J Vet Intern Med 2014;28:215–222

Expression and Activity of Collagenases in the Digital Laminae of Horses with Carbohydrate Overload-Induced Acute Laminitis

L. Wang, E.A. Pawlak, P.J. Johnson, J.K. Belknap, D. Alfandari, and S.J. Black

Background: Matrix metalloproteinases (MMP) are hypothesized to degrade structurally important components of the laminar extracellular matrix (ECM) in horses with laminitis.

Objective: To compare levels of expression of stromelysin-1 (MMP-3), collagenases (MMP-1, -13), and membrane type-MMPs (MMP-14, -15, -16), and the distribution of their ECM substrates, in laminae of healthy horses and horses with carbohydrate overload laminitis.

Animals: Twenty-five adult horses.

Methods: Gene and protein expression were determined in extracts of laminae using real-time quantitative polymerase chain reaction and Western blotting after sodium dodecylsulfate polyacrylamide gel electrophoresis. Distribution of MMP-13 and ECM components was determined using indirect immunofluorescent microscopy of nonfixed frozen sections. ECM morphology was assessed by hematoxylin and eosin staining.

Results: Of the genes studied, only those encoding MMP-1 and -13 were upregulated in CHO-induced laminitis; MMP-1 at Obel grade (OG)1 lameness and MMP-13 at OG3 lameness. Laminar MMP-1 was present as 52 kDa proenzyme only. MMP-13 was present as pro- (61 kDa) and processed (48 kDa) enzyme. MMP-13 localized to the basal epithelium of the secondary epidermal laminae and its increased expression were accompanied by the appearance in secondary dermal laminae (SDL) of multiple foci that were devoid of collagen I, fibronectin, chondroitin and keratan sulfate glycosaminoglycans, and eosin-staining material.

Conclusions and Clinical Relevance: MMP-13 is upregulated in laminae of horses with CHO-induced OG3 lameness and, by degrading components of the ECM, may contribute to the formation of ECM-free lesions (gaps or tears) that appear in the SDL with OG3 lameness.

Key words: CHO-laminitis; Equine; Matrix metalloproteinases.

The digital laminae connect the distal phalanx to the inner hoof wall and suspend the horse's axial skeleton within the hoof capsule. The laminae are composed of interdigitated folds of keratinized epidermal and connective dermal tissue, both with interdigitated secondary projections called SEL and SDL.[1] These join at a basement membrane, which is attached by anchoring filaments to hemidesmosomes on basal epithelial cells.[2] During CHO-induced laminitis, the junction between the epidermal and dermal layers of the laminae is compromised by physiologic changes in basal epithelial cells[3,4] and by degradation and reorganization of the abutting extracellular matrix (ECM).[5–7] The resulting partial or complete separation of the epidermal and dermal laminae frees the distal phalanx to rotate and sink within the hoof capsule causing severe pain and lameness.[8]

Abbreviations:

CHO	carbohydrate overload
CS	chondroitin sulfate
ECM	extracellular matrix
H&E	hematoxylin and eosin
IF	immunofluorescence staining
KS	keratan sulfate
MMP	matrix metalloproteinase
OG1	Obel grade 1
OG3	Obel grade 3
RT-qPCR	real-time quantitative polymerase chain reaction
SDL	secondary dermal laminae
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SEL	secondary epidermal laminae

Gelatinases (MMP-2 and MMP-9) have been a focus of interest in laminar pathophysiology for over a decade after their detection in the digital laminae of healthy horses,[9] their increase in explant medium from cultured laminae of laminitic compared to healthy horses,[10] and in the serum of horses with CHO-induced laminitis compared to healthy horses.[9] More recently, it has been shown that only catalytically inactive pro-MMP-9 is increased in laminae from horses with naturally acquired and experimentally induced (black walnut extract and CHO) laminitis[11,12] indicating that MMP-9 does not play a decisive role in laminar injury. In addition, whereas MMP-2 gene expression is significantly increased in laminae of horses with naturally

From the Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, MA (Wang, Pawlak, Alfandari, Black); the Department of Veterinary Medicine and Surgery, University of Missouri, Columbia, MO (Johnson); and the Department of Veterinary Clinical Sciences, The Ohio State University, Columbus, OH (Belknap). L. Wang and E.A. Pawlak are equal first authors.

Corresponding author: Dr S.J. Black, Department of Veterinary and Animal Sciences, University of Massachusetts, 427G ISB, 661 N. Pleasant St., Amherst, MA 01003; e-mail: sblack@vasci.umass.edu.

Submitted April 25, 2013; Revised July 31, 2013; Accepted October 17, 2013.

10.1111/jvim.12252

occurring and CHO-induced OG3 lameness as determined by SDS-renaturable gelatin zymography,[12] this finding varies greatly among horses,[12] and increases are not accompanied by an increase in native gelatinase activity in laminar extracts (Black, unpublished observations), or cryosections,[9] consistent with inhibition by tissue inhibitors of metalloproteinases (TIMPS).[13]

Although gelatinases may not be responsible for laminar failure, their increased expression in laminitic laminae is likely to be a harbinger of dysregulation of other MMPs. We therefore hypothesized that other MMPs are involved in laminar injury and here examined possible contributions of stromelysin-1 (MMP-3), collagenases-I and -III (MMP-1 and MMP-13), and membrane type MMPs (MMP-14, MMP-15, and MMP-16) to laminar damage in CHO-induced laminitis. We focused on these MMPs because they have the potential, if increased, to destabilize the laminae by degrading ECM components that provide tensile strength (interstitial collagens), connect collagen and proteoglycan elements of the ECM to each other and to cell-associated integrins (fibronectin), form the fibrous sheet of the basement membrane (Type IV collagen and laminin), tether hemidesmosomes of epithelial cells to the basement membrane (Type VII collagen), and prevent stretch deformation of the ECM (elastin).[14,15] We show here that genes encoding the MMPs are expressed in the laminae of healthy horses consistent with involvement of their products in dynamic modeling of laminae during normal growth and repair. Importantly, MMP-13 gene and protein expression are substantially increased in laminae of horses with CHO-induced OG3 lameness, and this increase is accompanied by the appearance in SDL of multifocal lesions that are devoid of Type I (and Type III) collagen I, fibronectin, chondroitin sulfate (CS) and keratan sulfate (KS) glycosaminoglycans and eosin-staining material. We propose that these represent gaps or tears in the ECM and arise under ambient force in regions of tissue that have been subjected to increased degradation by MMP-13.

Materials and Methods

Laminitis Induction and Tissue Collection

Laminae were harvested from 25 horses ranging from 3 to 12 years of age. All animals were thoroughly evaluated for health and gait before the start of the study. Horses were housed and cared for according to an institutionally approved protocol at the University of Missouri, College of Veterinary Medicine. Carbohydrate gruel comprised of 85% cornstarch and 15% wood flour was administered to 17 horses by nasogastric tube at 17.6 g/kg body weight, whereas 8 horses received water as a control. All horses were placed under general anesthesia at the onset of fever (developmental [DEV] group, ≥102°F, n = 6), at the onset of Obel grade I (OG1, n = 6) lameness, or upon developing Obel grade III (OG3, n = 5) lameness, and laminar tissue was collected as previously described.[14,15] Samples for immunofluorescence microscopy were embedded in OCT medium[a] and frozen over dry ice, whereas samples for RNA extraction and subsequent gene expression analysis were snap frozen in liquid nitrogen. Tissue processing was completed within 5 minutes of collection from the hoof, and samples analyzed herein were collected from the right front hoof and stored at −80°C until use.

RNA Extraction

RNA was isolated from samples of snap-frozen laminar tissue using a total RNA extraction kit[b] according to the manufacturer's instructions. Isolated RNA was quantified using spectophotometry[c] and consistently yielded $A260:280$ and $A260:230$ ratios near 2.0. Integrity was confirmed by gel electrophoresis.

Real-Time Quantitative PCR

cDNA was synthesized[d] for n = 8 control, n = 6 DEV, n = 6 OG1, and n = 5 OG3 animals. Primer sets for MMP-1, -3, -13, -14, -15, and -16 were designed against the equine sequence or, in cases where equine sequences have not been annotated, against homologous human sequences as predicted by extensive alignment screening against the equine database (Table 1). RT-qPCR reactions were run in triplicate using SYBR Green[e] according to the manufacturer's instructions. Ct values were averaged and analyzed using the ΔΔCt method.[16] We determined the expression patterns of glyceraldehyde 3-phosphate dehydrogenase (GapDH), β actin, β2 microglobulin, and TATA Box binding

Table 1. Primers used for RT-qPCR analysis MMP gene expression.

Domain Name	Sequence (F-Forward 5′; R-Reverse 5′)	Gene ID	Amplicon Length (Homology) (bp)	Primer Efficiency (%)	R^2
MMP-1	F-ATAACTACGATTCGGGGAGAAG-3′; R-TCTATGGGAAACCTCATAAGCA-3′	Equine 100033896	153	104	0.97
MMP-3	F-CTTTTGATGGACCTGGAAAAGT-3′; R-GAGTGATAGAGACCCAGGGAAT-3′	Equine 100034195	169	91	0.98
MMP-13	F-GTCCCTGATGTGGGTGAATAC-3′; R-ACATCAGACCAAACTTTGAAGG-3′	Equine 100009711	152	93.3	0.99
MMP-14	F-CCTGATAAGCCCAAAAACC-3′; R-CTTCCTCTCATAGGCAGTGTT-3′	Equine 100034170	213	104	1.00
MMP-15	F-TTTTCAAAGGTGACCGCTACT-3′; R-GTCCTCTTGGAAGAAGAAGGTG-3′	Equine 100062872	158	103	0.99
MMP-16	F-TGGTTACAAAAGTACGGCTACC-3′; R-CCTCTTGTCTGGTCAGGTACAC-3′	Equine 100049862	191	92	0.98

protein in laminar samples from control horses and horses with OG1 and OG3 lameness after administration of starch gruel. Expression of these genes did not differ significantly between groups. However, expression patterns of β2 microglobulin and GapDH had the lowest variation within groups and we normalized gene expression to GapDH as previously reported.[4] All primer pairs were designed to span introns in the respective genes, confirmed to amplify a single band (Fig S1), and the product validated by sequencing.

Protein Extraction

Approximately 0.35 g snap-frozen segments of laminae were pulverized and immediately homogenized in 10 mL of extraction buffer (50 mM Tris pH 7.0, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.5% NP-40 containing 10 μM E64, 1.5 μM pepstatin A and 1 mM phenylmethanesulfonyl fluoride)[f] on ice, extracted overnight, and concentrated by precipitation. Protein concentration was determined by Bradford colorimetric assay.[g]

SDS-PAGE and Western Blotting

An aliquot (30 μg protein content) of extract was boiled in reducing Laemmli (5 mM 2-mercaptoethanol) sample buffer,[g] subjected to SDS-PAGE in a 4% (w/v) polyacrylamide stacking gel with a 10% (w/v) polyacrylamide gel, and transferred to a polyvinyl membrane.[h] The membrane was blocked with 5% dry milk in PBS with 0.05% Tween-20[f] for 1 hour, washed with PBS with 0.1% Tween-20 for 30 minutes and then incubated with primary antibodies (Table 2) overnight at 4°C. After incubation with primary antibodies, the membranes were washed twice in PBS with 0.1% Tween-20 for 30 minutes and incubated with secondary antibodies conjugated with horseradish peroxidase. Detection was performed using enhanced chemiluminescence,[g] digitally imaged, and analyzed with associated software.[i]

Immunofluorescent Localization

Proteins of interest were localized in 10 μm sections of unfixed, snap-frozen laminar tissue using indirect immunofluorescence staining according to a previously described protocol.[17,18] Briefly, after blocking with 5% bovine serum albumin in PBS with 0.001% Tween-20, tissues were incubated with primary antibodies (Table 2) for 1 hour at room temperature. Antibody specificity was validated by dot blotting (Fig S2). After washing, sections were incubated for 1 hour at room temperature with appropriate fluorophore-conjugated secondary antibodies (Table 2) and counterstained with a DNA intercalating dye[j] to detect nuclei. Slides were imaged on an inverted fluorescent Zeiss 200M microscope with an apotome grid (Zeiss MOT200 with Zeiss Apotome and AxioVision v. 4.8[k]).

Hematoxylin and Eosin Staining

Ten micrometer sections of unfixed, frozen tissue from a representative control, OG1 and OG3 animal were dehydrated in ethanol and progressively rehydrated through several changes of solution and stained with Gill's hematoxylin #3 and a 0.5% alcoholic solution of eosin Y.[f] Slides were imaged on a standard light microscope.

Statistical Analysis

All gene expression and protein expression data were not normally distributed ($P < .05$) as determined by Column Statistics using the D'Agostino and Pearson Omnibus Normality Test. Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons test therefore was used to evaluate statistical significance. In all tests, $P < .05$ was considered significant.

Results

The expression of genes encoding MMP-1, -3, -13, -14, -15, and -16 was analyzed by RT-qPCR using primers described in Table 1. Gene expression was detected in all cases in the laminae of healthy horses (Fig S1) and horses with CHO-induced laminitis (data not shown). However, expression of genes encoding only 2 of the MMPs was found to be significantly increased in laminae of the laminitic horses, namely MMP-1 (Fig 1A) and MMP-13 (Fig 1B).

To determine whether increases in MMP-1 and MMP-13 gene expression are accompanied by increases in protein expression, extracts of laminae were subjected to SDS-PAGE and Western blotting. MMP-1 in extracts of laminae from healthy and laminitic horses resolved as a single polypeptide of 52 kDa corresponding to pro-MMP-1,[19,20] and its expression was increased in the laminae of horses with OG3 lameness but not with OG1 lameness compared with that in laminae of healthy horses (Fig 2A). Propeptide free MMP-1 was not detected in any extract under the conditions used. MMP-13 resolved as polypeptides of 61 and 48 kDa, corresponding respectively to pro-MMP-13 (61 kDa) and MMP-13 (propeptide free 48 kDa enzyme)[19,21] in extracts of laminae from healthy and laminitic horses (Fig 2B). Both pro-MMP-13 and

Table 2. Antibodies used for Western blot (WB) and immunofluorescence (IF) protein analysis and localization.

Primary Antibody	Dilution	Company	Secondary Antibody
Rabbit monoclonal (EP1247Y) to MMP-1, residues 115–140	1 : 1000 (WB)	Abcam ab 52631	Donkey anti-Rabbit IgG-HRP Jackson Immuno Research, West Grove, PA, USA #711-035-152 (WB)
Rabbit polyclonal to MMP-13, residues 250–350	1 : 1000 (WB)1 : 100 (IF)	Abcam ab84594	Donkey anti-Rabbit IgG-HRP Jackson Immuno Research #711-035-152 (WB) Donkey anti-Rabbit IgG-Dylight 594 Jackson Immuno Research #711-515-152 (IF)
Mouse monoclonal to collagen I	1 : 200 (IF)	Abcam ab6308	Donkey anti-Mouse IgG-Dylight 594 Jackson Immuno Research #715-515-150
Rabbit polyclonal to fibronectin	1 : 200 (IF)	Abcam ab2413	Donkey anti-Rabbit IgG-Dylight 594 Jackson Immuno Research #711-515-152

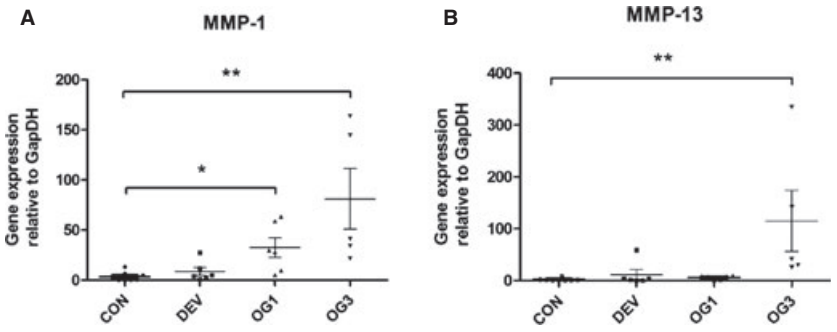

Fig 1. Increase in gene expression of MMP-1 and -13 during acute laminitis. RT-qPCR analysis of laminar gene expression of **(A)** MMP-1, **(B)** MMP-13 in control (n = 8), developmental (n = 6), OG1 (n = 6), and OG3 (n = 5) animals. *Indicates $P < .05$; **Indicates $P < .01$.

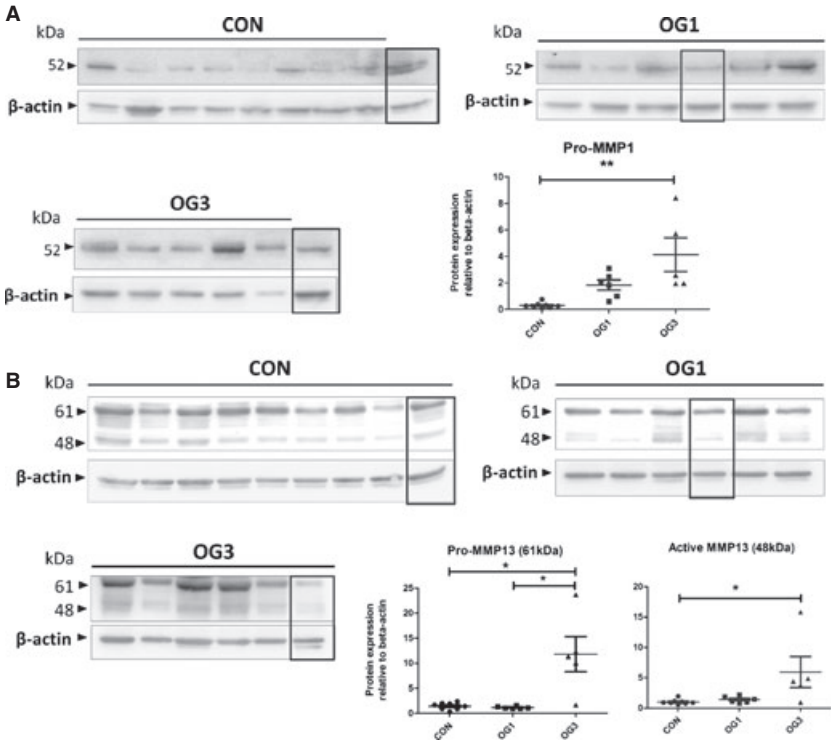

Fig 2. Increase in protein expression of MMP-1 and -13. Western blot analysis of protein expression of MMP-1 **(A)** and MMP-13 **(B)** in laminar extracts from control (n = 8), OG1 (n = 6), and OG3 (n = 5) animals. The "Box" signifies a common sample run in all 3 gels to normalize experimental variation between gels. The 52 kDa band corresponds to the proform of MMP-1 and the 61 kDa band corresponds to the proform of MMP-13 whereas the 48 kDa band corresponds to the active form of MMP-13. *Indicates $P < .05$; **Indicates $P < .01$.

MMP-13 were increased in the laminae of horses exhibiting OG3, but not OG1 lameness relative to laminae of healthy horses (Fig 2B).

MMP-13, which is stained red with specific antibody, localized to the basal epithelial cell layer in laminae of both healthy and laminitic horses (Fig 3A–C). This MMP has wide substrate specificity, degrading Types I, III, and IV collagen, fibronectin and several other ECM components.[21] Type I collagen was distributed throughout the SDL of healthy horses (Fig 3D, control) and horses with OG1 lameness (Fig 3E, OG1), but dispersed to the edges of the SDL in horses with OG3 lameness (Fig 3F, OG3) leaving the appearance of foci (example indicated by *, panel F) from which it was absent. Fibronectin exhibited a similar staining pattern as Type I collagen being widely distributed throughout the SDL in laminae of healthy horses (Fig 3G) and horses with OG1 lameness (Fig 3H), but dispersed to the edges in some regions of SDL in horses with OG3 lameness (Fig 3I) again leaving the appearance of foci in the ECM that were devoid of fibronectin (example indi-

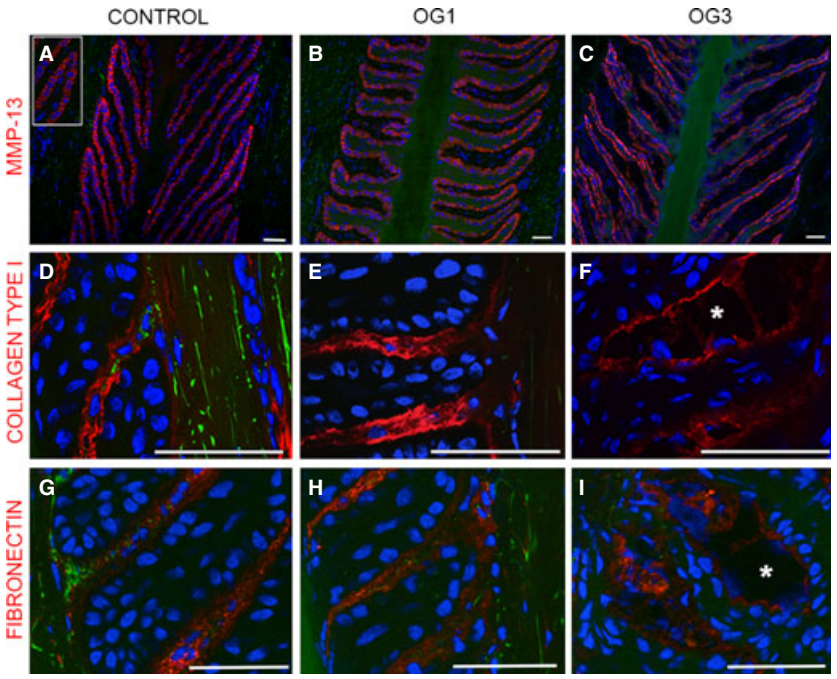

Fig 3. Distribution of MMP-13 and its substrates in the secondary dermal laminae. Immunofluorescent staining (red) of MMP13 (**A–C**, 20× objective), collagen type I (**D–F**, 63× objective) and fibronectin (**G–I**, 63× objective) in tissue sections from control (n = 6), OG1 (n = 6), and OG3 (n = 4) animals. Representative images of control (A, D, G), OG1 (B, E, H), and OG3 (C, F, I) are shown. Autofluorescent (putatively collagen) is shown in green, nuclei in blue. Scale bars 50 μm. *Indicates example foci from which Type I collagen or fibronectin is absent.

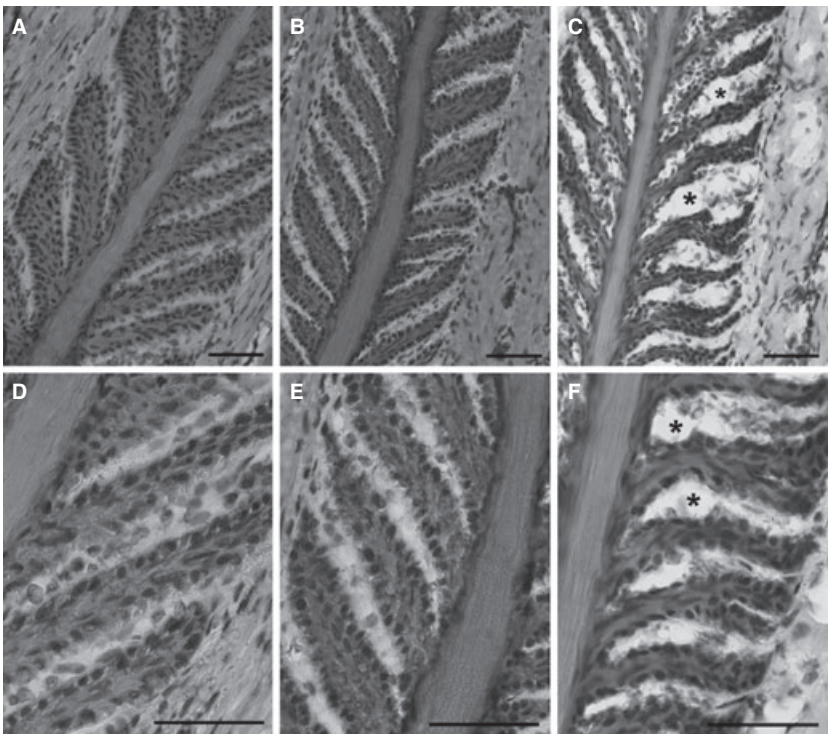

Fig 4. Loss of extracellular matrix elements in the secondary dermal laminae in OG3 Stage Laminitis. Hematoxylin and eosin staining of 10 μm sections of laminae from a representative animal from **A** and **D**) control group, **B** and **E**) OGI group, and **C** and **F**) OG3 group. Upper row imaged with 20× objective. Bottom row imaged with 40× objective. Scale bars 50 μm. *Indicates example foci from which H&E staining is absent.

cated by *, panel I). The specificity of antibodies used for these stains was confirmed by dot blotting (Fig S2).

To further characterize the foci devoid of Type I collagen and fibronectin that appear in the dermal ECM of horses with OG3 lameness, laminae from healthy and laminitic horses were stained with antibodies specific for CS and KS glycosaminoglycans. These stains identified laminitis-associated dispersion of the glycosaminoglycans to the edges of the SDL in horses with OG3 lameness (indicated by * in Fig S3C, F). Dispersion of these components was not accompanied by a major loss of basement membrane laminin, determined by pan-laminin staining (green; Fig S3, panel F). Staining with H&E showed that cells of the SDL are cocooned in amorphous eosin-staining material (putatively proteinaceous material) that completely fills the SDL in healthy horses (Fig 4 A,D), and in horses with OG1 lameness (Fig 4 B,E), but is absent from multiple foci in the SDL of horses with CHO-induced OG3 lameness (examples indicated by *, Fig 4 C,F), presumably corresponding to the foci devoid to collagen I, fibronectin, CS and KS glycosaminoglycans shown in Fig 3 and Fig S3.

Discussion

We show here that genes encoding MMP-1, -3, -13, -14, -15, and -16 are expressed within the laminae of healthy horses consistent with possible involvement of their products in dynamic modeling of the laminar ECM to accommodate growth and repair. Expression of genes encoding MMP-3, -14, -15, and -16 was similar in the laminae of healthy horses and horses with CHO-induced laminitis up to OG3 lameness, whereas expression of genes encoding MMP-1 and -13 was significantly increased in the laminitic horses; MMP-1 at OG1 and OG3 lameness, and MMP-13 only at OG3 lameness. Despite increase in its expression, MMP-1 is unlikely to contribute to pathologic remodeling of laminar ECM in CHO-induced laminitis because only the inactive form, 52 kDa pro-MMP-1,[19,21] was detected in extracts of laminae. In contrast, both pro- (61 kDa) and processed (active propeptide-free; 48 kDa) forms of MMP-13[19,21] were significantly increased in the laminae of horses with OG3 lameness relative to that in the laminae of healthy horses.

MMP-1 and MMP-13 are known to be produced by activated fibroblasts, epithelial cells and inflammatory leukocytes in other systems.[21] In the laminae of healthy horses and horses with CHO-induced laminitis, MMP-13 was shown to localize predominantly to laminar basal epithelial cells. Thus, increased MMP-13 gene and protein expression join that of ADAMTS-4,[3] and the suppression of the canonical Wnt signaling pathway,[4] as indicators of altered physiology of laminar basal epithelial cells in horses with CHO-induced OG3 lameness. Although lack of an appropriate specific antibody precluded immunolocalization of MMP-1, expression of the gene encoding this protein has been demonstrated in laminar basal epithelial cells by laser capture microscopy and RT-qPCR analysis (Belknap, personal communication). However, in the absence of immunostaining, we cannot ascribe increased production of pro-MMP-1 in laminae of horses with CHO-induced laminitis to the laminar basal epithelial cells.

Expression and activation of MMPs are tightly controlled on several levels, including regulation of transcription, enzymatically mediated cleavage of propeptide and inhibition by protease inhibitors. Transcription factors Fos/Jun and AP-1 mediate enhanced expression of MMP-1, MMP-13, and ADAMTS-4 in response to inflammatory factors such as interleukin-1 and tumor necrosis factor alpha[22,23] consistent with an inflammatory component of laminitis.[24,25] However, although laminar inflammation[24,25] and increased ADAMTS-4[4,22] gene expression are detected as early as the DEV (fever) stage of CHO-induced laminitis, increased laminar MMP-1 gene expression is first detected at OG1 lameness, whereas that of MMP-13 becomes increased between OG1 and OG3 lameness. Thus, it is unlikely that there is a direct relationship between MMP-13 expression and laminar inflammation.

Laminar basal epithelial cells line the junction of the SEL and SDL where they produce, and attach to, fibrillar proteins of the basement membrane. Secretion of the basement membrane proteins occurs at the apical surface of the basal epithelial cell, and that is also likely to be the case with pro-MMP-13. Secreted pro-MMP-13 is converted to MMP-13 by extracellular removal of its regulatory propeptide.[21] This can be mediated by several proteases including MT1-MMP (MMP-14).[26,27] Like the other membrane type MMPs, MMP-14 is processed by a furin during intracellular transport, and is placed onto the cell membrane as an active enzyme. The mechanism of MMP-13 activation by MMP-14 is not resolved but is independent of TIMP 2,[27] thus, distinguishing it from activation of MMP-2. MMP-14 has been localized to the laminar basal epithelial cells of the equine digital laminae by immunohistochemistry.[28] Thus, conversion of pro-MMP-13 to MMP-13 may occur at the apical surface of the laminar basal epithelial cells which would result, when the MMP locally is in excess of TIMPs, in cleavage of substrates in the basement membrane (collagen IV and laminin) and the abutting ECM of the SDL (Types I and III collagens, proteoglycans, and fibronectin).

In support of this possibility, basement membrane collagen IV and laminin have been shown to decrease in horses with CHO (oligofructose)-induced lameness[7]; the expression of genes encoding MMP-1 and MMP-13 is increased in the laminae of these horses by 24 hours after treatment (Pawlak, Pollitt and Black, unpublished data). In addition, Type I collagen is shown here to be substantially redistributed in the SDL of horses with CHO-induced OG3 lameness resulting in the appearance of multiple foci from which the collagen is absent. This is also the case with Type III collagen (Pawlak and Black, unpublished data). In addition, fibronectin was shown here to undergo similar redistribution in the SDL of horses with CHO-

induced OG3 lameness, again resulting in the appearance of multiple foci from which the protein is absent. Accompanying immunofluorescent staining of CS and KS glycoaminoglycans in sections of laminae from horses with CHO-induced OG3 lameness, and histochemical (hematoxylin and eosin) staining of cell nuclei and proteinaceous material in the sections also identified multiple foci in the SDL from which these materials were absent. Although general stains for ECM or basement membrane were not used in this study, the presence of foci in the SDL that lack multiple ECM components and occur with increased MMP-13 expression in laminae of horses with CHO-induced OG3 lameness is consistent with ECM degradation by MMP-13, and possibly with resultant focal tearing of affected regions under ambient force. This pathology has not been reported previously. A primary reason that it may have been missed in earlier investigations is that its detection is contingent on using snap-frozen OCT-embedded tissue because paraformaldehyde (PFA)-fixation and paraffin embedding, or PFA-fixation and sucrose impregnation of laminae before sectioning cause the gaps to collapse (Pawlak and Black, unpublished data).

A precise timeline for increased MMP-13 and appearance of putative gaps or tears in the SDL of horses with CHO-induced laminitis is not available. Both were absent at OG1 lameness and present at OG3 lameness. In addition, we have no direct evidence of a causal association between increased MMP-13 production in the laminitic tissue and damage to the SDL. Nevertheless, this is an attractive possibility and worthy of further investigation.

Footnotes

[a] Tissue-Tek O.C.T.; Sakura Finetek USA, Inc, Torrance, CA
[b] Stratagene Absolutely RNA Kit; Stratagene, La Jolla, CA
[c] NanoDrop 1000; Thermo Scientific, Wilmington DE
[d] qScript cDNA SuperMix; Quanta BioSciences, Gaithersburg, MD
[e] SYBR Premix Ex Taq; Applied Biosystems, Foster City, CA
[f] Sigma-Aldrich, St. Louis, MO
[g] BioRad Life Sciences, Hercules, CA
[h] Millipore, Billerica, MA
[i] G:Box with Gene Tools software; Syngene, Frederick, MA
[j] 4′,6′-Diamidino-2-phenylindole dihydrochloride (DAPI); Acros Organics, Thermo Fisher Scientific, Fair Lawn, NJ
[k] Carl Zeiss MicroImaging, Inc, Thornwood, NY

Acknowledgments

Supported by D08EQ-054 and D13EQ-004 from the Morris Animal Foundation; 35204-18313 from USDA NRI CSREES and MAS00907 from USDA CSREES to SJB. E. Pawlak was supported by a Lotta Crabtree Fellowship and a graduate fellowship from AQHA. D. Alfandari was supported by NIH DE016289.

The authors thank Dr Wesley Autio for statistical advice, and Drs Hannah Galantino-Homer, Susan Eades and Christopher Pollitt for helpful discussion.

Conflict of Interest Declaration: The authors disclose no conflict of interest.

References

1. Pollitt CC. The anatomy and physiology of the suspensory apparatus of the distal phalanx. Vet Clin North Am Equine Pract 2010;26:29–49.

2. Pollitt CC, Daradka M. Equine laminitis basement membrane pathology: Loss of type IV collagen, type VII collagen and laminin immunostaining. Equine Vet J Suppl 1998;26:139–144.

3. Wang L, Pawlak E, Johnson PJ, et al. Effects of cleavage by a disintegrin and metalloproteinase with thrombospondin motifs-4 on gene expression and protein content of versican and aggrecan in the digital laminae of horses with starch gruel-induced laminitis. Am J Vet Res 2012;73:1047–1056.

4. Wang L, Pawlak EA, Johnson PJ, et al. Impact of laminitis on the canonical Wnt signaling pathway in basal epithelial cells of the equine digital laminae. PLoS One 2013;8:e56025.

5. French KR, Pollitt CC. Equine laminitis: Cleavage of laminin 5 associated with basement membrane dysadhesion. Equine Vet J 2004;36:242–247.

6. French KR, Pollitt CC. Equine laminitis: Loss of hemidesmosomes in hoof secondary epidermal lamellae correlates to dose in an oligofructose induction model: An ultrastructural study. Equine Vet J 2004;36:230–235.

7. Visser MB, Pollitt CC. The timeline of lamellar basement membrane changes during equine laminitis development. Equine Vet J 2011;43:471–477.

8. Eades SC. Overview of current laminitis research. Vet Clin North Am Equine Pract 2010;26:51–63.

9. Mungall BA, Pollitt CC. Zymographic analysis of equine laminitis. Histochem Cell Biol 1999;112:467–472.

10. Pollitt CC, Pass MA, Pollitt S. Batimastat (BB-94) inhibits matrix metalloproteinases of equine laminitis. Equine Vet J Suppl 1998;26:119–124.

11. Loftus JP, Belknap JK, Black SJ. Matrix metalloproteinase-9 in laminae of black walnut extract treated horses correlates with neutrophil abundance. Vet Immunol Immunopathol 2006;113:267–276.

12. Loftus JP, Johnson PJ, Belknap JK, et al. Leukocyte-derived and endogenous matrix metalloproteinases in the lamellae of horses with naturally acquired and experimentally induced laminitis. Vet Immunol Immunopathol 2009;129:221–230.

13. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. Biochim Biophys Acta 2010;1803:55–71.

14. Garner HE, Coffman JR, Hahn AW, et al. Equine laminitis of alimentary origin: An experimental model. Am J Vet Res 1975;36:441–444.

15. Johnson PJ, Kreeger JM, Keeler M, et al. Serum markers of lamellar basement membrane degradation and lamellar histopathological changes in horses affected with laminitis. Equine Vet J 2000;32:462–468.

16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. Methods 2001;25:402–408.

17. Pawlak E, Wang L, Johnson PJ, et al. Distribution and processing of a disintegrin and metalloproteinase with thrombospondin motifs-4, aggrecan, versican, and hyaluronan in equine digital laminae. Am J Vet Res 2012;73:1035–1046.

18. Hayes NV, Newsam RJ, Baines AJ, et al. Characterization of the cell membrane-associated products of the Neuregulin 4 gene. Oncogene 2008;27:715–720.

19. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 2006;69:562–573.

20. Peake NJ, Khawaja K, Myers A, et al. Levels of matrix metalloproteinase (MMP)-1 in paired sera and synovial fluids of juvenile idiopathic arthritis patients: Relationship to inflammatory activity, MMP-3 and tissue inhibitor of metalloproteinases-1 in a longitudinal study. Rheumatology (Oxford) 2005;44:1383–1389.

21. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. Circ Res 2003;92:827–839.

22. Vincenti MP, Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: Integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res 2002;4:157–164.

23. Arner EC. Aggrecanase-mediated cartilage degradation. Curr Opin Pharmacol 2002;2:322–329.

24. Faleiros RR, Johnson PJ, Nuovo GJ, et al. Laminar leukocyte accumulation in horses with carbohydrate overload-induced laminitis. J Vet Intern Med 2011;25:107–115.

25. Leise BS, Faleiros RR, Watts M, et al. Laminar inflammatory gene expression in the carbohydrate overload model of equine laminitis. Equine Vet J 2011;43:54–61.

26. Knauper V, Will H, Lopez-Otin C, et al. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. J Biol Chem 1996;271:17124–17131.

27. Knauper V, Bailey L, Worley JR, et al. Cellular activation of proMMP-13 by MT1-MMP depends on the C-terminal domain of MMP-13. FEBS Lett 2002;532:127–130.

28. Kyaw-Tanner MT, Wattle O, van Eps AW, et al. Equine laminitis: Membrane type matrix metalloproteinase-1 (MMP-14) is involved in acute phase onset. Equine Vet J 2008;40:482–487.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Validation of RT-qPCR primers.

Fig S2. Validation of collagen and fibronectin antibody specificity by dot blot.

Fig S3. Loss of glycosaminoglycans from the SDL during CHO-induced laminitis.