Cloning and expression of equine insulin-like growth factor binding proteins in normal equine tendon

Linda A. Dahlgren, DVM, PhD, and Alan J. Nixon, BVSc, MS

Objectives—To define a portion of the nucleotide sequences of each of the 6 insulin-like growth factor (IGF) binding proteins (IGFBPs) in horses and describe patterns of messenger RNA (mRNA) and protein expression for IGFBPs in normal equine tendons.

Animals—7 horses.

Procedure—Total RNA was extracted from the tensile region of normal superficial digital flexor tendons and reverse transcribed into complementary DNA (cDNA). The cDNA was amplified via PCR, and products representing portions of each IGFBP were cloned and sequenced. Nucleotide sequences were used to deduce the amino acid sequences, and both nucleotide and predicted amino acid sequences were compared with those published for bovine, human, mouse, and ovine IGFBPs. Gene expression was quantitated by real-time PCR assay, and protein expression was evaluated by western ligand blot (WLB).

Results—Clones ranged in size from 262 to 522 bp and had high degrees of sequence homology with other mammalian species. Sequence homology was highest between bovine and equine IGFBPs (86% to 95%) and amongst the IGFBP-5 sequences from the various species (92% to 95%). Message for IGFBP-2 to -6, but not IGFBP-1, was expressed in normal tendon. Protein expression for IGFBP-2, -3, and -4 was detected by WLB in normal tendon and markedly increased in damaged tendons.

Conclusions and Clinical Relevance—Results provide basic information and tools needed for further characterization of the role of the IGF system in tendon healing and may lead to the ability to potentiate the response of healing tendon to exogenous IGF-I via concurrent manipulation of IGFBPs. (Am J Vet Res 2005;66:300–306)

Tendinitis is a common injury of athletic individuals of many species that can be recalcitrant to treatment. Intratendinous administration of anabolic growth factors such as insulin-like growth factor (IGF)-I may improve the healing response of tendon by enhancing the cellular response to injury. Insulin-like growth factor-I is produced in a variety of tissues, including normal and injured tendons. Exogenous IGF-I treatment augments the healing process in injured tendon through the stimulation of extracellular matrix synthesis and cell proliferation. The biological effects of IGF-I are exerted through the complex network of the IGF system, which includes 2 ligands (IGF-I and IGF-II), 2 IGF receptors (type I and type II), a family of secreted IGF binding proteins (IGFBPs), and IGF proteases. Insulin-like growth factor-I binds to the type I IGF receptor located in the target cell membrane, triggering a series of phosphorylation events that propagate a signal from the cell membrane to the nucleus. The end result of this complex signal transduction pathway is the modulation of gene expression and the triggering of anabolic responses within the cell.

The IGFBPs are a family of 6 structurally related, high-affinity binding proteins that regulate the bioavailability and activity of IGF-I within the body. The IGFBPs differ in size and in their binding properties for IGF-I and IGF-II, posttranslational modifications, and biological functions. The primary structure of the IGFBPs consists of 3 domains of approximately equal size. The N- and C-terminal domains are characterized by a high degree of conservation and contain a series of conserved cysteine residues that are involved in intradomain disulfide bond formation. The conserved domains also play an important role in a variety of protein-protein interactions and contain major IGF binding sites. The N- and C-terminal domains are joined by a weakly conserved linker region that gives each binding protein a distinct function. This region is unique to each binding protein and contains the cleavage sites for proteolysis and the sites for posttranslational modifications (glycosylation and phosphorylation).

The IGFBPs function to restrict the access of IGF-I to the receptor and control the insulin-like side effects of IGF-I. Another important action of the IGFBPs is to protect IGF-I from pericellular proteases, increasing the half-life of IGF-I from 10 minutes to 10 hours. The biological activities of the IGFBPs facilitate the storage of IGF-I in the extracellular space, making the IGF-I peptide readily available to respond to changes in the cellular environment. In addition to IGF-dependent functions, the IGFBPs exert IGF-independent effects via distinct cell surface receptors. Through these diverse biological roles, individual IGFBPs may inhibit or potentiate the effects of IGF-I.

Most of the circulating IGFBPs are produced in the
the IGFBPS would have unique nucleotide sequences, but with a high degree of homology to other mammalian species, and that the IGFBPs would have a unique pattern of expression in tendon tissue.

Materials and Methods

Tissue harvest—The tensile region of the superficial digital flexor tendons from 7 healthy adult horses was used for this study. The horses were donated to the Cornell University Hospital for Animals for reasons unrelated to this study. The horses were euthanatized for use in unrelated studies. Tendons were harvested under ribonuclease-free conditions and snap-frozen in liquid nitrogen for gene expression studies or rinsed in protease inhibitors and snap-frozen in liquid nitrogen for biochemical analysis.

RNA isolation and gene cloning—Frozen tendon specimens were cooled to −196°C and pulverized in a freezer mill, and total RNA was isolated by use of the guanidinium chloride-phenol extraction method and column purification. First-strand complementary DNA (cDNA) was synthesized by use of oligo(dT) priming and a commercially available reverse transcriptase kit. Regions of high sequence homology were identified as targets for cloning on the basis of mammalian sequence data (Appendix). Primers were designed that spanned these regions by use of the appropriate human sequence data for each binding protein (Table 1). Polymerase chain reaction was performed by use of a short-cycle PCR technique, which included denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and amplification for 30 cycles. Two microliters of cdNA was amplified with 1 unit of DNA polymerase in a final volume of 25 µL containing 10X PCR assay buffer, 0.2mM deoxyribonucleotides (dNTPs), and 2µM of each primer. The PCR products were cloned into a plasmid vector by use of a commercial kit. Colonies verified to contain the correct insert were grown for 18 hours in culture for automated DNA sequencing. A consensus sequence was generated from forward and reverse sequencing of 4 independent clones for each binding protein and was compared with those published for bovine, human, mouse, and ovine. Alignment reports were generated by use of the Clustal V Method. These sequences were subsequently used for primer and probe design for gene expression studies.

Gene expression—Gene expression for IGFBP-1 through -6 was quantitated by fluorescence-based real-time PCR assay by use of a 1-step reverse transcriptase PCR technique. The primers and dual-labeled fluorescent probe ([6-carboxy-fluorescein] 6-FAM as the 5′ label [reporter dye] and TAMRA [6-carboxy-tetramethylrhodamine] as the 3′ label [quenching dye]) were designed by use of a commercially available software program (Table 2). Total copy number of mRNA was obtained for each gene of interest from a previously validated standard curve constructed with six 10-fold serial dilutions of samples having a known copy number of the plasmid DNA insert. Standard curves were processed simultaneously with the samples. These values were then standardized to 18S ribosomal RNA expression (as determined by mass) and reported per nanogram of RNA. Cross-reactivity between primer-probe pairs of each IGFBP was

<table>
<thead>
<tr>
<th>Target</th>
<th>5′ Primer</th>
<th>3′ Primer</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-1</td>
<td>CCAAGAAGCTGCAAGAAGAAG</td>
<td>GATCTTCTCCACACTCAAG</td>
<td>346</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>CCGTCGAAGACCTTCAGCTCG</td>
<td>GCCTCCCTGTCTCTGTATTAG</td>
<td>423</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>CGCTCTGAGGAAATGCAGAGT</td>
<td>GTGAGTAGCACACCACGAG</td>
<td>501</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>CCGACGAAGCTCCCTCAGTGG</td>
<td>CTTCGCGCGCGGCTCTGCTTG</td>
<td>522</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>TGGCCTGCGCGCTGCTCAGAAG</td>
<td>CGGAGATCGGCTGCTCTTG</td>
<td>309</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>GGAGCTTAACCCCAAGACAG</td>
<td>CTTGGGATGATGGCACTG</td>
<td>252</td>
</tr>
</tbody>
</table>

Table 2—Primer and probe sequences used for real-time PCR assay quantification of gene expression of IGFBP-1 to -6 and 18S ribosomal RNA in tendon samples from healthy horses.

<table>
<thead>
<tr>
<th>Target</th>
<th>5′ Primer</th>
<th>3′ Primer</th>
<th>Probe</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-1</td>
<td>TTAATCTGCGCAATCCGACAGAAGAAGA</td>
<td>CCAACCAATGCGAGCACTTC</td>
<td>CCAACCAATGCGAGCACTTC</td>
<td>85</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>CCACCTGAGAACCCTTCACCTTGC</td>
<td>TGAACCAATGCGAGCACTTC</td>
<td>TGAACCAATGCGAGCACTTC</td>
<td>74</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>GAAAGGCTGGCGAGAAGG</td>
<td>TCTGGGTTGTGCTCTACGAGA</td>
<td>TGAACCAATGCGAGCACTTC</td>
<td>68</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>CGGCGGATCTACGGAAGAACCT</td>
<td>CTGGGCTGACAGCTGACCT</td>
<td>TGAACCAATGCGAGCACTTC</td>
<td>93</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>GGTGTTGCTGAGCCAGAAGAAGA</td>
<td>CTTGTGTCTGAGACTGCTTCT</td>
<td>TGAACCAATGCGAGCACTTC</td>
<td>69</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>GGTGTCGCAAGGACGACAGAGAG</td>
<td>TGGTGATGCGCTGCTGCGCTG</td>
<td>TGAACCAATGCGAGCACTTC</td>
<td>116</td>
</tr>
<tr>
<td>18S</td>
<td>CCGCTTGGTGGACCTAGTAAACC</td>
<td>CCGTTCGGGACGACGGGCT</td>
<td>TGAACCAATGCGAGCACTTC</td>
<td>115</td>
</tr>
</tbody>
</table>
tested with the different plasmid templates from each of the standard curves.

**Protein expression**—Protein expression for the IGFBPs was investigated by use of western ligand blot (WLB) techniques. Frozen tendon specimens were cooled to −196°C, pulverized, lyophilized, and solubilized in nonreducing SDS-PAGE loading buffer. Tendon samples were separated on 12% SDS-PAGE gels under nonreducing conditions. Blotting was performed as described, with modifications. Proteins were transferred to a 0.45-μm nitrocellulose membrane at 15 V for 18 hours at 4°C in a sealed bag. After transfer, the membranes were blocked and probed for 2 to 3 hours at 4°C in a sealed bag with 4×106 counts/min each of iodine 125-labeled IGF-I and IGF-II. Membranes were washed, dried, and exposed to radiographic film at −70°C for 1 to 7 days. Autoradiographs were generated and scanned on a flatbed scanner to quantitate protein expression by use of pixel intensity as a measure of band intensity.

**Western immunoblot (WIB) techniques** were used to verify the identification of bands from the WLBs. Tendon samples were separated and transferred to nitrocellulose, as described for WLB. Membranes were incubated with primary antibodies (caprine anti-human IGFBP-1, IGFBP-3, IGFBP-4, and IGFBP-5; rabbit anti-bovine IGFBP-2; and rabbit anti-human IGFBP-6) according to the manufacturer’s instructions. Secondary antibodies (rabbit anti-goat IgG, donkey anti-rabbit IgG) conjugated with horseradish peroxidase were selected on the basis of the species specificity of the particular primary antibody. Enhanced chemiluminescence detection reagents were used for immunodetection.

**Results**

**RNA isolation and cloning**—Equine clones ranging in size from 262 to 522 bp were obtained for IGFBPs 1 to 6 by use of standard cloning and sequencing techniques. Successful PCR amplification using the primer pairs for IGFBP-4 required the use of a commercial kit designed to facilitate PCR amplification of G-C-rich sequences. Initial attempts to amplify a product using several primer pairs for IGFBP-1 were not successful in producing a product of the correct size. Equine liver was selected as a particularly IGFBP-1-rich tissue on the basis of the available literature and was successfully used to obtain the desired PCR product by use of the described IGFBP-1 primer pair (Table 1).

The 6 partial clones varied in location based on area of highest sequence homology selected for primer design. All clones were located within the mature peptide domain of the proteins. Alignment of the predicted amino acid sequences for the 6 cloned equine IGFBPs demonstrated the 3 distinct structural regions, N-terminal domain, linker region, and C-terminal domain, characteristic of the IGFBPs (Figure 1). Clones for IGFBP-1, -2, -3, and -6 contained partial sequences for the linker region plus the C-terminal domain. The clone for IGFBP-5 contained partial sequence for the N-terminal domain plus the linker region. The clone for IGFBP-4 contained partial sequence for all 3 domains. The N- and C-terminal domains contained the well-conserved cysteine residues characteristic of these conserved domains. In addition, highly conserved glycine and glutamine residues were detected in the equine proteins.

The nucleotide sequences for the cloned regions of IGFBPs 1 to 6 (Figure 2) had a high degree of sequence homology, compared with those published for bovine, human, mouse, and ovine IGFBPs (Table 3; Appendix). For each binding protein, sequence homology was highest between equine and bovine (86% to 95%), followed by ovine (83% to 94%), human (75% to 95%), and mouse (69% to 92%). Mean sequence identity across species was highest for IGFBP-5 (92% to 95%), followed by IGFBP-4 (89% to 95%), IGFBP-2 (89% to 93%), IGFBP-3 (81% to 94%), IGFBP-6 (69% to 90%), and IGFBP-1 (75% to 86%). The predicted amino acid sequences were compared between equine, bovine, human, and ovine for each of the partial binding proteins cloned. Amino acid sequences were highly conserved between the species and to a similar degree to those described at the nucleic acid level.

**Gene and protein expression**—Results of realtime PCR were consistent with results of the cloning of the 6 IGFBPs. There were no detectable levels of...
Figure 2—Partial nucleotide and predicted amino acid (aa) sequences for equine IGFBP-1 to -6 from cloned regions. The shaded regions indicate differences between the equine (Eq) and human (Hu) sequences. The nucleotide count for the partial equine sequences is listed on the right.
mRNA for IGFBP-1 in equine tendon. Positive controls using liver and kidney RNA amplified well by use of the described primer-probe sets (Table 2). Mean copy number per nanogram of RNA ($\pm$ SD; n = 7 horses) for the 5 remaining IGFBPs in normal equine tendon was as follows: IGFBP-2 ($1.0 \pm 0.6$), IGFBP-3 ($6.0 \pm 5.3$), IGFBP-4 ($19.4 \pm 3.9$), IGFBP-5 ($7.5 \pm 2.4$), IGFBP-6 ($332.1 \pm 70.7$).

Protein content of the IGFBPs was universally low in normal tendon (Figure 3). Only 1 IGFBP (apparent molecular weight [MW], 32 kd) was readily detectable above background amounts in normal tendon and was confirmed to be IGFBP-2 by WIB. The IGFBPs of apparent MWs of 24 and 38 to 42 kd were also expressed in extremely low amounts just above background in normal tendon and were confirmed to correspond with IGFBP-4 and -3, respectively. The characteristic doublet of bands described in the literature was evident for IGFBP-3 in equine tendon. Mean pixel intensities of tendon samples ($\pm$ SD, n = 5 horses) for the scanned WLBs of tendon samples were as follows: IGFBP-2 ($24.4 \pm 3.8$), IGFBP-3 ($19.0 \pm 1.4$), and IGFBP-4 ($22.1 \pm 2.2$).

**Discussion**

To the authors’ knowledge, this is the first study characterizing the pattern of gene expression and protein production of the IGFBPs in tendon of any species. Messenger RNA encoding 5 of the 6 IGFBPs (IGFBP-2 to -6) was expressed in equine tendon tissue. Protein production for IGFBP-2, -3, and -4 was detected by WLB, with IGFBP-2 being the most abundant. Although all cell types synthesize at least 1 binding protein, each cell type appears to express a different combination of IGFBPs. The individual IGFBPs produced in a specific tissue are most likely a function of the specialized nature of each cell type in the body.

In the study reported here, the pattern of expression for IGFBP mRNA and protein in tendon tissue is most similar to those described for bovine endothelial cells. The lack of association between the pattern of message expression and protein production has been described by other researchers. This disparity may have been caused by variation in the efficiency of PCR reactions for the various IGFBP mRNAs or lack of translation into protein for a variety of cellular and environmental reasons. Additionally, limitations in the sensitivity of the WLB assay to low protein content may have obscured expression profiles for IGFBP-5 and -6.

The lack of expression of IGFBP-1 in tendon is consistent with findings of other reports in bovine endothelial cells, human vascular smooth muscle cells, ovine growth plate cartilage, and human gastric cancer cells, all of which expressed little or no IGFBP-1. The IGFBP-1 is expressed primarily in the liver and kidney and functions in metabolism and reproduction. The importance of the lack of expression in tendon is not known; however, it is presumed to simply be a function of the specialized nature of tendon fibroblasts.

The IGFBPs are a highly conserved family of proteins that retain a range of diverse biological functions, a contrast made possible by the combination of the 2 conserved domains and the diverse linker region. The high degree of structural homology reflects the important regulatory processes in which the IGFBPs are involved. The 6 cloned mammalian IGFBPs share an overall 50% protein sequence homology within a species and approximately 80% nucleotide sequence homology between species. In the study reported here, the partial sequence information available from the clones is consistent with that described in other mammalian species. The equine clones had the characteristic pattern of conserved cysteine residues in the N- and C-terminal domains. In addition, 4 of the 6 equine clones included the C-terminal region that contained...
tains the conserved glycine and glutamine residues that appear to have a conserved IGF-I binding function.\textsuperscript{10}

As expected, for each individual binding protein there is a high degree of homology between the partial equine sequences and those from other mammalian species. Nucleotide sequences ranged from 69% to 95% homology. Amino acid alignments ranged from 65% to 98% homology. The IGFBP-5 had the highest degree of sequence homology (97% to 98% at the amino acid level). This finding is consistent with findings of other reports.\textsuperscript{10,11} Bovine sequence was most similar to that of the horse (approx 92% homologous). Mouse sequence was least similar (approx 85%). Despite the moderately high degree of homology, there are species differences in the amino acid sequences of the proteins. These differences may prove problematic with respect to the cross-reactivity of antibodies between species, complicating the use of various commercial assays in equine studies.

The biological actions of the IGF system are diverse, and the IGFBPs play an important role in regulating this system. The binding proteins lend a degree of tissue specificity to the system through the specialized role of the different IGFBPs and the posttranslational modifications that serve to further modify their biological activities.\textsuperscript{12} For these reasons, the pattern of expression of the IGFBPs in tendon tissue is important in understanding the role of the IGF system in tendon physiology and repair.\textsuperscript{4} Concentrations of the IGFBPs are low in normal equine tendon. Results of a study\textsuperscript{12} in injured equine tendon suggest that the IGFBPs in tendon are highly responsive to injury and are upregulated as a part of the normal healing response. This finding is consistent with that in other tissues.\textsuperscript{18,19} Investigations into the role of the IGFBPs in tendon healing will expand our knowledge of the overall role of the IGF system in the tendon healing process. Manipulation of the IGFBPs and related proteins in coordination with adjunctive IGF-I treatments may have the potential to result in a more robust anabolic response and enhance the tendon repair process.

References


### Appendix

GenBank accession numbers for nucleic acid and amino acid sequences used for sequence homologies in Table 3.

<table>
<thead>
<tr>
<th>IGFBP</th>
<th>Bovine Nucleic acid</th>
<th>Human Nucleic acid</th>
<th>Mouse Nucleic acid</th>
<th>Ovine Nucleic acid</th>
<th>Bovine Amino acid</th>
<th>Human Amino acid</th>
<th>Mouse Amino acid</th>
<th>Ovine Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP-1</td>
<td>XS4879</td>
<td>XM_004688</td>
<td>CA68770</td>
<td>AF327650</td>
<td>P24591</td>
<td>P226959</td>
<td>P226959</td>
<td>AAG48350</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>AF074854</td>
<td>M35410</td>
<td>L05439</td>
<td>S44612</td>
<td>AA004862</td>
<td>NP_000588</td>
<td>NP_032388</td>
<td>S44612</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>AF305712</td>
<td>X84876</td>
<td>X81581</td>
<td>AF327651</td>
<td>P20959</td>
<td>P22692</td>
<td>P22692</td>
<td>AA04851</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>SS2270</td>
<td>NM_001526</td>
<td>NM_010517</td>
<td>AF327653</td>
<td>Q05716</td>
<td>NP_000590</td>
<td>NP_032370</td>
<td>S77394</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>SS2857</td>
<td>AF055033</td>
<td>NM_010518</td>
<td>AF327652</td>
<td>Q05717</td>
<td>NP_000590</td>
<td>NP_032388</td>
<td>AF327653</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>SS2774</td>
<td>AF297519</td>
<td>NM_008344</td>
<td>AF327653</td>
<td>Q05718</td>
<td>NP_002169</td>
<td>NP_032370</td>
<td>AAG48353</td>
</tr>
</tbody>
</table>