Implication of Adam Related Metalloproteases in Equine Laminitis

IMPLICATION OF ADAM RELATED METALLOPROTEASES IN EQUINE LAMINITIS

A Thesis Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Animal Biotechnology & Biomedical Sciences

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DEDICATION

To Stephen Peter Lynch

ACKNOWLEDGMENTS

My greatest appreciation goes to my advisor, Dr. Dominique Alfandari, without whom none of this would have been possible. I would also like to thank my committee members for their help in guiding me through this process and for their excellent ideas. I would also like to thank my family for always being there as my support network, lending advice and giving me that extra push when necessary.

I would also like to thank the members of my lab, Helene Cousin, Kate McCusker, and Russ Neuner who taught me more than they may ever realize. They also made my stay in this lab overly enjoyable and I thank them for that.

Lastly I would like to thank my girlfriend Michelle Misuraca for always being there and for dealing with my studies over the last several years. There were several times when I wish I had time to spend with her and she completely understood that my studies came first. Her understanding and support through this process is one thing that motivated me more than anything.

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CHAPTER I

MATERIALS AND METHODS

Acquisition of Hoof Lamellar Samples

This has been described (Johnson, Kreeger et al. 2000; Loftus, Belknap et al. 2006). For BWE and starch induced laminitis, horses were anesthetized (Johnson, Kreeger et al. 2000; Loftus, Belknap et al. 2006; Loftus, Belknap et al. 2007), the distal aspect of the right fore limb disarticulated from each horse at the level of the metacarpophalangeal joint and acquisition of lamellar tissue from each hoof accomplished within 5 minutes using a band saw, Horses were euthanized immediately after removal of the hoof. For clinical samples, lamellae were isolated with 10 minutes of euthanasia. Blocks of lamellar tissue (approximately 5 mm X 5 mm) were obtained by sharp dissection and either placed in 10% formalin or immediately frozen by submersion in liquid nitrogen and stored at -80° C until processed. In all cases blocks of lamellar tissue were obtained from the mid-point between the level of the coronary band
and the ground- bearing surface on the dorsal aspect of the hoof.

BWE Samples

Archived samples of laminar tissue from previous studies (Waguespack, Cochran et al. 2004; Waguespack, Kemppainen et al. 2004; Black, Lunn et al. 2006) were used. Briefly, healthy horses were administered either 6 L of water as a control (n = 10) or BWE (n = 10) (2 g heartwood/kg body weight, prepared as described (Eaton et al., 1995) via nasogastric intubation. Horses were euthanized at 1.5 hours post-induction, after a 30 % drop in the baseline leukocyte count (3 to

5 hours after BWE), or at the onset of Obel grade 1 lameness (typically around 10-12 hours after BWE). All animal protocols were approved by the Institutional Animal Care and Use Committees (IACUCs) of the Ohio State University or Auburn University.

CHO Samples

Carbohydrate-treated animals: Archived samples of lamellae from a previous study (Johnson, Kreeger et al. 2000) were used. Briefly, healthy horses were administered starch at a concentration of 17.6 grams of starch per kg of body weight via a nasogastric tube as described or water as a control (Johnson, Kreeger et al. 2000). Horses were euthanized when they developed Obel grade III laminitis (experimental group) typically 36 to 48 hours following administration of starch, or at equivalent time points for control animals. All animal protocols were approved by the IACUC of the University of Missouri.

Clinical Samples

These were obtained from horses euthanized at the University of Missouri School of Veterinary Medicine Equine Clinical Center. Animals were grouped as follows: Those presenting “acute clinical laminitis” had not previously shown signs of this disease; those presenting “chronic clinical laminitis” had a history of the condition and were chronically lame but not in an acute episode; those presenting “chronic aggravated laminitis” had a history of the condition and were experiencing a debilitating episode. Control clinical samples were from horses euthanized for a variety of conditions unrelated to laminitis and had no history of laminitis.

RNA Isolation and cDNA Synthesis

Black Walnut Extract laminae were pulverized using a tissue homogenizer. Total RNA was then extracted from the tissue using the guanidinium isothiocynate method as described in (Alfandari, Whittaker et al. 1995). RNA was quantified at OD260 and 1μg of each sample was run on a 1% agarose gel with 0.1μg/ml ethidium bromide to ensure RNA integrity. Using 0.5 g of frozen laminae tissue and 10 ml of guanidinium isothiocynate solution we obtained 25-160 μg of RNA depending on sample. 10 μg of this RNA was treated by RNAse free DNAse 1 (Promega) prior to cDNA synthesis reaction using oligo dT and MMLV reverse transcriptase in a 50 μl reaction volume (Promega). Before addition of the MMLV, 10 μl of the reaction mix was removed and saved for the no-RT control PCR and to control for complete genomic DNA digestion. This cDNA was then diluted in sterile H2O 1:10 and 1 μl of this dilution was used for each 20 μl RT-qPCR reaction. Total RNA was also extracted from frozen equine testis tissue using a tissue homogenizer. Using 100 mg of tissue we were able to extract approximately 70 μg of total RNA. cDNA synthesis was done using the same method used for the BWE treated animals. The cDNA for the clinical and Carbohydrate Overload samples were obtained as previously described (Waguespack, Cochran et al. 2004; Waguespack, Kemppainen et al. 2004).

RT-qPCR and Data Processing

The quantitative PCR data was obtained using the Roche Lightcycler 2.0. Each primer set was tested in order to determine the melting curve of the amplification product which was then used to determine the optimal fluorescence reading temperature. The reading temperature for each primer set was selected at 2-4°C before complete dissociation of the specific amplification product. Following each reaction, PCR products were run on 2% agarose gel with 0.1μg/ml ethidium bromide to confirm that the proper size fragment was amplified for each product. Cycling conditions were as follows: 1 cycle at 95°C for 5 minutes, 45 cycles of 60°C for 30s, 72°C for 30s, single fluorescence reading at predetermined temperature, 95° C for 15s, 1 cycle for melting curve using a stepwise increase of temperature from 65°C to 95°C with a constant fluorescence reading. In order to determine the most stable control gene for comparison using the ΔΔCt method we used the GeNorm computer program (Ghent University). We tested the expression patterns of Glyceraldeyhde 3-phosphate dehydrogenase (GAPDH), β-Actin, and α2-Microglobulin and the most stable control gene in both the BWE and CHO/Clinical laminitis sets was determined to be GAPDH and was used as our control. Each sample was then run in triplicate and their Ct values were averaged and analyzed using the ΔΔCt method of analysis (Livak and Schmittgen 2001). The results from this analysis for the control animals were averaged separately for animals from the BWE subset (n=3) and animals from the CHO/Clinical laminitis subsets (n=4). Experimental results for each animal and gene were then divided by the average

of the control animals to produce a final fold increase controlled both to GAPDH and to animals that did not show signs of laminitis.

Cloning of ADAMs

Testis cDNA was used as template to amplify ADAM related metalloprotease as well as TIMPs by RT-PCR using the minimally degenerated oligonucleotides found in Table 3. Cycling conditions for all genes were: 1 cycle at 95°C for 4 minutes, 35 cycles of 94°C 30 seconds, 55°C 30 seconds, 72°C 1 minute, 1 cycle 72°C for 5 minutes. Using equine testis cDNA as a template each genes correct product size was amplified. These PCR products were cloned into pCR4-TOPO (Invitrogen) and sequenced either by the university of Massachusetts Amherst genomic facility or the Genewiz company. These sequences were then analyzed using BLAST and upon confirmation of each genes identity, specific primers were designed for real time quantitative PCR. Degenerate oligonucleotides were also employed to clone full-length constructs of both MMP9 and ADAM-TS4.

CHAPTER II

HISTORY OF LAMINITIS

Introduction to Laminitis

Laminitis is defined as an inflammation of the laminae, especially in the hoof of the horse (Merriam Webster). It is a disease that has affected the equine industry since as early as 350 B.C. when Aristotle termed it 'Barley Disease'. Since this time there have been many treatments developed for the disease, including treatment with anti-inflammatory drugs, NSAIDs, and ice baths. All of these treatments are capable of helping to treat the symptoms, but none target the underlying systemic issues. In recent years work has been done to understand exactly how the equine hoof responds to laminitis and what structures are being affected. It is critical to determine what the molecular pathologies are during the initiating and developmental stages of laminitis in order to develop a treatment that has any potential for success.

Anatomical Evolution of the Horse

The horse evolved over a 60 million years into an animal that man kind has relied on to help spread onto every corner of the planet. The original animal was termed *Hyracotherium* and its unique characteristics allowed for its development into the animal that we now know as the modern *Equus Caballus* (Short 1975). The forelimbs of the modern horses predecessor had five padded toes, named p1 through p5, while the hind limbs had only three padded toes, named p1 through p3.

As evolution selected for the strongest of this animals traits, the forelimbs drastically changed. Over a period of about 50 million years the horse had evolved into an animal known as *Pliohippus* which had evolved such that the only weight bearing toe was p3 (Short 1975). Over the next 10 million years the horse evolved slightly further and the only clearly digit that is visible to date is the p3 bone. Both p4, the metatarsal bone, and p2, the metacarpal bone are still present and are termed the splint bones. These bones play an important role in supporting the knee and hock in *Equus Caballus* (Short 1975).

Evolution has clearly shown that perhaps the most important bone in a horses body is the p3 digit. This bone is solely responsible for the support of the animals entire mass, which averages around 1000 pounds. It also has to be taken into account that the force of impact on the forelimbs greatly exceeds that of the animals body weight. Inflammation and degradation of the sensitive laminae, which connects the p3 bone to the inner wall of the hoof is the major cause of laminitis and a great deal of work has been done to study how this connection is made. The connection between the keratinized hoof wall and the p3 digit can be divided into three sections, the coronary papillae, secondary lamellae, and terminal papillae, as seen in Figure 1.

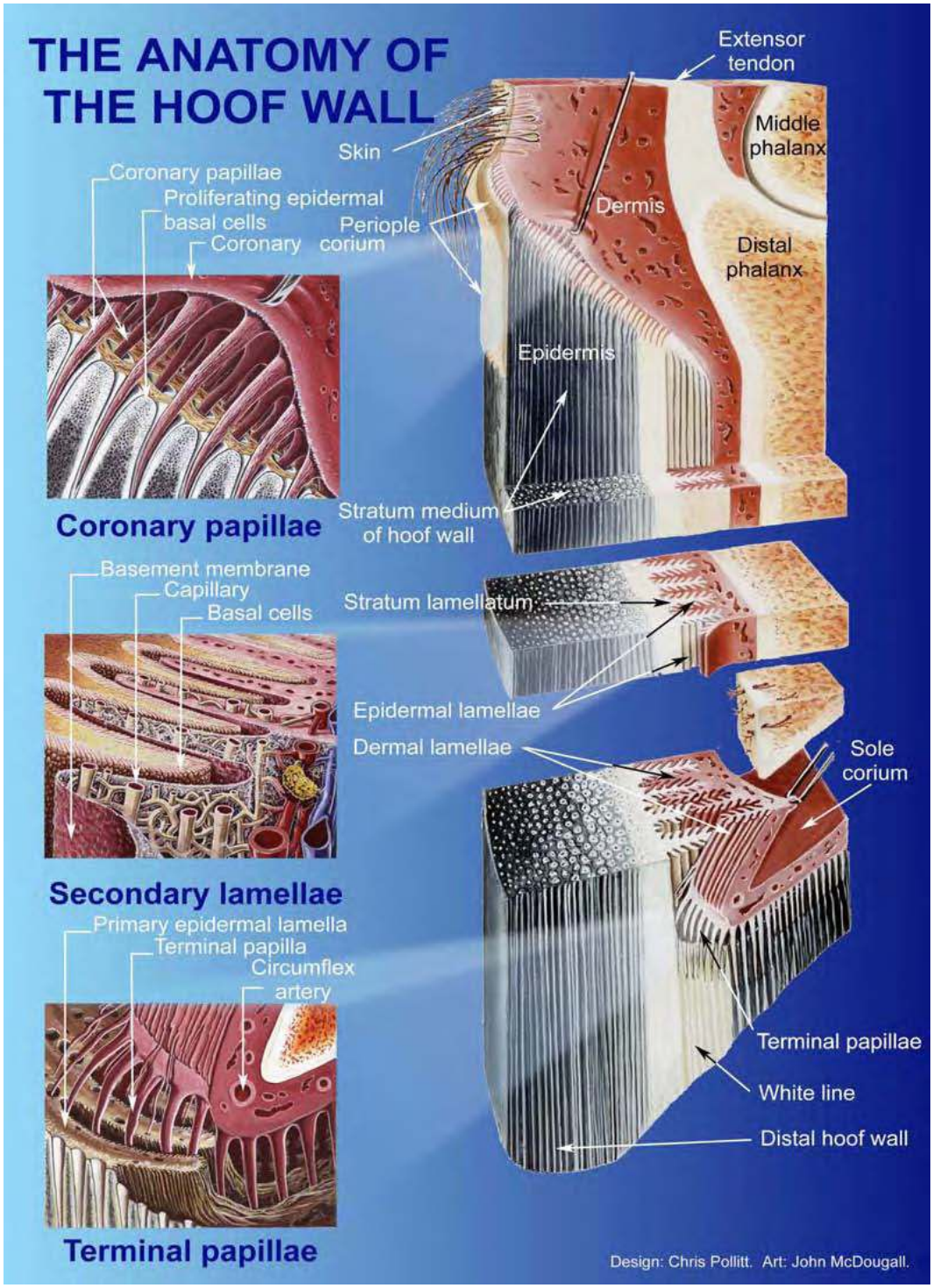


Figure 1: Anatomy of the Hoof Wall (Daradka and Pollitt 2004; Pollitt and Daradka 2004; Keller, Galloway et al. 2006)

The corium, located at the top of the hoof wall is a highly vascular area consisting of tough connective tissue with a network of arteries, veins, and capillaries. It is essential for nourishment of the hoof and it plays a major role in the connection of the p3 digit with the inner hoof. Each coronary papillae that shoots out from the corium is responsible for providing nutrients to an individual hoof wall tubule (Kasapi and Gosline 1998). As epidermal basal cells produce keratinocytes they are targeted in a downward path towards the bottom of the hoof wall (Daradka and Pollitt 2004). This is how the hoof is able to grow constantly through the life of a horse.

Moving distally in the hoof, the next major region of importance for support is the secondary lamellae. Within this region is the *stratum lamellatum* which has several hundred projecting primary epidermal lamellae. These epidermal lamellae are shaped like long rectangles and they are incorporated into the *stratum lamellatum* like a key in a lock. It is believed that the structure of the epidermal lamellae is intended to increase the surface area of the lamellae which suggests its potential role in the suspension of the animals weight (Sarratt and Hood 2005). Overall the entire surface area of the inner hoof wall has been calculated to average $1.3m^2$ which is equal to that of the surface area of a humans entire skin (Sarratt and Hood 2005). With this amazing surface area the animal is able to deal with the great deal of stress placed on the hoof in both active and stationary activities.

In the location where the epidermis and dermis meet, there is a very tough sheet of extracellular matrix known as the basement membrane. This structure

attaches the cells of the lamellar epidermis to type I collagen on the surface of the p3 bone (Pollitt and Daradka 1998). Filaments of collagen IV run in the dorsal-ventral axis and are covered in laminin, these two together form the *laminin densa* (Pollitt 1994). Type I collagen and *laminin densa* interweave and are found at a high concentration in the basement membrane (Pollitt 1994) which is not surprising considering the weight bearing load of the hoof.

Conflicting Hypothesis on the Cause of Laminitis

At this point in time there are two main hypotheses on how laminitis manifests itself. The first hypothesis suggests that constriction of the blood vessels supplying nutrients to the collagen rich laminae become restricted, causing cell death in the laminae (Hood 1999). The second hypothesis proposes that a local inflammation of the laminae recruits leucocytes that activate MMPs and initiate the destruction of the laminae (Johnson, Tyagi et al. 1998; Mungall, Pollitt et al. 1998). This second hypothesis has been supported in recent findings where horses induced with Black Walnut Extract showed proteolytic fragments of collagen in their blood (Johnson, Kreeger et al. 2000). Other recent work has shown an infiltration of neutrophils into the laminae which correlates with an increased level of MMP-9 protein (Loftus, Belknap et al. 2006). This would most likely occur because neutrophils have been observed to store MMP-9 in their tertiary granules (Chakrabarti and Patel 2005).

Regardless of the initial triggering mechanism the end result is always the destruction of the collagen rich laminae. It is interesting to note that both ischemia

and inflammation can result in the activation of metalloproteases capable of degrading the collagen rich laminae.

Natural Laminitis Description

Causes of natural laminitis have such a great range that it is very hard to pin point what started the disease. Many of the potential causes are outlined in Table 1.

Cause	Reasoning
Carbohydrate Overload	If a horse is given grain in excess or eats grass under stress and has accumulated excess of non-structural carbohydrates it may be unable to digest the carbohydrates in the foregut. Excess carbohydrates move to the hindgut and ferment in the cecum. This causes an increase in lactic acid levels and proliferation of bacteria capable of producing endotoxins. Endotoxins are absorbed into the bloodstream resulting in a decreased circulation, especially in the hoof.
Insulin Resistance	Not fully understood but may be triggered by sugar and starch imbalance.
Nitrogen Compound Overload	Rapid fluctuation of non-protein nitrogens. The natural metabolic processes become overloaded resulting in liver disturbance and toxic imbalance.
Hard Ground	Hard surfaces increase concussion on horses feet, potentially causing laminitis.
Lush Pastures	Same concept as carbohydrate overload.
Colic	Release of endotoxins into the blood stream

Table 1: Causes of Laminitis

The progression of laminitis has been divided into four stages. The developmental (prodromal) stage begins when an initial injury occurs until there is an appearance of the clinical signs of laminitis (Hood, Grosenbaugh et al. 1993). The duration of this phase depends mainly on what factor caused the initial injury, for example Black Walnut ingestion will cause a shorter developmental stage than colic. The next stage is the acute phase lasting from when clinical signs

are observed until there is evidence of mechanical collapse of the distal phalanx within the hoof capsule, or 72 hours, whichever comes first (Hood 1999). If there are clinical signs over 72 hours but there is not structural failure of the hoof the disease is classified as sub acute. Once the horse develops structural failure, for example rotation or sinking of the distal phalanx, then the disease is considered in the chronic phase.

These stages are also linked to the Obel grading system of laminitis, which was developed by Obel in 1948 (Obel N. *Studies on the histopathology of acute laminitis.* Uppsala, Sweden: Almquist and Wiskells, 1948.). This system grades a horse symptom from 1 through 4, with 4 being the worst prognosis. At grade 1 a horse alternately and incessantly lifts the feet, lameness is not evident at a walk, but at a trot a short stilted gait is noticed. Grade 2 is defined as a horse that walks with a stilted gait but can still have a foot lifted. The main difference at grade 3 is that a horse moves much more reluctantly and will resist lifting of a foot. At the most severe grade, grade 4, the horse refuses to move unless forced to do so.

Induction models of Laminitis

Development of an experimental model for natural laminitis is essential in attempting to study the molecular and pathological events leading up to the degradation of the laminae. There are two current models used for the experimental induction of laminitis, these are the Black Walnut (BWE) and Carbohydrate Overload (CHO) models. Each model has its own benefits and drawbacks, which have been explored extensively since their discovery.

Induction of laminitis by treatment of Black Walnut extract via nasogastric intubation was developed in the 1980's (Minnick, Brown et al. 1987). It was known in the horse industry that animals exposed to shavings from the black walnut tree, *Juglans Nigra*, became laminitic. In order to confirm this hypothesis Minneck soaked 2g/kg of black walnut shavings in water and administered it to the animals via nasogastric intubation. In this experiment, horses developed edema of the coronary band and 8/10 horses demonstrated clinical signs of laminitis within 12 hours of administration (Minnick, Brown et al. 1987). Measurements revealed at least a 30% decrease in white blood cell counts 4 hours after administration of BWE, and an increase in white blood cells, packed cell volume, and hyperglycemia starting at 8 hours post administration (Galey, Whiteley et al. 1991). These changes in white blood cells are similar to that of animals with endotoxemia but when blood from BWE induced horses was analyzed for endotoxins there were none present (Eaton, Allen et al. 1995).

Another aspect of natural laminitis that is clearly necessary for any relevant induction model is the inflammatory response. This is because it has been noted that hoof wall temperature is increased in animals with both natural and induced cases of laminitis (Baxter 1994). Work has been done to look at the expression of IL-1β, an inflammatory cytokine, in the laminae of horses administered BWE after approximately 3 hours (Fontaine, Belknap et al. 2001). The results showed an increase in IL-1β which provided evidence of an inflammatory response occurring early in the development of BWE laminitis. Other pro-inflammatory cytokines such as IL-6 and IL-8 have been observed to be

increased in the CHO and BWE models of laminitis, once again indicating that laminitis in accompanied by an inflammatory response (Belknap, Giguere et al. 2007). These results have only been investigated in induction models of laminitis and it is critical to see if these same results are found in animals with naturally occurring laminitis.

The most commonly used methods for induction of laminitis by Carbohydrate Overload was developed in 1975 (Garner, Coffman et al. 1975). A laminitis inducing ratio of 85% corn startch and 15% wood cellulose flour was administered at a dose of 17.6g/kg body weight via a stomach tube. It has been observed that there is an alteration in cecal flora, lactic acidosis, and endotoxemia after administration but the exact mechanism of laminitis induction is still unknown (Garner, Moore et al. 1978; Moore, Garner et al. 1979; Krueger, Kinden et al. 1986; Sprouse, Garner et al. 1987; Weiss, Evanson et al. 1998; Weiss, Evanson et al. 2000). Obel Grade 3 lameness was observed approximately 40 hours post CHO treatment. Increased packed cell volume, leukocytosis, and hyperproteinemia were all observed in these animals indicating a close relation to natural laminitis (Coffman and Garner 1972; Harkema, Robinson et al. 1978; Moore, Garner et al. 1981; Fagliari, McClenahan et al. 1998).

Examination of the hoofs histology in the CHO model has shown that there is an initial alteration in the digital vasculature, including swelling of endothelial cells and mild edema formation (Hood, Grosenbaugh et al. 1993). After 6-12 hours of administration a leukocyte infiltration occurs that dissipates as inflammatory cells are able to migrate to the epidermal layer. Microvascular

thrombi and accompanying severe edema formation are seen after 24 hours, and hemorrhage occurs in the primary dermal layer after 72 hours (Hood, Grosenbaugh et al. 1993). It has also been observed that after 48 hours of CHO treatment the lamellar basement membrane disintegrates and attachment of the basement membrane to basal cells of the epidermis is gone (Pollitt 1996).

Treatment

To date there is no cure for laminitis but there are several treatment options. In many cases rest and corrective shoeing is what an owner will do to curb a laminitic episode. Shoes will typically have pedal bone support to keep the p3 bone in the proper position to aide readhesion. In many other cases analgesics and vasodilators are used to relieve pain, reduce inflammation, and improve blood flow to the hoof. It is thought that increasing blood flow to the hoof area will help to speed the influx of repair enzymes to the area. Since there is no cure these treatments are only supplementary to the animals own immune system which overall has to take on the task of repairing any damage to the laminae.

CHAPTER III

HISTORY OF ADAMs AND RELATED METALLOPROTEASES

ADAM discovery

The ADAM family of proteins was initially discovered as a cellular family of proteins that share a common domain organization with the previously discovered snake venom metalloproteases (Weskamp and Blobel 1994; Wolfsberg, Primakoff et al. 1995).

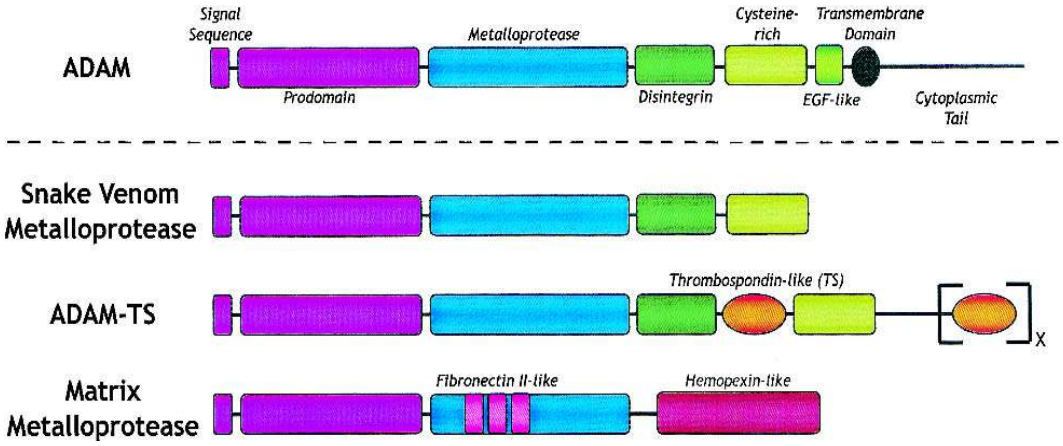
Domain Organization

ADAMs are members of the zinc protease superfamily which is subdivided based on their catalytic sites primary structure. This group of metalloproteases belongs to the metzincin subfamily which is further divided into adamlysins (matrixins belong to this family as well MMPs and ADAM-TS) (Wolfsberg, Primakoff et al. 1995). The typical organization of an ADAM from n-termini to c-termini is a signal sequence, a prodomain, metalloprotease domain, disintegrin domain, cystine rich repeats, an EGF like domain and a transmembrane domain with a cytoplasmic tail afterwards (Seals and Courtneidge 2003).

The ADAM-TS subfamily share a very similar organization to that of the ADAMs with a signal sequence, prodomain, metalloprotease domain, disintegrin domain followed by a single thrombospondin like domain followed by a cysteine rich domain (Seals and Courtneidge 2003). This is then followed by varying numbers of thrombospondin like repeats depending on the exact ADAM-TS being discussed.

MMPs, a group of metalloproteases belonging to the same family as the adamlysins share a similar domain organization to that of ADAMs. Typical organization for the MMPs is a signal sequence, a prodomain, metalloprotease domain with fibronectin type II like repeats, and a hemopexin like domain (Seals and Courtneidge 2003). A schematic representation of these domains can be found in Figure 2.

TIMPs, the natural Tissue Inhibitors of Metalloproteases have four family members, TIMP1-4. This family of proteins has two domains, an N-terminal domain and a C-terminal domain (Woessner 2001). Each domain contains three disulfide bridges and most of the biological functions reside in the N-terminal domain (Brew, Dinakarpandian et al. 2000).



(Seals and Courtneidge 2003)

Figure 2 – Domain Organization of ADAM Family of Metalloproteases
Domains of the ADAMs and related metalloproteases. ADAMTS family members have variable number of thrombospondin-like (TS) repeats. MMP shown is representative of the gelatinase class (MMP-2 and 9).

Expression, Regulation, and Localization

The expression pattern of the ADAM family of proteins is greatly varied with many of these proteins being expressed in the testis (Seals and Courtneidge

2003). These proteins are thought to be synthesized in the rough ER and then go on to mature in the late Golgi compartment (Lum, Wong et al. 1999; Hougaard, Loechel et al. 2000; Howard, Maciewicz et al. 2000). It is while the protein is in the late Golgi that the pro domain is thought to be cleaved, making the protein competent to function. Cleavage of the pro domain can also occur at the cell surface by furin-like proteases (Srour, Lebel et al. 2003). Metalloprotease activity of ADAMs 10, 17, and 19 can occur within intracellular compartments (Skovronsky, Moore et al. 2000; Shirakabe, Wakatsuki et al. 2001). With all of these differences in localization it is presumed that the localization and activity of ADAMs depends on the cell type, substrates, and which ADAM is interacting.

The ADAM-TS family of proteins has been shown to localize within the ECM. This localization is mediated via the thrombospondin (TSP) repeats which can bind to ECM components. The tissue distribution for each ADAMTS family member is wide spread in normal adult tissues. A table of the ADAM-TS genes of interest in laminitis and their tissue localizations in humans can be found below in Table 2. The regulation of this gene family is not very well understood but there is some evidence suggesting that many of these family members are regulated by growth factors, hormones and inflammatory cytokines (Miles, Sluka et al. 2000; Wang, Wang et al. 2003; Kashiwagi, Enghild et al. 2004).

Gene	Fetal Tissue	Normal Adult Tissue
ADAM-TS1	Kidney, Lung	Heart, Placenta, Liver, Skeletal Muscle, Kidney, Thyroid, Adrenal Cortex, Stomach, Bladder, Aorta, Colon, Prostate, Spinal Cord
ADAM-TS4	Not tested	Bladder, Brain, Ovary, Heart, Skeletal muscle, Uterus, Stomach, Spinal cord
ADAM-TS5	Not tested	Bladder, Cervix, Oesophagus, Placenta, Uterus

Table 2: Localization of ADAMTS Gene Expression. Adapted from (Porter, Clark et al. 2005)

Matrix Metalloproteases expression is mostly ubiquitous and therefore these enzyme are involved in many physiological processes (tissue remodeling in pregnancy, wound healing, and angiogenesis) and pathological conditions (cancer, arthritis, periodontitis) (Yan and Boyd 2007). These metalloproteases are also capable of degrading the ECM components. This activity is what allows the MMP family to remodel and repair defective tissues to allow for normal growth. MMPs are primarily regulated by their natural inhibitors, TIMPs (Cawston, Billington et al. 1999). This family of metalloproteases has been shown to localize to the cartilage of patients with arthritis, suggesting their role in inflammatory diseases (Koolwijk, Miltenburg et al. 1995). Their presence is typically linked to tissues undergoing remodeling. This is also the case in the joints of horses with

osteochondritis dissecans, a disease in which there is a loss of blood supply to the area of bone directly beneath the joint (Al-Hizab, Clegg et al. 2002).

TIMP expression is widespread. TIMP2 expression is constitutive and widely expressed in many tissue types while the rest of the family members are inducible proteins and are known to exhibit tissue specificity (Brew, Dinakarpandian et al. 2000). TIMP1 is enriched in reproductive organ systems; TIMP3 is enriched in the heart, kidney, and thymus (Leco, Apte et al. 1997; Crocker, Pagenstecher et al. 2004).

Substrate Activities

ADAMs have many substrates and are known to be involved in activities such as the shedding of cytokines and cytokine receptors, processing of growth factors and their receptors. There have been 30+ ADAMs identified to date and 19/30 contain the zinc-binding active consensus site of HExxHxxGxxH (Nagase and Kashiwagi 2003). One of the best studied ADAMs is ADAM17, better known as TNFα converting enzyme, TACE (Black 2002). TNFα is one of the most predominant proinflammatory cytokines studied in modern science. Originally ADAM17 was found the be the only ADAM capable of processing TNFα in a TNFα peptide cleavage assay (Black, Rauch et al. 1997; Moss, Jin et al. 1997). It was then seen that TNFα processing still occurred in ADAM17 deficient mice, but when an ADAM inhibitor was added to culture this processing stopped, suggesting that other ADAMs were processing TNFα (Reddy, Slack et al. 2000). It was later found that both ADAM9 and ADAM10 cleave TNFα peptides *in vitro* (Lunn, Fan et al. 1997; Rosendahl, Ko et al. 1997; Roghani, Becherer et al. 1999;

Amour, Knight et al. 2000). Even though ADAM17 is not the sole ADAM responsible for TNFα processing it is still known as an important element because its expression is increased in the inflammatory response (Patel 1999; Colon, Menchen et al. 2001). ADAM10 and 17 are also known to cleave the chemoattractant fractalkine which is known to function in both adhesion and chemoattraction (Rossi and Zlotnik 2000).

ADAMs are also believed to have a role in the degradation of the extracellular matrix (ECM). ADAM 10 is known to cleave collagen IV in vitro, and ADAM15 is capable of cleaving collagen and gelatin in vitro (Chubinskaya, Cs-Szabo et al. 1998; Martin, Eynstone et al. 2002). It has also been proposed that ADAM15 could degrade ECM either directly through its metalloprotease activity or indirectly through proteolytic activation of MMPs (Martin, Eynstone et al. 2002). ADAM13 also has a known role in ECM modification in xenopus cranial neural-crest migration partly due to its ability to cleave fibronectin from the ECM (Alfandari, Cousin et al. 2001).

ADAMTS proteins are known to function in inflammation, angiogenesis, and development (Tang and Hong 1999). Aggrecan, one of the major proteoglycans of cartilage, is the primary target of many ADAMTS proteins termed aggrecanases. When aggrecan is removed from cartilage the collagen is left open to attack by the MMP (Pratta, Scherle et al. 2003). The aggrecanase family is made up of ADAMTS-1, 4, 5, 8, 9, and 15. Of these aggrecanases ADAMTS4 and 5 are the most studied and the cleavage fragments of aggrecan are known (Sugimoto, Takahashi et al. 1999; Tortorella, Pratta et al. 2000;

Tortorella, Malfait et al. 2001). These two are also capable of cleaving the chondroitin sulfate proteoglycans brevican and versican (Matthews, Gary et al. 2000; Sandy and Verscharen 2001).

The natural substrate of the TIMP family of proteins are other metalloproteases such as ADAM, ADAMTS and MMPs. TIMP1 and TIMP2 are able to bind to both pro-MMP9 and pro-MMP2 via their C-terminal hemopexin like domain (Apte, Olsen et al. 1995; Gomez, Alonso et al. 1997; Olson, Gervasi et al. 1997; Olson, Bernardo et al. 2000). Binding via the C-terminal domain is the most common way in which TIMPs interact with the pro-MMPs to regulate them. TIMP2 and TIMP4 can bind pro-MMP2 and inhibit MMP2 and MT1-MMP activity (Hernandez-Barrantes, Shimura et al. 2001). The C-terminal domain of TIMP3 mediates its binding to the ECM while the other members in the TIMP family are only found in soluble forms (Langton, Barker et al. 1998). TIMP3 is also able to inhibit several members of the ADAM and ADAMTS families including ADAM10, ADAMTS4 and 5 (Smith, Kung et al. 1997).

The Matrix Metalloprotease (MMP) family substrate activities are categorized into six categories. These include the collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and then the 'orphan' MMPs which do not fall into any of the above categories. Each subsets name essentially identifies its substrate. The main family of interest in laminitis is the gelatinase family due to their known presence in laminitis animals (Kyaw-Tanner and Pollitt 2004; Loftus, Belknap et al. 2006).

The gelatinases, made up of MMP2 and MMP9, have an additional fibronectin domain inside the catalytic domain. This domain is potentially responsible for their ability to digest denatured collagen or gelatin (O'Farrell and Pourmotabbed 1998). MMP2 is capable of degrading types IV, V, VII, and X collagen, elastin, fibronectin, laminin, and several chemokines. This MMP is also capable of activation of proMMP1 and proMMP9 (Yu, Sato et al. 1998). MMP9 also has a type V collagen like sequence between the catalytic and hemopexin like domains. This MMP is capable of degrading denatured collagen, types IV, V, and XI collagen, aggrecan, and elastin (Visse and Nagase 2003).

CHAPTER IV

IMPLICATION OF ADAMs AND RELATED METALLOPROTEASES IN EQUINE LAMINITIS

Functions of MMPs in Laminitis

Matrix Metalloproteases were first identified as proteins of interest in laminitis in 1996 when C.C.Pollitt suggested that the expression and activation of MMPs was the mechanism causing the separation of the epidermal and dermal laminae (Pollitt 1996). Johnson *et al* (Johnson, Tyagi et al. 1998) then analyzed laminae tissue samples from laminitic and non-laminitic horses. This group discovered that there was gelatinase activity at 92 kDa, 72 kDa, and 66 kDa which corresponds to MMP9, and MMP2 respectively. This discovery both confirmed what Pollitt had suggested and initiated a new hypothesis on the overall events leading to the degradation of the laminae. Previous to this study, work had focused mainly on the histopathological abnormalities within the epidermal laminae and not in abnormalities developed in the collagen of the basement membrane and the ECM of the dermis.

Since this discovery there has been much focus on the involvement of MMPs in both natural and experimentally induced laminitis. The activation of endogenous MMP2 and MMP9 activity has been shown to reduce the tensile strength of laminar explants in vitro (Mungall, Pollitt et al. 1998). MMP2 has also been shown to be localized to the cytoplasm of the basal and parabasal cells which are in close proximity to the lamellar basement membrane using *in situ* hybridization. Along with that result it was shown that MMP2 mRNA is

significantly elevated in laminitic horses using qPCR (Kyaw-Tanner and Pollitt 2004). Studies have also been done on the concentrations of collagen IV, a known target of MMP2 and 9, in horses with laminitis. The results showed that there was an increase in collagen IV in serum of horses with natural laminitis which matched up with elevated MMP tissue activity indicated by more gelatin degradation in a gelatin zymography assay (Johnson, Kreeger et al. 2000). In the BWE model of laminitis MMP9 increase has been correlated to immigration of neutrophils into the laminae (Loftus, Belknap et al. 2006). Since neutrophils are known to store mature MMP9 in their tertiary granules it is not necessary for its transcription in order to observe its accumulation (Chakrabarti and Patel 2005). While these are promising data, it is essential to note that gel zymogram do not reflect the true activity of MMP in vivo as the SDS-PAGE removes the prodomain artificially activating the enzyme. While these experiments are indicative of the relative abundance of each MMP, more experiments are needed to detect active MMP in situ.

Role of ADAMs in Laminitis

ADAMs are known to have a prominent role in both inflammatory disease and cartilage degradation (Moss and Lambert 2002; Nagase and Kashiwagi 2003). ADAMs are known to be up regulated at the site of inflammation and their roles as sheddases is potentially critical during this process. ADAM17 (TACE) is the most studied ADAM in inflammatory diseases and the exact cleavage sequence is known (Black, Rauch et al. 1997; Itai, Tanaka et al. 2001; Moss and Lambert 2002). ADAM10 is also known to process pro-TNFα, suggesting it also may

complement the activities of ADAM17 (Lunn, Fan et al. 1997). Both ADAM10 and 17 are also able to cleave the chemoattractant fractalkine which functions in both adhesion and chemoattraction (Rossi and Zlotnik 2000). These two ADAM are therefore likely candidate to be increased in the model of laminitis derived from inflammation.

The ADAM-TS family of proteases also has a potential role in laminitis disease progression based on their ability to cleave and process aggrecan, a proteoglycans found in many ECMs (Nagase and Kashiwagi 2003; Porter, Clark et al. 2005; Gendron, Kashiwagi et al. 2007). ADAMTS-1, 4, and 5 are all known aggrecanases and their presence in the laminae could be a critical step is determining the molecular pathology of laminitis. The presence of these proteases would suggest that laminitis may bear closer resemblance to arthritis than to any other human diseases and could have implication in the development of a disease.

The TIMP family of inhibitors is also an interesting target, as the members of this family are able to inhibit all of the ADAMs mentioned above. The affinity of certain TIMPs is higher for certain ADAMs so it will be interesting to determine how the expression levels are changed in each individual case.

The goal of this gene selection is to evaluate how the overall proteolytic activity may change during the onset and evolution of laminitis.

Working Model of Proteolytic Cascade Leading to Degradation of Collagen and Aggrecan

We have used the knowledge accumulated on MMP and ADAM to draw a diagram that describes how they may be involved in the degradation of the ECM Laminae**.** (Figure 3),. Upon the initial damage it is possible that either ADAM10 or ADAM17 could be activated. These molecules are capable of processing pro-TNFα to its active form TNFα (Rosendahl, Ko et al. 1997). Once this potent cytokine has been activated it is capable of activating several pathways which all ultimately lead to the potential degradation of the laminae. The first of these pathways is the activation of the ADAMTS family of aggrecanases which could degrade aggrecan present in the laminae. The second pathway that could be activated is the MMP pathway. Both MMP2 and 9 are capable of degrading type IV collagen which is known to be present in the laminae (Kuwano, Ueno et al. 2005). The activation of both families of these proteases could lead to the separation of the laminae, causing laminitis disease progression. On the other hand, the TIMP family of protein is able to inhibit many of these pathways at several points. TIMP3 is capable of inhibiting both ADAM10 and 17, as well as the ADAMTS family of aggrecanases (Smith, Kung et al. 1997) . TIMP1 is capable of inhibiting MMP9 and TIMP2 is capable of inhibiting both MMP2 and 9 (Apte, Olsen et al. 1995; Gomez, Alonso et al. 1997; Olson, Gervasi et al. 1997).

In order to test this model we have cloned and sequence members of the ADAM, ADAMTS, TIMP and MMP family of proteins. We the used these

sequence to study variation in gene expression during the progression of natural and artificially induced laminitis.

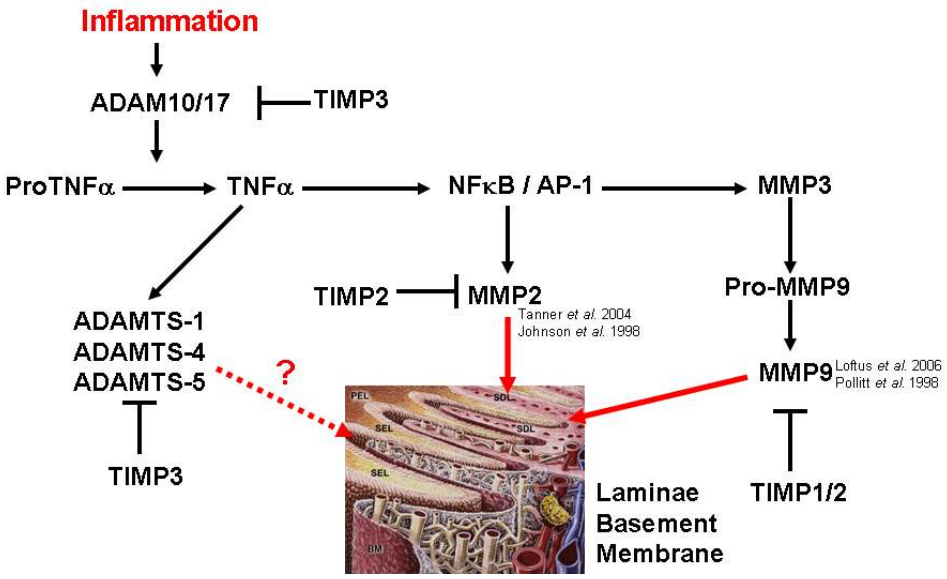


Figure 3: Model of a proteolytic cascade that could lead to the development of laminitis.

CHAPTER V

CLONING OF ADAMs

Cloning and Sequencing of Equine ADAM10, 17, TS4, TS5 and MMP9

In order to study the role of the ADAM family of proteins in equine laminitis, ADAM10, ADAM17, ADAM-TS4, and ADAM-TS5 needed to be cloned. To accomplish this, multiple protein sequence alignments were done for each gene between several mammalian species in order to determine regions of homology (Figures 4-7). Degenerated oligonucleotide primers were designed for each genes most homologous region and are denoted by the boxed sequences in Figures 4-7. Each of these figures represents a ClustalW alignment of the equine sequence with three other mammalian species. Identical residues are denoted by an asterix (*), conservative changes by a double point (:). Also these figures display the locations of the real time PCR primers which are shown with arrows. Since ADAMs are known to be expressed in the testis (Wolfsberg, Straight et al. 1995; Kim, Kang et al. 2006) cDNA was made from equine testis tissue. Each primer set amplified its expected size bands, which were cloned into pCR4-TOPO (Invitrogen) for sequencing. These results, compared with the human sequences showed that ADAM10 is 95% identical, ADAM17 is 91% identical, full length ADAM-TS4 is 90.8% identical, and ADAM-TS5 is 91% identical. Each cloned sequence corresponded to a different region of domains, which can be seen in Figures 4-7.

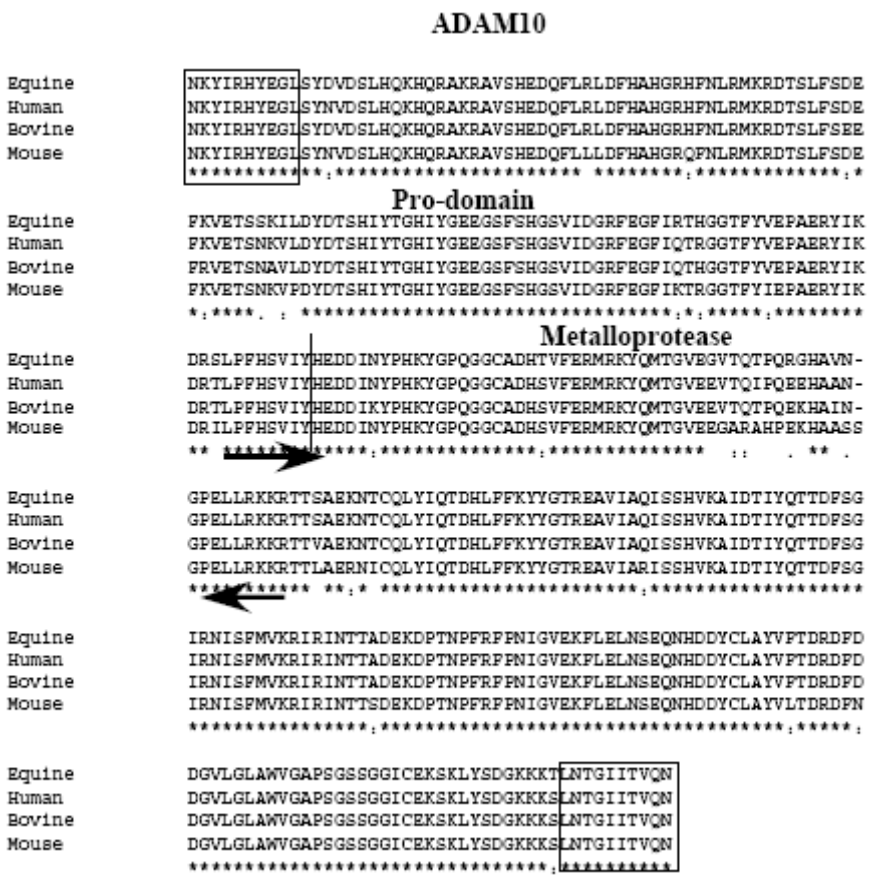


Figure 4: Amino Acid Sequence Comparison of Equine ADAM10

Single letter amino acid sequence from equine, human, bovine, and mouse ADAM10 were aligned using ClustalW software. Identical residues denoted by an asterisk (*), conservative changes are indicated by a double point (:). The sequence corresponding to the degenerate oligonucleotides are indicated in boxes. Position corresponding to the oligonucleotide primers used for qRT-PCR indicated by arrows. The domains are separated by vertical lines.

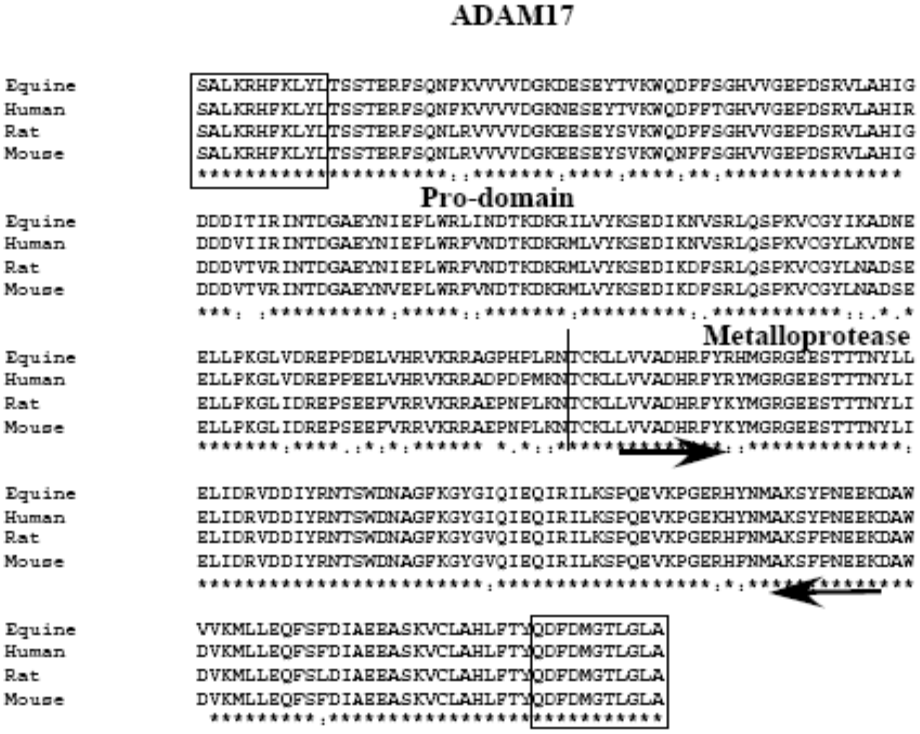


Figure 5: Amino Acid Sequence Comparison of Equine ADAM17

Single letter amino acid sequence from equine, human, rat, and mouse ADAM17 were aligned using ClustalW software. Identical residues denoted by an asterisk (*), conservative changes are indicated by a double point (:). The sequence corresponding to the degenerate oligonucleotides are indicated in boxes. Position corresponding to the oligonucleotide primers used for qRT-PCR indicated by arrows. The domains are separated by vertical lines.

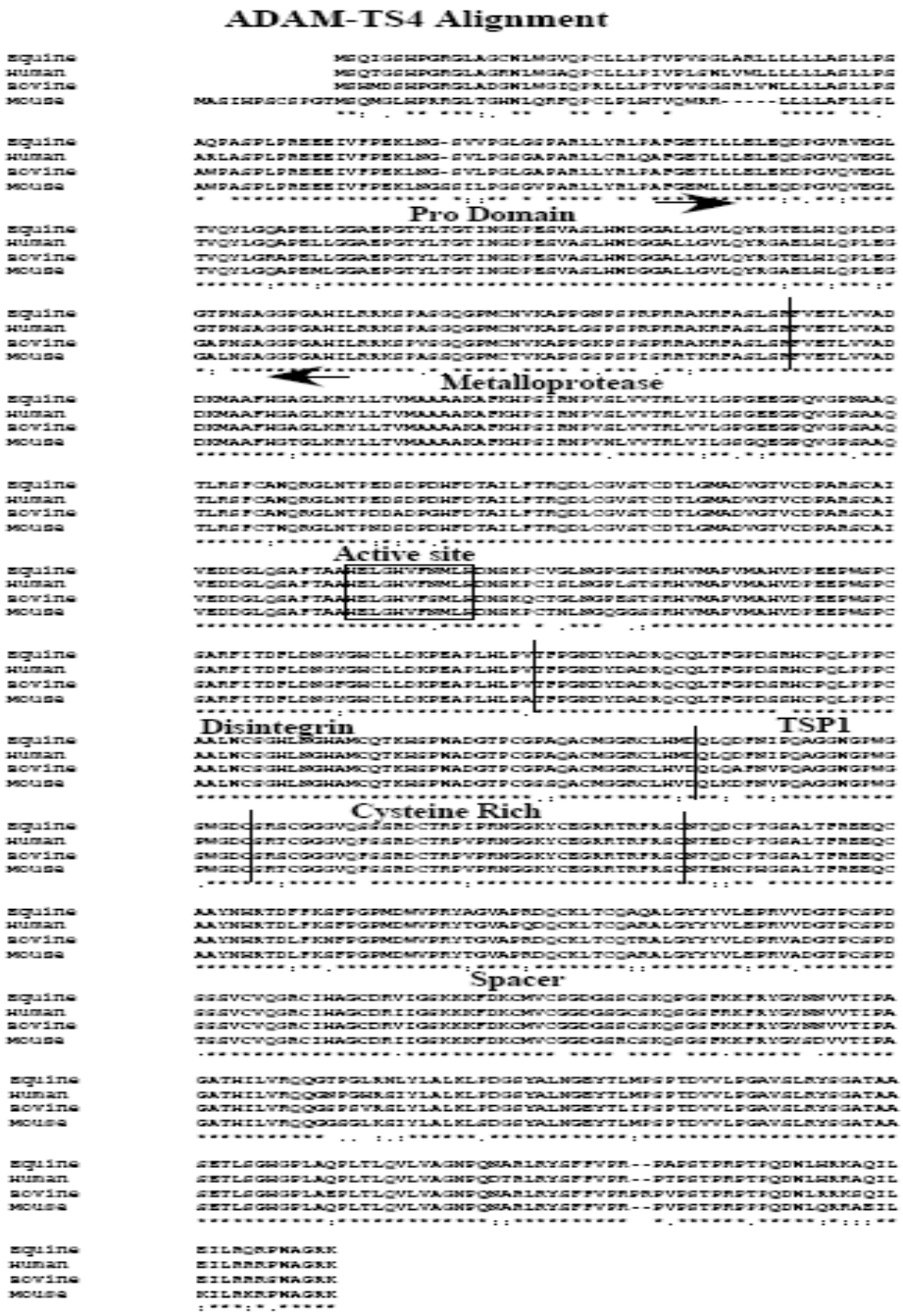


Figure 6: Amino acid sequence comparison of Equine ADAMTS-4

Single letter amino acid sequence from equine, human, bovine, and mouse ADAMTS-4 were aligned using ClustalW software. Identical residues denoted by an asterisk (*), conservative changes are indicated by a double point (:). The sequence corresponding to the metalloprotease active site is indicated in a box. The positions corresponding to the oligonucleotide primers used for qRT-PCR are indicated by arrows. The domains are separated by vertical lines.

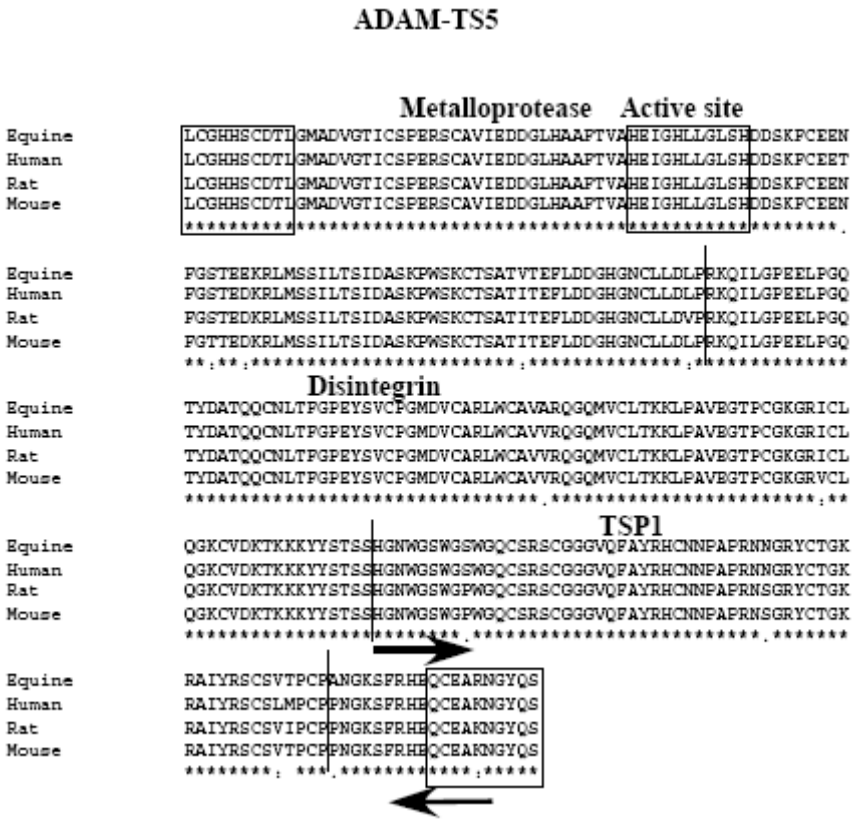


Figure 7: Amino acid sequence comparison of Equine ADAMTS-5

Single letter amino acid sequence from equine, human, bovine, and mouse ADAMTS-5 were aligned using ClustalW software. Identical residues denoted by an asterisk (*), conservative changes are indicated by a double point (:). Sequence corresponding to degenerate oligonucleotides is indicated in a box. Active metalloprotease site is also labeled and presented in a box. Position corresponding to oligonucleotide primers used for qRT-PCR is indicated by arrows. The domains are separated by vertical lines.

The specific fragments obtained by amplification are shown in Figure 8. The positions of these primers can also be seen in Figures 4-7. For each of these primer sets, PCR amplification was performed using no-RT controls, Genomic DNA, and cDNA samples to test 1) that the cDNA did not contain contaminating genomic DNA, 2) if the primer could amplify from genomic DNA.

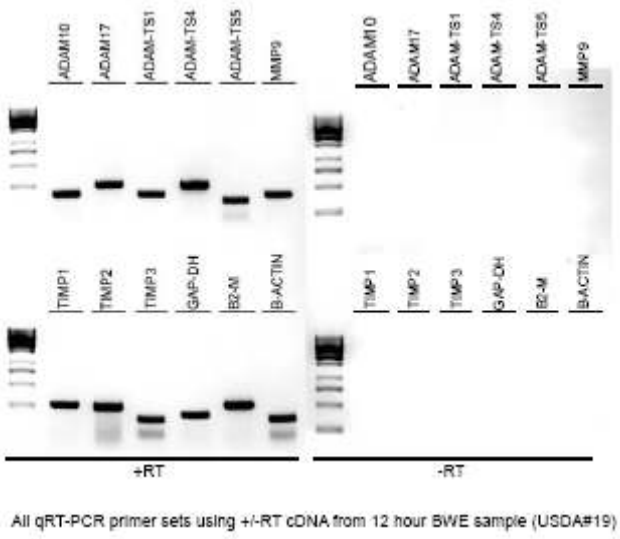


Figure 8: PCR Amplification of +/-RT cDNA Using Specific Oligonucleotide qPCR Primers

Degenerate primers and those used for the amplification of full length and partial clones can be found in Table 3. A table that shows the primer efficiencies, slope and R^2 values, as well as the specific melting temperatures of the amplification product as determined by running a melting curve can be found in Table 4.

	Forward	Reverse	Size bp
ADAM10	CCGTTTCACTCTGTTATTTATCAT	AGGGATTTGTAGGGTCTTTCTCAT	397
ADAM17	TGGCAGGACTTCTTCAGCGGACAC	TTTCTTCATTTGGATAACTTTTTG	571
ADAM-TS1	TGAAAAGCAGGAAAAAGATGAGAAT	AAGGAGGAACGAATGGTAGGAGGTA	436
ADAM-TS4	GCTGTGCTATTGTGGAGGATGATGG	CCAGGGAAAGTCACAGGCAGATG	507
ADAM-TS5	ACAGAAGAGAAGCGCTTAATGTCTTCCA	CCCTCTTTCCTGTGCAGTAGCGGCCATT	504
TIMP1	ACCTTACAGCGGCGTTATGAGAT	ATAGGAATGGGAAAGAGGGTGAA	495
TIMP2	AGGTGGACTCTGGGAACGACATC	GCTCTTCTTCTGGGTGGTGCTCA	246
TIMP3	CCCTTTGGCACACTGGTCTACAC	GAGAAGAATGGCAAAAGCAGGAG	864
MMP9	CGCCCCCTGCCACTTCCCCTTCACC	GAGGCGCCCATCACTGCGGCCCTCT	408
GapDH	TTGTCATCAACGGAAAGGCCATCA	ACGGAAGGCCATGCCAGTGAGCTT	456
β2-Microglobulin	CAGGTTTACTCACGTCACCC	CTGGTTAGAGGTCTCGATCCC	240
β-Actin	GGGAAATCGTGCGTGACAT	AGCACTGTGTTGGCGT	616
qPCR			
ADAM10	CCGTTTCACTCTGTTATTTATCAT	TCTTCAGGAGTTCTGGACCATTA	191
ADAM17	CAGACCATCGCTTTTACAGACAC	TTTCTTCATTTGGATAACTTTTTG	252
ADAM-TS1	GGTGCAAGCTCATCTGTCAA	TCCATTTCCTCCGCAAATAC	194
ADAM-TS4	GCCTTTGGGGAGACGCTGCTACTA	GATGTGAGCCCCAGGTCCCCCAGC	282
ADAM-TS5	AACTGGGGGTCCTGGGGGTCCTGG	CATTTCTTGCCTCACACTGCTCAT	158
TIMP1	GTCTCCGGCATTCTGTTGTT	TAGCGGGGGTGTAGACAAAC	244
TIMP3	CCCTTTGGCACACTGGTCTACAC	GTTGCAGAGTCCTGTGTACATCTT	192
MMP9	CGCCCCCTGCCACTTCCCCTTCACC	CCGTCCTGGGTGTAGAGTTTCTC	208
β-Actin	CGACATCCGTAAGGACCTGT	GTGGACAATGAGGCCAGAAT	192
GapDH	GATTGTCAGCAATGCCTCCT	AAGCAGGGATGATGTTCTGG	194

Table 3: Primer Pairs Used for Specific Gene Amplification and for qRT-PCR

	Efficiency	Slope	R^2	Size	Melting Temp
ADAM10	99.5	-3.33	.923	191	80
ADAM17	89.9	-3.59	.884	252	80
ADAMTS1	106.8	-3.172	.964	194	81
ADAMTS4	93.7	-3.482	.972	282	86
ADAMTS5	102.1	-3.273	.903	158	85
TIMP1	85.1	-3.914	.926	244	88
TIMP2	88.1	-3.644	.991	246	86
TIMP3	91.5	-3.542	.972	192	82
MMP9	90.9	-3.561	.93	208	88
GAPDH	92.9	-3.505	.911	194	87

Table 4: qRT-PCR Amplification Efficiencies

Testis cDNA was diluted in 10 fold increments in sterile H_2O from a 1:1 to a 1:100 dilution to create templates for the generation of a standard curve for each gene tested. Efficiencies between 85-110% along with a slope between -3.0 and -4.0 and an R^2 value between 0.90 and 1 were acceptable as being equal in efficiencies.

	Forward	Reverse	Size in bp
ADAM10	TAAATAAATACATTAGACATTATGAAGGAT	GAGAGSCCATAGTTCTGAACAGTRATRATT	1032
ADAM17	TCAGCYYTGMAAAGGCAYTTTAAATTATACTT	TAAGCYARTCCAAGWGTTCCCATATCAAAATC	805
ADAM-TS4	CACCCTGGGKATGGCWGAYGTKGGCACMRT	TTGCATYGGTCTCGRGGGGMCACWCCTGWR	868
ADAM-TS5	ATTTATGTGGGCATCATTCATGTGACYACY	AGACTGATARCCATTYYTKGCYTCACACTG	817
FL MMP9 deg	ATGAGCCYCYKGCAGCCCYTGGT	CTAGTCCTCAGGGCACTMCAGGA	2288
FL MMP9 spec.	ATGAGCCCCTGGCAGCCCTTGGTCC	GACCCCTAGTCCTCAGGGCACT	2288
FL ADAM-TS4 deg	ATGTCCCASAYRGRCTCGCATCC	GCCGGGATTGTGAGGTTATTTCC	2514
FL ADAM-TS4 spec.	ATGTCCCAGATAGGCTCGCATCCC	GCCGGGATTGTGAGGTTATTTCC	2514

Table 5: Oligonucleotide Primer Sets Used for Cloning ADAMs

CHAPTER VI

EXPRESSION OF ADAM RELATED METALLOPROTEASES IN BWE INDUCED LAMINITIS

Animals Used

For the BWE experiments, archived samples of laminar tissue from previous studies were used (Waguespack, Cochran et al. 2004; Waguespack, Kemppainen et al. 2004; Black, Lunn et al. 2006).

Real Time PCR Results

In the BWE model of laminitis, Table 6A, it was observed that ADAM10 and ADAM17 were more likely to be decreased than increased. ADAM10 expression was decreased in 5/8 animals with 2 animals showing no change and one animal showing a slight increase at the 3 hour time point. ADAM17 was decreased in 3/8 animals and no change was seen in 4/8 animals with one animal showing a slight increase in expression at the 3 hour time point. ADAM-TS1 was slightly increased in 5/8 animals with the remainder showing no changes in expression. ADAM-TS4 was the most induced gene of this study and every animal showed at least a slight increase with most animals (6/8) showing a large increase in expression. ADAM-TS5 was decreased in this study in 3/8 animals with the remainder showing no change. MMP9 reduction seemingly occurred at the early time point of 1.5 hours with 2/2 animals showing a decrease. TIMP1 expression was not seen to change other than 1/8 animals showing a 2.13 fold increase. TIMP2 expression was reduced in 5/8 animals with no trend towards

either early or late reduction. Expression of TIMP3 was not changed at the early time point, however 5/6 animals at the 3 hour and 12 hour time points showed a reduction in expression.

CHAPTER VII

EXPRESSION OF ADAM RELATED METALLOPROTEASES IN CHO INDUCED LAMINITIS

Animals Used

For the CHO experiments, archived samples of lamellae from a previous study were used (Johnson, Kreeger et al. 2000).

Real Time PCR Results

The CHO model of laminitis, Table 6B, was grouped into animals which either showed a response to treatment (CHO-R), and those which did not (CHO-NR). Animals which were in the CHO-R group showed no changes in either ADAM10 or ADAM17 expression, while there was an increase in expression of ADAM-TS1. ADAM-TS4 was greatly increased in 3/3 animals in the CHO-R group, and also in 2/3 animals in the CHO-NR group. ADAM-TS5 expression was also increased in both the CHO-R and CHO-NR groups with 5/6 animals showing an increase. MMP9 was consistently induced in all 6/6 animals in the CHO study. TIMP1 expression was also increased in 5/6 animals in the CHO-R and CHO-NR groups. TIMP2 was decreased in the CHO-R group while there was no change seen for the animals in the CHO-NR group. TIMP3 was increased in all of the horses in the CHO-NR group while there were 2/3 horses with an increase in the CHO-R group, with the remaining animal showing a decrease in expression.

CHAPTER VIII

EXPRESSION OF ADAM RELATED METALLOPROTEASES IN NATURAL LAMINITIS

Animals Used

The natural laminitis samples were obtained from horses which were euthanized at the University of Missouri School of Veterinary Medicine Equine Clinical Center.

Real Time PCR Results

Natural cases of laminitis, Table 6B, share the best correlation of gene expression with the CHO model. This model had animals grouped into either acute cases in which the animals had not previously shown signs of laminitis, animals in the chronic subset had a history of laminitis but had not had any acute episodes, and animals in the chronic aggravated subset had a history of laminitis and were experiencing a debilitating episode. All horses, regardless of classification, showed an increase in ADAM-TS4 expression and 9/11 showed a decrease in TIMP2 expression. ADAM10 expression was decreased in both the chronic and chronic aggravated animals (4/8) with the remainder showing no change in expression. ADAM17 showed no change in expression, with the exception of one clinical animal having a decrease. ADAM-TS1 only had 3/11 animals with slight increases while ADAM-TS5 expression was increased in acute cases as well as 5/8 of the chronic and chronic aggravated cases. MMP9 expression was greatly increased in the acute cases of laminitis while there were 6/8 animals with an increase in the chronic and chronic aggravated cases of

laminitis. Expression of TIMP1 was increased in acute cases while there was either no change or a decrease in expression in the chronic and chronic aggravated cases. Expression of TIMP3 was increased in all animals in the clinical cases and expression ranged from 3.2 to 31.76 fold increase.

A

	Horse #	Age	Breed	ADAM10	ADAM17	ADAMTS1	ADAMTS4	ADAMTS5	MMP9	TIMP1	TIMP2	TIMP3
Control	4703	9y	N Walking Hors	1.03	0.66	1.03	1.06	1.05	1.09	1.01	1.12	0.77
	USDA#5-#15	9-10y	StdBred	1.00	1.26	1.02	1.78	1.03	1.01	1.02	1.05	1.24
	Set 2 - C	N/A	N/A	1.01	1.98	0.54	1.52	1.27	1.90	1.24	1.55	1.58

	Horse #	Age	Breed	ADAM10	ADAM17	ADAMTS1	ADAMTS4	ADAMTS5	MMP9	TIMP1	TIMP2	TIMP3
BWE 1.5h	22	5y	StdBred	0.41	1.88	4.34	48.72	0.97	0.24	2.13	0.45	0.53
	25	12y	StdBred	1.22	1.24	2.09	2.25	0.93	0.10	0.97	1.11	1.69

	Horse #	Age	Breed	ADAM10	ADAM17	ADAMTS1	ADAMTS4	ADAMTS5	MMP9	TIMP1	TIMP2	TIMP3
BWE 3h	4716	N/A	Old Palimino	2.66	0.43	1.34	24.60	0.16	0.55	0.57	0.41	2.10
	17	6y	StdBred	0.52	0.80	6.48	102.77	1.25	0.51	1.76	1.00	0.11
	Set 2 - 3	N/A	N/A	0.49	4.14	1.48	13.61	0.13	5.75	1.20	0.44	0.03

	Horse #	Age	Breed	ADAM10	ADAM17	ADAMTS1	ADAMTS4	ADAMTS5	MMP9	TIMP1	TIMP2	TIMP3
BWE 12h	USDA#19-#9	8-9y	StdBred	0.11	0.23	4.98	22.11	0.34	0.35	1.82	0.04	0.01
	USDA#1-#11	15y	StdBred	0.49	1.96	1.10	4.73	0.90	1.70	1.10	2.23	0.25
	Set 2 - 12	N/A	N/A	0.15	0.47	2.00	31.34	0.66	1.81	0.72	0.18	0.02

B

CHO	Horse #	Age	Breed	ADAM10	ADAM17	ADAMTS1	ADAMTS4	ADAMTS5	MMP9	TIMP1	TIMP2	TIMP3
Control	150321	14y	Appaloosa	0.49	0.23	0.98	1.63	1.55	0.70	1.44	1.45	2.79
	149978	20y	Thouroughbred	2.01	1.45	1.01	0.11	2.07	1.41	1.76	2.95	2.12
	149090	N/A	N/A	0.27	1.04	1.36	1.33	0.12	1.45	0.19	0.74	0.12
	147235	4y	Quarter Horse	0.72	2.80	0.74	1.58	2.00	0.19	0.97	0.43	0.24
	Horse #	Age	Breed									
CHO-NR	147660	4y	Saddlebred	1.86	1.05	2.12	3.87	3.55	2.26	1.03	1.13	25.09
	147369	11y	Quarter Horse	1.20	1.08	3.46	1.13	1.28	3.60	4.24	0.57	14.48
	147144	6y	Thouroughbred	1.42	2.09	1.80	17.84	4.45	2.60	2.04	0.52	4.02
	Horse #	Age	Breed									
CHO-R	N1942	3y	StdBred	1.51	1.85	3.81	30.41	5.97	6.62	17.64	0.44	25.27
	147421	5y	Morgan	0.56	1.77	4.80	91.99	18.13	4.90	6.12	0.07	0.24
	TBX04	3y	StdBred	0.44	1.08	2.09	53.08	3.82	5.28	4.58	0.64	2.83
Clinical	Horse #	Age	Breed									
Acute	KK82	N/A	No Info	0.51	1.90	1.91	271.22	11.34	81.76	5.78	0.19	4.57
	143914	N/A	No Info	0.99	1.42	2.33	10.46	2.21	32.75	1.68	0.32	2.62
	149056	3y	Thouroughbred	2.04	1.79	5.97	45.78	65.80	76.11	29.33	0.82	26.77
	Horse #	Age	Breed									
Chronic	146955	N/A	Mini Pony	1.61	0.83	0.67	8.28	1.04	10.90	1.18	1.14	31.76
	144728	22y	Quarter Horse	0.69	0.48	1.36	18.11	0.37	2.17	0.67	0.35	4.93
	145632	Old	Pony	0.40	1.14	1.96	3.20	2.22	1.64	0.27	0.33	5.49
	145329	22y	TN Walking Horse	0.55	1.44	0.91	2.73	3.86	4.92	0.41	0.31	15.64
	Horse #	Age	Breed									
onic agravated	AXT82	N/A	N/A	1.60	1.25	0.68	5.28	4.57	41.64	1.56	0.32	24.80
	149168	25y	Pony	0.36	1.19	2.40	14.55	0.67	0.38	0.50	0.22	3.20
	141644	3y	Quarter Horse	0.35	1.32	1.61	8.13	2.52	2.50	0.26	0.12	14.88
	143633	12y	Quarter Horse	0.41	0.95	1.17	333.14	5.48	9.13	0.32	0.31	5.05

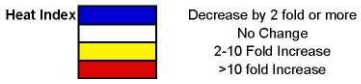


Table 6: Quantative Real Time PCR Results

Real-time quantative PCR was performed on cDNA samples of 32 horses. Each specific oligonucleotide primer pair was run in triplicate on three different days using a Roche Lightcycler 2.0. The relative value for each sample was obtained using the ΔΔCt method and controlled to expression of GAPDH. Each value was averaged from triplicates. Numbers between 2 and 10 are highlighted in yellow and correspond to a 2 to 10 fold increase. Numbers superior to 10 are in red and correspond to an increase greater than 10 fold. Values inferior to 0.5 are highlighted in blue and correspond to at least a 2 fold decrease. The horse number, age, breed, as well as the results for each of the selected genes are presented. BWE; Black Walnut Extract, CHO; Carbohydrate Overload.

CHAPTER IX

SUMMARY OF RESULTS

These results show that ADAMs which are known to be associated with inflammation, ADAM10 and ADAM17, are not significantly increased in horses with laminitis, regardless of the mode of induction (BWE, CHO, natural) and the extent of disease. These two genes also clearly act independently of one another, as it was observed that an increase in one did not necessarily result in an increase of the other.

The most consistently increased gene in this study, independent of the mode of induction, was ADAM-TS4 with 24/25 animals showing an increase which ranged from 2 fold to 333.14 fold increase. The CHO model, acute laminitis cases, and BWE 3 hour animals all had a significant increase over 10 fold. In many of these cases, the disease was well developed but the increase at the early BWE time point of 1.5 hours indicates that this ADAM is being induced before lameness occurs and then remains activated at very high levels throughout disease progression. TIMP2, the natural inhibitor of MMP2, was decreased in 16/25 horses, which could play a role in the eventual degradation of the laminae since MMP2 is known to be increased in horses with laminitis (Kyaw-Tanner and Pollitt 2004). When the observation is limited to horses with clinical signs (12 hour BWE samples, CHO-R, and all clinical cases), 14/17 horses showed a decrease in TIMP-2. The expression of TIMP1 seemed to be randomly increased or decreased with no real trends visible. TIMP3 expression was decreased in 5/8 animals in the BWE model while it was increased in 16/17 horses in the CHO and

clinical cases. This is an interesting fact considering that TIMP3 is an inhibitor of ADAM10, ADAM17, and ADAM-TS1, 4, and 5. This increase could be a response of the laminae to try and stop the progression of the disease.

When you look at each induction subset which was investigated the results are much more interesting considering that the BWE model is known to be a model of early and reversible laminitis while the CHO model is more like that of a clinical case of laminitis. In the BWE model Table 6A, ADAM-TS4 was the only gene that was induced in every animal. MMP9 was initially reduced in the 1.5 hour and 3 hour animals, while TIMP2 was reduced at the later time points of 3 hours and 12 hours. TIMP3 was also reduced in 5/8 horses and its reduction occurred mainly in the late stage animals. These results, which show that this model may in fact be an early and reversible form of laminitis, could help to make use of this model to understand how the reversion of disease is occurring. In this case, ADAMTS-4 increase would be at the onset of the development of the disease preceding all other MMP.

In the CHO induction model, Table 6B, there were horses which were responders, CHO-R, and horses which did not respond to the treatment, CHO-NR. The CHO-R group showed increased levels of ADAM-TS1, 4, 5, MMP9 and TIMP1 with a slight decrease in TIMP2 expression. The CHO-NR group had only 1/3 animals with an increase in ADAM-TS1, 2/3 animals with an increase in ADAM-TS4 and TS5 and all had a similar increase in MMP9. This result suggests that ADAM-TS4 and TS5 are directly linked to the progression of laminitis even with the increase of TIMP3 that was observed in 5/6 animals in this

group. TIMP2 was also reduced in 2/3 CHO-R animals which is similar to the result which was observed in the BWE model at the late time points.

In the clinical laminitis cases, Table 6B, there was a strong correlation of results to those observed in the CHO model. All animals in this group showed an increase of ADAM-TS4 expression from 2.73 to 333.14 fold induction. MMP9 was also increased in this group in 9/11 animals and a decrease in TIMP2 was observed for 9/11 animals as well. There was no change observed in expression of ADAM17 with the exception of one animal showing a slight decrease. ADAM10 expression was overall unchanged in the acute and chronic cases of laminitis while it was decreased in 3/4 of the chronic aggravated animals. TIMP3 expression in this group was seen to increase in all cases of the disease suggesting that it is potentially being increased in response to the influx of the ADAM-TS proteins.

A broad overview of these results indicates that there are several key targets for further study. ADAM-TS4, which is seen to have increased transcription in 24/25 animals, regardless of the induction method, is a likely target for the treatment of laminitis. The expression of TIMP2 also brings to question its importance in the progression of disease considering that it has decreased expression in 16/25 animals in this study. The high expression levels of MMP9 are also of interest in this disease, and have been the target of many studies in the past (Mungall, Pollitt et al. 1998; Mungall and Pollitt 1999; Mungall and Pollitt 2002; Loftus, Belknap et al. 2006; Loftus, Black et al. 2007). Recent work on MMP9 suggests that this accumulation of MMP9 is a downstream event

of the initial inflammation and activation of endothelial cells (Loftus, Black et al. 2007).

CHAPTER X

DISCUSSION

The initial focus of this project was on ADAM10 and ADAM17 (TACE) because they are both capable of processing TNFα which is one of the pro-inflammatory cytokines potentially playing a role in laminitis. The results obtained showed no real dramatic changes in the expression of these genes in all modes of induction. Increases observed in both TIMP1 and 3, inhibitors of these ADAMs, suggests that the activity of these proteins is likely reduced causing a decrease in TNFα processing. Another study by Belknap *et al* (Belknap, Giguere et al. 2007) where TNFα mRNA was measured by qPCR and no significant increase was observed, suggest that TNFα is not likely to be essential for the progression of the disease.

Previous work on laminitis has mainly targeted two proteins, MMP2 and MMP9. These two metalloproteases have been seen in multiple instances to be increased depending on the mode of induction of laminitis. MMP9 protein was shown to be induced up to Obel grade 1 lameness in the BWE model (Loftus, Belknap et al. 2006) and both MMP2 and 9 are induced in the CHO model (Kyaw-Tanner and Pollitt 2004). The expression of MMP9 mRNA was confirmed in the CHO model in this study but results are not concrete in the BWE model.It is possible that the difference observed for MMP9 expression in this study compared to the previous is the choice of gene selected for the normalization (GAPDH versus β2-Microglobulin). We are confident that the results obtained in this study are accurate since in the same conditions, the MMP9 mRNA

quantification for the CHO was confirmed by a similar increase in MMP9 protein level obtained from the same horses tested by Dr. Black and colleagues. . One interesting factor that was common throughout all modes of induction and disease progression is that TIMP2 was frequently reduced in 16/24 animals. TIMP2 is responsible for the inhibition of both MMP2 and MMP9 and its decreased expression could allow for an increase in MMP activity even in the absence of an increase in MMP mRNA and protein expression in the laminae to cause damage to the laminae (Gomez, De Lorenzo et al. 1999; Nagase, Visse et al. 2006).

The most obvious gene which was consistently upregulated independently of the method of induction was ADAM-TS4. This protein is considered to be one of the main factors involved in cartilage remodeling and osteoarthritis pathology (Malfait, Liu et al. 2002; Song, Tortorella et al. 2007). In cartilage ADAM-TS4 and 5 cleave aggrecan whose function is to provide a mechanical resistance to compressive loads. This resistance is provided by the overall negative charges on the polysaccharide side chains. These negative charges create an osmotic environment that allows for the high osmotic swelling pressure of cartilage. This pressure is counteracted by resistance of intact collagen fibers which in turn creates the high tensile strength.

Aggrecanase in cartilage is known to be induced by several cytokines such as IL-1, IL-6, and TNFα (Tortorella, Malfait et al. 2001). There are also reports of its induction by fibronectin fragments (Homandberg, Meyers et al. 1992; Stanton, Ung et al. 2002). All of the above cytokines have been observed to be induced in both a BWE and CHO induction model (Belknap, Giguere et al. 2007) and could be contributing factors to the

large increase of ADAM-TS4 that was observed. The induction of ADAM-TS4 by fibronectin or by the previously mentioned cytokines still remains to be determined.

The most potent natural inhibitor of ADAM-TS4 is TIMP3 (Hashimoto, Aoki et al. 2001). The remaining members of the TIMP family are known to have a higher affinity for the MMPs (Clegg, Coughlan et al. 1998; Kashiwagi, Tortorella et al. 2001; Cross, Chandrasekharan et al. 2005). There is clearly an association between the decrease in TIMP3 and disease progression in the BWE model (5/8 animals) but this is not as evident in the natural and CHO induced laminitis cases. There are several animals that had a poor prognosis and a drastic increase in TIMP3 expression. Even with the increased level of this inhibitor disease progression is able to continue suggesting that there may be more to treatment than finding a way to increase transcription of the TIMPs.

Aggrecan is able to protect collagen from degradation by MMPs, but there are other ECM proteins like fibronectin and thrombospondin that interfere with ADAM-TS4 access and degradation of aggrecan (Tortorella, Pratta et al. 2000; Hashimoto, Shimoda et al. 2004). This cooperation could explain why the development of laminitis needs activation of both ADAM-TS4 and MMPs. With such a focus on the treatment of arthritis in the past couple of years there have been several inhibitors developed. These include hydroxamates, P1' biphenylmethyl substituted inhibitors, to inhibit ADAM-TS proteases (Yao, Chao et al. 2002; Noe, Natarajan et al. 2005). MMP inhibitors have also been developed recently and one is highly selective for the inhibition of MMP-2 (Hayashi, Jin et al. 2007). With all of these tools available as potential treatments for laminitis

there is clearly a promising area of research to be pursued in both the development of a clearly defined pathway for laminitis and in finding a way to inhibit the proteases responsible for cartilage degradation without affecting other pathways for which these metalloproteases are critical.

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