

Chapter 5

Laminitis: Current Concepts

Introduction

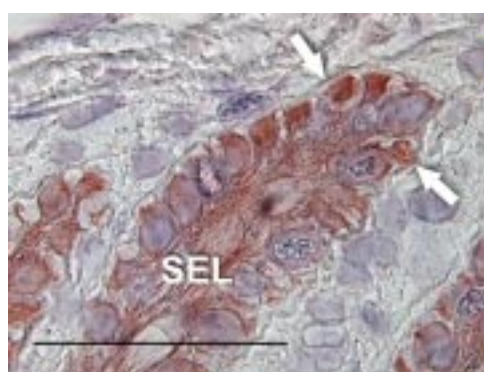
The prefix of the word *laminitis* correctly identifies the laminae, or more correctly, lamellae of the inner hoof wall as the focus of laminitis pathology. The suffix *-itis* implies a role for inflammation. The hoof wall lamellae are certainly inflamed in the acute phase. Tissue damage has occurred; there is pain, redness and swelling beneath the hoof wall. The mystery is why?

In acute laminitis, the tissue suspending the distal phalanx from the inner hoof wall fails, specifically at the junction between the connective tissue of the dermis or corium (the bone side) and the basal cell layer of the epidermal lamellae (the hoof side). This junction, the basement membrane zone, appears to be the weak link in an otherwise robust and reliable structure. In acute laminitis there is wholesale epidermal cell detachment from, and lysis of, the lamellar basement membrane and this leads to failure of the lamellar anatomy and, ultimately, failure of the suspensory attachment between hoof and distal phalanx. There is a good correlation between the grade of severity, as seen with the microscope (histopathology), and the degree of lameness (using the Obel grading system) shown by the horse. Thus, when the horse first starts to show the foot pain of laminitis, it means that the anatomy of the hoof wall lamellae is being destroyed. The worse the lameness, the worse the damage. Any activity that places stress on an already weakened lamellar attachment apparatus (such as forced exercise) will cause further damage and is contraindicated. The use of nerve blocks to eliminate pain will encourage locomotion and do more damage.

The laminitis process

The spectacular disintegration of the lamellar attachment apparatus, initiated during the developmental phase of laminitis, renders a normally robust and trouble-free epidermal/dermal system useless in a relatively short period of time. Logic indicates that somehow this normally tightly-controlled, metabolic process is thrown into disarray to cause the lamellar-specific lesion during the laminitis developmental period. Our evidence suggests that it is

the enzymatic remodeling of the epidermal lamellae, assumed to be essential if the continually-proliferating hoof wall is to move past the stationary distal phalanx, that is activated beyond control to destroy the lamellar attachment apparatus. The enzymes that destroy the key components of the lamellar attachment apparatus are metalloproteinase -2 and metalloproteinase -9 (MMP-2 and MMP-9). These enzymes are also found in a wide range of other remodeling tissues such as bone, joints and endometrium, as well as in metastasising malignant tumours (Figure 5.1).



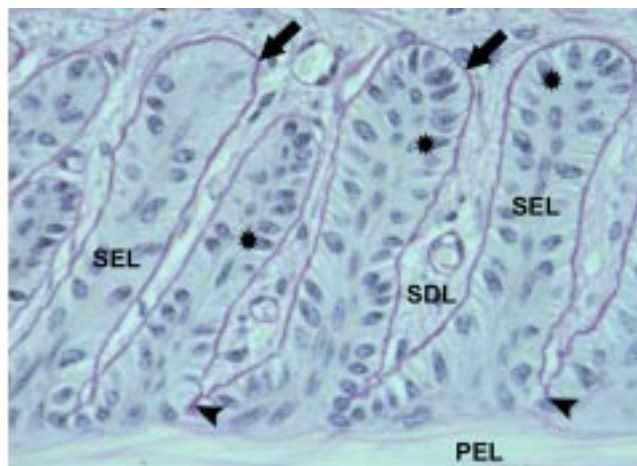
◀FIGURE 5.1 Micrograph showing immunolocalisation of matrix metalloproteinase (MMP-2) in the hoof wall lamellae. The brown stained cytoplasm shows the location of the enzyme MMP-2 in SEL basal and parabasal cells. MMP positive cytoplasm is closely associated with the basement membrane (arrows). The proteins responsible for anchoring basal cells to the basement membrane as well as the basement membrane itself are substrates of activated MMP-2. Excessive production and activation of basal cell MMP could cause the basement membrane lesion that characterises laminitis pathology. Bar = 5 µm.

Normal MMP activity is constantly responding to the stresses and strains of equine life as well as to constant growth. When called for, sufficient MMP is manufactured locally to release epidermal cell to cell, and cell to basement membrane attachment, as required, maintaining the correct shape and orientation of the lamellae. From time to time injury, to the basement membrane would require its lysis and reconstruction. The controlled release of specific MMP inhibitors keeps the remodeling process in equilibrium. The hoof lamellae and the hoof itself slowly migrate past the stationary basement membrane that is firmly attached to the connective tissue covering the upper surface of the distal phalanx.

The sequences of microscopic events that initiate laminitis follow a consistent pattern and the stages of histological laminitis can be identified according to the degree of severity of these changes. It was therefore possible to develop a grading system for the histopathology of laminitis, based on changes to several key parameters of hoof lamellar anatomy. At the AELRU, we created a laminitis assessment system, initially by staining lamellar tissues with periodic acid Schiff (PAS) and periodic acid silver methanamine (PASM) stains, and later with immunohistochemical methods using basement membrane (BM) specific

antibodies. Making the basement membrane clearly visible led to the realisation that laminitis was essentially a basement membrane lesion.

The normal anatomical characteristics that are assessed before allocating a laminitis grade to a section of lamellar hoof tissue are as follows. The tips of the secondary epidermal lamellae (SELs) are always rounded (club-shaped) and never tapered or pointed. The basal cell nuclei are oval in shape with the long axis of the oval at a right angle to the long axis of the secondary epidermal lamella. These parameters can be satisfactorily assessed using routine haematoxylin and eosin (H&E) staining of sections. The basement membrane penetrates deeply into the crypts between the SELs and outlines the wafer thin, but connective tissue filled, secondary dermal lamellae. The basement membrane is tightly adherent to the basal cells of each SEL. The PAS and PASM stains show this best (Figure 5.2).

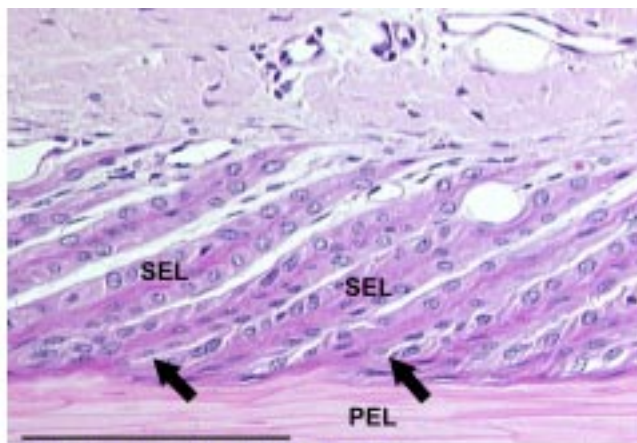


▲FIGURE 5.2 Micrograph of normal hoof lamellae stained to highlight the basement membrane. The basement membrane (arrowed) of each secondary epidermal lamella (SEL) shows as a dark magenta line closely adherent to the SEL basal cells. Between the bases of each SEL the BM penetrates deeply (arrowheads) and is close to the anuclear, keratinised, primary epidermal lamella (PEL). The SEL tips are rounded (club-shaped). The basal cell nuclei are oval in shape (stars) and positioned away from the BM at the apex of each cell. The long axis of each basal cell nucleus is at right angles to the the long axis of the SEL. The secondary dermal lamellae (SDL) are filled with connective tissue even at their very tips, between the SEL bases. These parameters of hoof lamellar anatomy form the basis of the histological grading system of laminitis histopathology. Stain = PAS. Bar = 10 μ m

A histological grading system for laminitis

As the developmental phase ends and the acute phase begins, the lamellar basal

and parabasal cells lose their normal shape and appear to slide over one another. The basal cell nuclei become rounded instead of oval and take up an abnormal position in the cytoplasm of the cell (**Figure 5.3**). Ultrastructural evidence shows extensive loss of hemidesmosomes and failure of the basal cell cytoskeleton and this is the reason for the sudden pathological changes in shape and behaviour of the SELs. They become stretched, long and thin, with tapering, instead of club-shaped, tips.

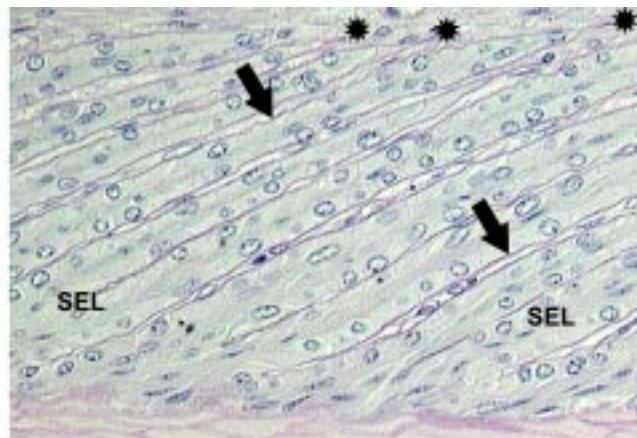


▲ **FIGURE 5.3** Grade 1 histological laminitis (H&E stain). The secondary epidermal lamellae (SELs) are longer and thinner than normal and the SELs have pointed instead of the normal rounded tips. The basal cell nuclei are no longer oval in shape and have become round and situated abnormally close to the basement membrane. The tips of the secondary dermal lamellae (arrowed) are still situated close to the primary epidermal lamella (PDL) which is normal. H&E stain. Bar = 10 μ m.

While this is going on, the BM of the SEL loses its attachment to the basal cells. This is first noticeable at the tips of the SELs where teat-shaped bubbles of loose BM form cell (**Figure 5.4**). To render this detectable by light microscopy the tissues have to be processed with special connective tissue stains such as the periodic acid Schiff (PAS) and periodic acid silver methanamine (PASM) stains.

Laminitis ultrastructure

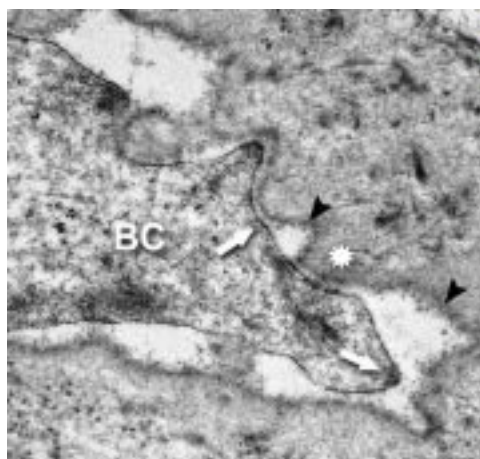
Examination of laminitis tissues with the electron microscope confirms the existence of lysis and separation of the lamellar basement membrane (**Figure 5.5**). Importantly the greater magnification shows widespread loss of basal cell hemidesmosomes and contraction of the basal cell cytoskeleton away from the inner cell surface. Electron microscopy shows



▲FIGURE 5.4 Grade 1 histological laminitis (PAS stain). Micrograph showing hoof lamellar tissues stained to highlight the basement membrane. The basement membrane (arrowed) is stained dark magenta. At the now tapered tips of the secondary epidermal lamellae (SELs) the basement membrane has lifted away (stars) from the underlying basal cells. Between the SEL bases the BM is in its normal position, close to the primary epidermal lamella (PEL). PAS stain. Bar = μm .

why the BM separates from the feet of the basal cells. The anchoring filaments that bridge the gap between the hemidesmosome and the lamina densa of the BM are no longer present.

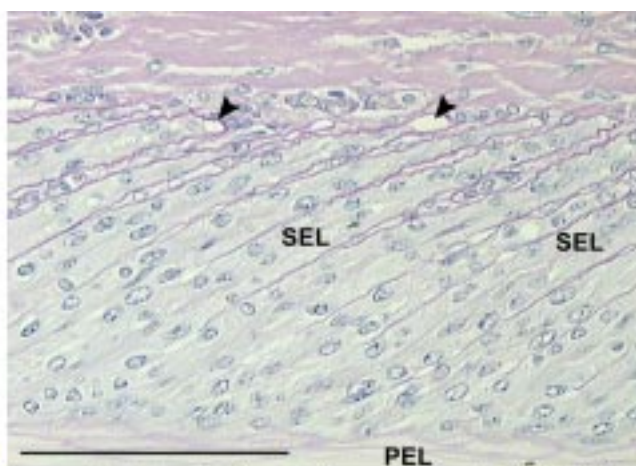
Loss of anchoring filament protein has been confirmed recently using immunohistochemistry. Preliminary results using antibody to laminin-5 (the major anchoring filament protein) show that laminin-5 is absent at the stage when the BM is lifting off the lamellar basal cells. Uncontrolled activation of lamellar MMP is the likely cause of the anchoring filament destruction.



◀FIGURE 5.5 Electron micrograph of hoof lamellar tip developing laminitis. The lamina densa (arrow heads) of the basement membrane is separating from the plasmalemma of the lamellar basal cell (BC). Some hemidesmosomes (star) appear undamaged but many others (arrows) have faded, are losing their anchoring filaments and are drawing away from the basement membrane.

Grade 2 histological laminitis

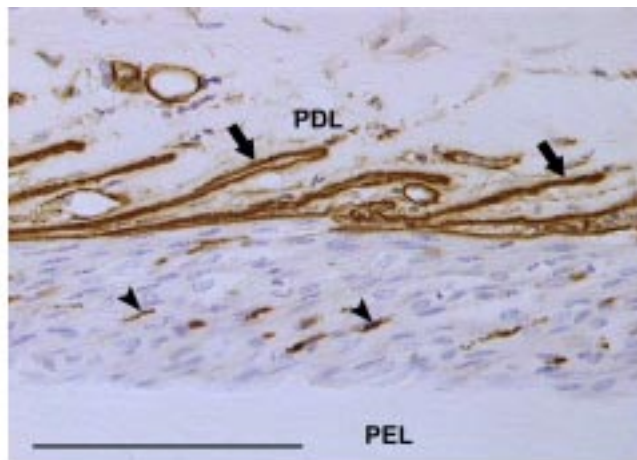
With the BM no longer tethered to the basal cells, it slips further away with each cycle of weight bearing by the horse. The lamellar basement membrane begins to disappear initially at the bases of the SELs (Figure 5.6). The BM retracts from between the SELs and takes with it the connective tissue. The BM-free epidermal cells appear not to be undergoing necrosis, at least initially, and clump together to form amorphous, BM-free masses, on either side of the lamellar axis.



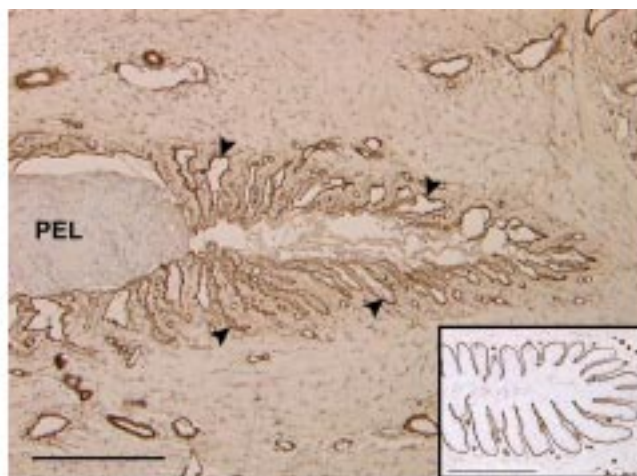
▲FIGURE 5.6 Micrograph showing hoof lamellar tissues (stained to highlight the basement membrane) with histological grade 2 laminitis. The basement membrane is stained dark magenta. At the tips of the now pointed secondary epidermal lamellae (SELs) the basement membrane (BM) has continued to lift from the underlying basal cells to form empty, teat-shaped, caps (arrowheads). The BM has disappeared from the crypts between the SEL bases. The lamellar BM is no longer close to the primary epidermal lamella (PEL). There is a reduced amount of connective tissue between the SELs. PAS stain. Bar = 10 μ m.

Grade 3 histological laminitis

In laminitis, the worse case scenario is a rapid and total BM separation from all the epidermal lamellae. Sheets of BM peel away to form aggregations of loose isolated BM in the connective tissue adjoining the lamellae. The epidermal lamellar cells are left as isolated columns with no connection whatsoever with the dermal connective tissue (Figure 5.7). The lamellar tips slide away from their basement membrane connective tissue attachments, at



▲FIGURE 5.7 Grade 3 histological laminitis (immunostain). Only remnants (arrowheads) of the basement membrane (BM) remain between the now disorganised secondary epidermal lamellae. Most of the lamellar epidermal cells have coalesced into an amorphous mass no longer effectively attached to any connective tissue. The remainder of the lamellar BM lies free, in strands (arrowed), among the connective tissue of the primary epidermal lamella (PDL). Type IV collagen immunostain. Bar = 10µm.



▲FIGURE 5.8 Grade 3 histological laminitis (immunostain). The basement membrane of a lamellar tip is highlighted by type IV collagen immunostaining. The tip of the primary epidermal lamella (PEL) has completely detached from its basement membrane. The PEL basal cells are now an unattached, amorphous mass. Collapsed tubes of basement membrane, now empty of epidermal cells, are still attached to connective tissue (arrowheads). The PEL has already moved 0.03 mm from its dermal compartment and soon the distance will be measured, using a tape measure, on a radiograph. The inset shows a normal lamellar tip, immunostained the same way. Type IV collagen immunostain. Bars = 10 µm.

first microscopically, but as the degree of separation increases the distance between hoof and distal phalanx becomes measurable in millimeters (**Figure 5.8**). This is manifest clinically as the “sinker”, the worst possible situation for a horse with laminitis.

Since the BM is the key structure bridging the epidermis of the hoof to the connective tissue of the distal phalanx, it follows that the wholesale loss and disorganization of the lamellar BM inexorably leads to the failure of hoof anatomy so characteristic of equine laminitis.

An additional component of lamellar anatomy that is affected is the lamellar capillaries. As the BM and the connective tissue between the SELs disappears so do the capillaries; they become obliterated, compressed against the edges of the primary dermal lamellae. Without capillaries in the lamellar circulation, blood bypasses the capillary bed through dilated arteriovenous shunts, and dramatically changes the nature of the foot circulation. A bounding pulse is detectable by finger palpation of the digital arteries. It also explains why the radioactive, capillary-sized particles (described in Chapter 4) bypassed the circulation at the beginning of the acute phase. The phenomenon of vascular shunting is now placed after the triggering of MMP production and occurs as a consequence of it.

An enzymatic theory of laminitis

The enzymatic theory of laminitis, based on lamellar MMP activation, challenges the alternative view that laminitis develops because the flow of blood is impeded to cause ischaemic necrosis of epidermal lamellae. Furthermore epidermal cell necrosis, intravascular coagulation and edema are not recognised in the author’s laboratory in sections made from tissues in the early stages of laminitis. The vessels in the primary dermal lamella, even the smallest, are predominantly open, without evidence of microvascular thrombi (blood clots). The gross anatomical appearance of freshly dissected laminitis tissue is one of dryness. Sometimes the lamellae just peel apart.

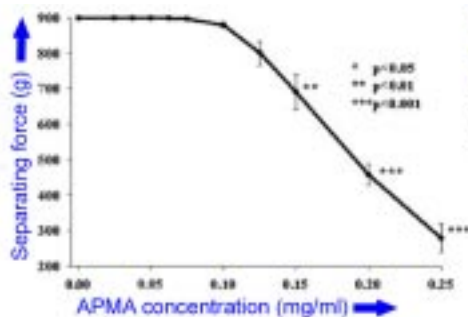
How do the trigger factors of laminitis reach the lamellae? There is now strong evidence, from three independent international laboratories, that the foot circulation during the developmental phase of laminitis is vasodilated. Laminitis does not occur if the foot is in a state of vasoconstriction during the developmental phase, suggesting that the trigger factors will only cause laminitis if they reach the lamellar tissues at a high enough concentration and over a long enough time period.

What are the trigger factors? Since the carbohydrate overload model of laminitis is characterised by endotoxin production it would seem a reasonable presumption that endotoxaemia should play a key role in initiating laminitis pathology. Tumour necrosis factor (TNF) along with other cytokines, such as interleukin, is expressed by mononuclear phagocytes within minutes of exposure to endotoxin. The cytokine cascade originating from an inflamed leaky bowel is responsible for most of the pathological effects of endotoxaemia. However, laminitis has never been triggered by the experimental administration of endotoxin into the bloodstream or the peritoneal cavity and the actual trigger factors of laminitis remain unidentified. What appears certain in the light of recent research is that the lamellar disintegration of laminitis is mediated by the inappropriate release of excess MMP. But what triggers MMP release?

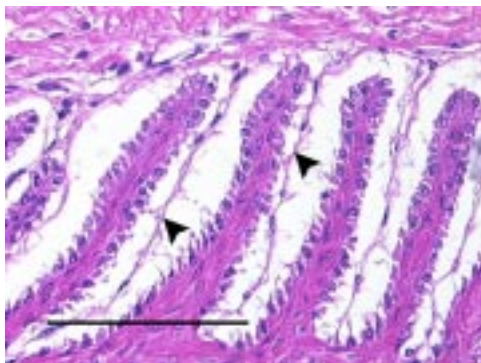
Laminitis *in vitro*

To answer this vital question it was necessary to develop an *in vitro* model for laminitis to enable us to study a range of putative trigger factors under controlled laboratory conditions. We were able to establish a test for separation of the dermal and epidermal lamellae using small explants of tissue taken from the inner hoof wall of normal, freshly killed, abattoir horses. After incubation for 48 h in tissue culture medium, in the presence of the laminitis trigger factor under investigation, each explant was subjected to tension. The force required to separate epidermal from dermal lamellae was recorded. When dermal-epidermal lamellar separation occurred readily (as occurs in field cases of laminitis) we considered the tissue to have developed *in vitro* laminitis. Lamellar explants can be cultured for up to 7 days in normal medium and no lamellar separation occurs. It is virtually impossible to separate normal lamellar explants. Normal explants resist a separating force of 900 grams. When a chemical known to activate metalloproteinases (we use a non-physiological MMP activator, the organo-mercurial compound, aminophenylmercuric acetate or APMA), was added to the explant tissue culture medium, the explants separated when only a small separating force was applied. In fact a dose-response curve, between the force required to separate explants and the concentration of metalloproteinase activator, can be constructed (**Figure 5.9**).

All explant tissues were fixed in formalin and examined histologically for evidence of separation. Histological sections showed a clear zone of complete separation between the basement membrane and the basal cells of the epidermal lamellae (**Figure 5.10**). This is a characteristic of *in vitro* laminitis and resembles the basement membrane lesion of natural



◀FIGURE 5.9 Explant separation induced by APMA. Explants incubated in D-MEM tissue culture fluid containing the metalloproteinase activator APMA reliably separated when APMA concentrations exceeded 0.15 mg/ml. Mean \pm standard error. Significance as indicated.

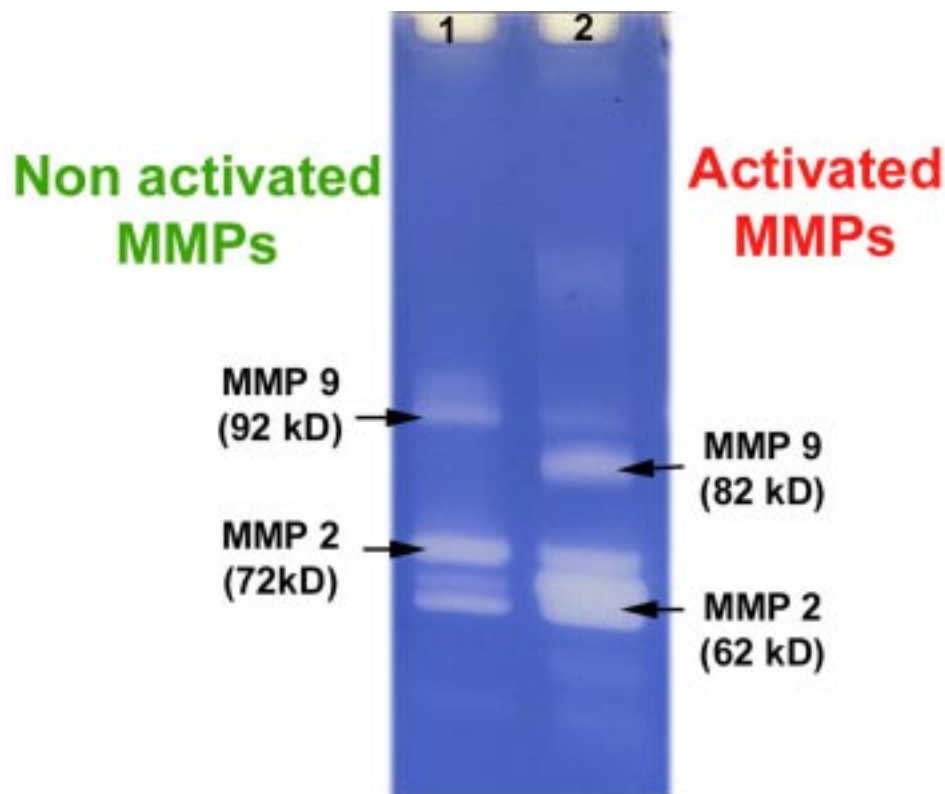


◀FIGURE 5.10 Micrograph of lamellar hoof explant with *in vitro* laminitis. Cultured for 48 hours in tissue culture fluid with the MMP activator APMA added the basement membrane (arrowheads) of the secondary epidermal lamellae is no longer attached to the basal cells. Activation of lamellar MMPs causes this *in vitro* lesion that resembles natural laminitis. H&E stain. Bar = 10 μ m.

in vivo laminitis. The model has become a potent tool that enables our laboratory to screen a large number of potential, natural, laminitis trigger factors without having to perform experiments with live horses. The presence or absence of MMP activation in the explant tissue culture medium was detected zymographically using gelatin polyacrylamide electrophoresis (Figure 5.11). Analysis of the culture medium from normal hoof explants shows that explants produce two MMPs (gelatinases) of molecular weight 92 and 72 kDa. A small amount of the active forms of the MMP-2 is also present in normal horses. Incubation of normal hoof explants with APMA results in the activation of MMP-9 and MMP-2.

Laminitis and metalloproteinase activity

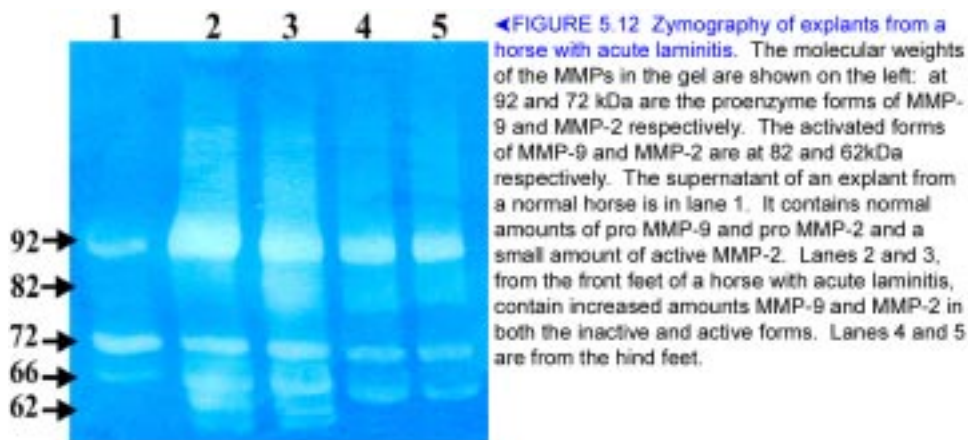
Lamellar explants from horses with acute laminitis, cultured in medium the under the same conditions contained not only increased amounts of inactive MMP-2 and MMP-9, but large increases in the amounts of activated MMPs (Figure 5.12). It was concluded that there is increased production of active MMP in lamellar tissues affected by laminitis.



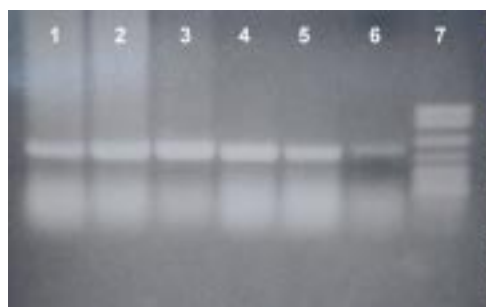
▲FIGURE 5.11 Zymography of normal lamellar explants. The tissue culture fluid, in which explants were cultured, was applied to lanes in a polyacrylamide gel containing 0.1% gelatin. After electrophoresis and overnight incubation, the gel was stained for protein with Coomassie Blue G-250. Because the gel contains protein (the soluble collagen gelatin) the entire gel stains blue, except where gelatin has been digested by MMP activity. Thus the clear areas reveal the existence of MMP-9 and MMP-2 in lamellar hoof tissue. Proteins of known molecular weight (not shown) are electrophoresed at the same time to determine MMP molecular weights in kiloDaltons (kD). Lane 1 shows the MMPs contained in a normal explant. There is pro-MMP-9 but no active MMP-9, a large band of pro-MMP-2 and some active MMP-2. Lane 2 shows the effect of MMP activation with APMA. The pro-MMP-9 has been converted to active MMP-9 and a similar conversion of pro-MMP-2 has occurred. Cleavage of a 10 kD fragment from pro-MMP 9 and 2 activates the enzyme.

Laminitis causes increased gene transcription of lamellar MMP

Destruction and detachment of the lamellar basement membrane are the key lesions of acute laminitis. The genes controlling hoof lamellar MMP-2 and MMP-9 activity are significantly upregulated in tissues affected by acute laminitis, thus providing firm circumstantial evidence that MMP activation is a pivotal event in the development of laminitis (Figure 5.13).



◀FIGURE 5.12 Zymography of explants from a horse with acute laminitis. The molecular weights of the MMPs in the gel are shown on the left: at 92 and 72 kDa are the proenzyme forms of MMP-9 and MMP-2 respectively. The activated forms of MMP-9 and MMP-2 are at 82 and 62kDa respectively. The supernatant of an explant from a normal horse is in lane 1. It contains normal amounts of pro MMP-9 and pro MMP-2 and a small amount of active MMP-2. Lanes 2 and 3, from the front feet of a horse with acute laminitis, contain increased amounts MMP-9 and MMP-2 in both the inactive and active forms. Lanes 4 and 5 are from the hind feet.



◀FIGURE 5.13 Lamellar gene (m-RNA) expression of equine specific MMP-2 in hoof tissues from 5 horses with laminitis (lanes 1-5) and from a normal horse (lane 6). Semi-quantitative RT- polymerase chain reaction (PCR) shows that there is a much higher level of MMP-2 gene expression than normal, in the lamellar tissues of the five horses with laminitis. Lane 7 shows digoxigenin (DIG) labeled molecular weight markers that confirm the identity of MMP-2.

Metalloproteinase inhibitors

The activity of tissue MMPs has recently been shown to correlate strongly with the degree of malignancy and invasiveness of lethal human tumours, such as malignant melanoma, breast and colon cancer. Research in this field has generated a wide range of chemical agents capable of inhibiting MMP activity both *in vitro* and *in vivo*. We have shown that one of these (Batimastat or BB-94, British Biotech, Oxford) blocks the activity of the laminitis MMPs *in vitro* and has the potential to be a useful tool in the prevention and management of acute laminitis. We are conducting trials to test whether MMP inhibitors can prevent or ameliorate field cases of laminitis.

The search for natural laminitis trigger factors

We have used the *in vitro* laminitis explant model to investigate most of the

proposed trigger factors of equine laminitis. The equine lamellae have tested resistant to virtually all known cytokines, tissue factors and prostaglandins. Gram negative bacterial endotoxin, extract of black walnut (*Juglans nigra*) and even anaerobic culture conditions fail to induce lamellar separation or significant MMP activation. There is one notable exception however. A factor present in the supernatant of cultures of *Streptococcus bovis* isolated from the equine caecum activates equine hoof MMP-2 and causes lamellar separation. During grain overload *S. bovis* is the principal microorganism responsible for the rapid fermentation of carbohydrate to lactic acid in the equine hindgut. In the presence of virtually unlimited substrate, its population explodes exponentially. We are currently investigating the role of the *S. bovis* MMP activator in natural cases of equine laminitis. If it crosses the mucosal barrier of the hindgut and enters the circulation it may be a “cause” of laminitis (at least in the carbohydrate overload model) that has escaped previous consideration.

Key Points

- A grading system for the histopathology of laminitis was developed based on the consistent pattern of histological changes to the secondary epidermal lamellae, basal cells and basement membrane that occur with the onset of laminitis. Grades 1-3 histological laminitis reflect increasing separation of the basement membrane, along with its connective tissue, from between the secondary epidermal lamellae.
- As the basement membrane and connective tissue retract, the capillaries of the lamellae are damaged, resulting in extensive changes to the circulation in the foot as blood bypasses the capillary bed through dilated arteriovenous shunts.
- An *in vitro* model for laminitis using hoof explants, followed by zymographic analysis of enzymes, showed that activation of MMP-2 and MMP-9, by laminitis or APMA, resulted in separation of epidermal and dermal lamellae. Increased gene transcription of MMP-2 and MMP-9 was present during laminitis.
- Histological examination of explants treated with APMA showed that the separation produced *in vitro* resembled the basement membrane lesion of laminitis *in vivo*.
- The presence of an MMP inhibitor, BB-94, blocked the activity of the MMPs *in vitro*.

- Factors present in the supernatant of cultures of *Streptococcus bovis* activated MMP-2 and caused lamellar separation.