 8.

Tissue amounts of PDGF-B mRNA were determined (Figure 6). Expression of mRNA for PDGF-B decreased steadily during the 24-week period. Copy number of PDGF-B expression for week 24 was significantly lower, compared with values for control horses at week 0 and values for experimental horses at 1 week after induced injury.

Discussion

The complete coding sequences of equine PDGF-A and -B were determined and compared to human sequences. Equine PDGF-B nucleotide and amino acid sequences had similar homology and total length with those of human PDGF-B. However, equine PDGF-A had a 70-bp deleted segment in the same location as that for the human short-form variant of PDGF-A. This discrepancy may have been attributable to the fact that there are 2 variant splice forms of the human nucleotide sequence (ie, a
It is unknown whether horses have both variants of PDGF-A. It is possible that the PDGF-A long-form variant was cloned and not chosen for sequencing because a 70-bp difference was overlooked during gel electrophoresis of PCR products. However, flanking primers to this region failed to yield a doublet on gel electrophoresis, which suggested that an alternate variant form was unlikely or minimally expressed. To confirm whether the long-form variant exists, it would be necessary to clone PDGF-A by use of primers designed for areas within the 70-bp segment.

Temporal expression of the PDGF-A gene in tendons with collagenase-induced lesions appeared to have no discernible pattern. The amounts of PDGF-A mRNA expression remained relatively stable during the 24-week period, with the amounts in normal tendons of control horses at week 0 higher than amounts in experimental horses at weeks 1 through 4. Expression of PDGF protein was not determined because of a lack of equine-specific assay reagents. It has been suggested that there is substantial early increase as a result of platelet attraction to tendinitis lesions with subsequent platelet degranulation and release of growth factors. One possible reason for the PDGF-A mRNA amounts that were lower than expected during weeks 1 through 4 was poor PDGF mRNA production by tendon fibroblasts. Platelets are the first cells to migrate to an injured area (almost immediately after injury), and they become activated and release growth factors into those areas. It appears from the lack of changes in PDGF expression by tenocytes that these growth factors would have to be released from the platelets and not by the tendon fibroblasts and support structures. It should be mentioned that in addition to tenocytes, PDGF mRNA can be produced by macrophages and endotenon cells, both of which were found in the experimental system. The lack of gene expression of PDGF has been observed in other healing tissues. It has been determined in wounds in the skin of mice and damaged Achilles tendons of rats that there are no significant differences in PDGF gene expression during healing.

Expression of PDGF-B mRNA in the lesions decreased over time during the 24-week period. Amounts of PDGF protein during wound healing are highest soon after injury and remain high for several weeks during healing, before tapering off to expected values for healthy tissues. The gradual decrease of PDGF-B gene expression during the 24-week period seemed logical because PDGF is most active during the healing process, when it stimulates cell proliferation and metabolic regulation. This correlates with histologic evidence from normal and injured tendons. Increased cellularity after injury, compared with cellularity for normal tissue, is evident for several weeks but then tapers off. Unexpectedly, the expression of PDGF-B in normal tendons of control horses was higher than expression in tendons during acute tendinitis and in healing tendons, which makes it unlikely that PDGF-B gene expression was induced by the injury. Indeed, it could be hypothesized that exogenous PDGF protein from platelet degranulation downregulated the production of constitutive PDGF mRNA by tendon fibroblasts. This hypothesis also implies that PDGF initiates a negative feedback loop that diminishes the initial signal for PDGF gene expression, possibly through stimulation of endogenous inhibitory substances, such as β fibroblast interferon. It has been suggested that PDGF protein induces a positive autocrine effect, thereby increasing PDGF gene expression. Culture of human mesangial cells revealed that addition of PDGF-BB increased expression of PDGF-A and -B mRNA. However, cell-specific effects vary, and a study that used rat osteoblasts revealed that PDGF-BB did not enhance amounts of PDGF-B mRNA. Analysis of results of
those studies indicates tissue variation in the regulation of PDGF-A and -B genes. Nevertheless, the PDGF system during acute injury is confounded by a rich source of protein, but not mRNA, coming from platelets. Because platelets do not have a nucleus, they have no opportunity to mount a local autocrine response. In addition, the amount of protein could have varying effects on PDGF gene expression. It is also possible that PDGF protein can elicit positive and negative feedback responses, which would be determined on the basis of the sampling time and amount of protein in an injured area.

Two other growth factors (IGF-I and TGF-β1) examined in the collagenase-induced model of acute tendinitis had temporal differences in mRNA and protein expression during the 24-week period. The IGF-I mRNA and protein concentrations increased early during healing, then peaked at 4 weeks and decreased through 24 weeks. Expression of TGF-β1 mRNA increased markedly immediately after injury, compared with values for the control horses, and steadily decreased during the 24-week period. Expression of TGF-β1 protein increased more gradually after induced injury, compared with values for uninjured control horses, and reached a maximum at week 4. Because the decreases in PDGF mRNA expression became consistently greater as the interval after the acute injury increased, examining concentrations of PDGF protein would be of interest because of the hypothesized use of IGF-I and TGF-β1 as secondary messenger systems for the promotion of tendon healing.

Although more research centered on the control of constitutive PDGF gene expression and feedback effects are necessary, the beneficial role of PDGF during healing is undisputed. Development of a protein assay specific for equine PDGF would enable gene expression to be correlated with protein concentrations in platelets, macrophages, and fibroblasts in healing and normal tendons. The source of PDGF in healing tissue, whether intrinsic, extrinsic, or a combination of both, could then be determined. Knowledge of the temporal protein concentrations would also enable investigators to design exogenous PDGF treatments and evaluate the most opportune time for their use.

References


a. 6750 Freezer Mill, Spex CertiPrep, Metuchen, NJ.

b. Trizol, Gibco-Life Technologies, Grand Island, NY.
c. RNeasy, Qiagen, Valencia, Calif.
d. Superscript first-strand synthesis, Invitrogen, Carlsbad, Calif.
e. 9600 GeneAmp PCR system, Perkin-Elmer Inc, Boston, Mass.
f. Taq Gold DNA polymerase, Applied Biosystems, Foster City, Calif.
g. pCRII-TOP0, Invitrogen, Carlsbad, Calif.
h. X-gal, Sigma-Aldrich Corp, St Louis, Mo.
i. Qiagen spin minikit, Qiagen, Valencia, Calif.
j. DNA sequencing facility, Bioresource Center, Cornell University, Ithaca, NY.
k. MegAlign, DNASTAR Inc, Madison, Wis.
l. EdiSeq, DNASTAR Inc, Madison, Wis.
m. Collagenase, Sigma-Aldrich Corp, St Louis, Mo.

n. Absolute quantitative PCR, ABI PRISM 7900 HT sequence detection system, Applied Biosystems, Foster City, Calif.
o. Primer Express software, version 2.0, Applied Biosystems, Foster City, Calif.
Appendix 1

Primer sequences used to develop equine-specific clones of PDGF-A and -B for use in transformation and sequencing.

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<th>Forward primer</th>
<th>Reverse primer</th>
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Appendix 2

Primer and probe sequences for real-time quantitative PCR analysis of gene expression for PDGF-A and -B.

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