

Equine laminitis basement membrane pathology: loss of *type IV* collagen, *type VII* collagen and laminin immunostaining

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Abbreviations:

BM	=	basement membrane
Ep	=	epidermal cells
E	=	erythrocytes
PEL	=	primary epidermal lamella
PDL	=	primary dermal lamella
PMNs	=	polymorphonuclear leucocytes
SDL	=	secondary dermal lamella
SEL	=	secondary epidermal lamella
V	=	blood vessels
MMP	=	matrix metalloproteinase
TNF- α	=	tumour necrosis factor - α
TGF- β 1	=	transforming growth factor - β 1

Summary

Disintegration of the basement membrane (BM) of the equine hoof lamellae and failure of the BM to remain attached to the basal cells of the secondary epidermal lamellae (SEL) is one of the earliest pathological events to occur in acute laminitis. Changes in the lamellar basement membrane were investigated by immunolabelling the key structural components of the BM, *type IV* collagen, *type VII* collagen and laminin in the lamellar BM of horses 48 h after the induction of laminitis. Lamellar tissues were harvested from 2 normal horses and 2 horses with acute laminitis. Immunostaining with antibody raised against human epitopes for *type IV* collagen, *type VII* collagen and laminin successfully stained the basement membranes of horse hoof lamellar tissues. Vascular tissue did not immunostain with *type VII* collagen antibody. Normal BM stained as a fine dark brown line and the lamellar BM was adhered to the basal cells of the SELs with no evidence of lamellar separation. At least 2 changes to the lamellar BM occurred in acute laminitis: loss of attachment of lamellar epidermal basal cells to their underlying BM and disintegration of the lamellar BM. In some sections from feet affected by acute laminitis, there was widespread separation of the SELs from their BM without loss of BM immunostaining and in others there was extensive loss of BM immunostaining. In lesions characterised by lamellar separation, the epidermal basal cells at the tips of the primary epidermal lamellae appeared to have slipped away from their

BM and were an amorphous clump of epidermal cells devoid of immunostained BM. The BM from which they had separated remained in its original position in the dermis and was clearly outlined by all 3 antibodies. In other areas, however, virtually all the BM immunoreactivity at the PEL tips was absent. Only the occasional distorted SEL tip and fragments of BM retained sufficient immunostaining to allow anatomical identification. Numerous polymorphonuclear leucocytes (PMNs) invariably surrounded the tips of lamellae showing large scale loss of immunoreactivity and many PMNs had penetrated the lamellar BM and were within the epidermal compartments. PMNs were less frequent in the midlamellar region. Immunostaining of the BM of many SELs was absent in the midlamellar region. In some lamellae loss of BM immunostaining had occurred only at the bases of the SELs and fragments of immunostained BM were present in the zones of lysed BM suggesting that BM lysis was incomplete at the time of tissue fixation. In other lamellae, lysis of the BM was complete; there was no immunostained BM between SELs and the bulk of the epidermal cells of each PEL were an amorphous column of cells on either side of the central keratinised axis of the PEL. The lamellar BM which remained appeared as immunostained strands of unattached BM along the edges of the PDLs. Activation of BM degrading metalloproteinases (MMPs) occurs in acute laminitis and it seems likely that uncontrolled MMP activity is responsible for the loss and disorganisation of lamellar BM demonstrated in this study.

Introduction

Disintegration of the basement membrane (BM) of the hoof lamellae and failure of the BM to remain attached to the basal cells of the secondary epidermal lamellae (SEL) is one of the earliest pathological events to occur in acute laminitis (Pollitt 1996). The attenuated, distorted appearance of the BM in histochemically stained sections examined with the light microscope, led to speculation that loss of collagen and glycoprotein from the lamellar basement membrane was part of the mechanism initiating collapse of the lamellar anatomy (Pollitt 1996).

The molecular components of the BM can also be identified using immunohistochemical methods. Laminin has been identified in the equine lamellar BM as well as in adjacent dermal blood vessels using a mouse monoclonal antibody raised

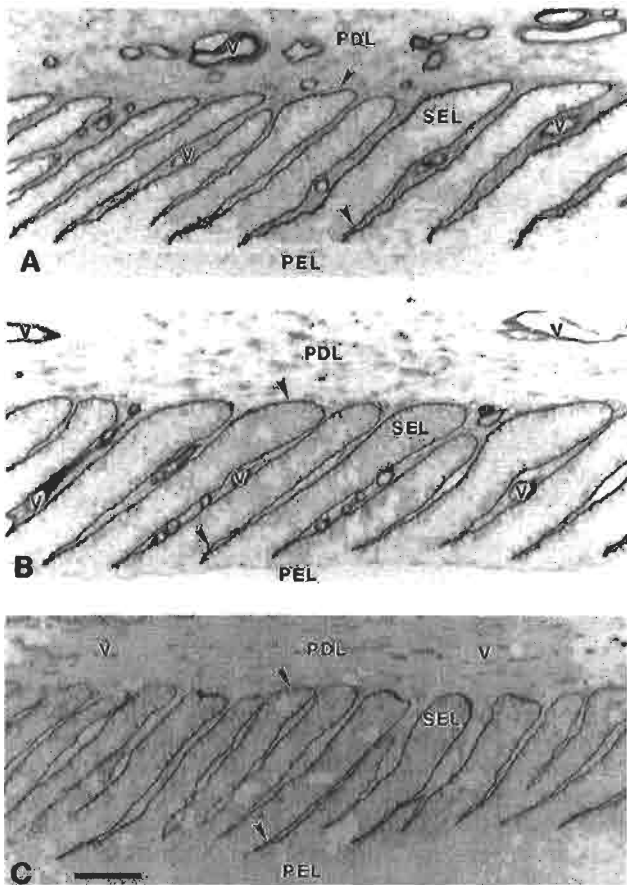


Fig 1: Normal lamellae: type IV collagen (A), laminin (B) and collagen type VII (C) immunostaining. The basement membranes (arrowheads) of the epidermal lamellae and the blood vessels (V) of the dermal lamellae are clearly immunostained as a fine dark brown line in A and B. The blood vessels did not immunostain in C. Nuclei of the epidermal basal cells and dermal fibroblasts are counterstained with haematoxylin. The basement membrane is closely adhered to the basal cells of the lamellar epidermis and there is no evidence of separation. Bar = 100 μ m.

against human laminin (Pollitt 1994). Type IV and type VII collagen have been located in the epidermal BM of many species (Rigal *et al.* 1991; Germain *et al.* 1995; Blankenship and Given 1995) but not the horse. Immunolabelling can be used to establish changes in the amount of type IV collagen, type VII collagen and laminin in the epithelial BM. For example, immunoreactivity for laminin and type IV collagen in the BM lining the epithelium of the uterine lumen is progressively lost during implantation of the blastocyst in mice (Blankenship and Given 1995). Similarly, in a temporal study of regeneration of the epidermo-dermal junction after full thickness skin wounding in pigs, type IV collagen and laminin appear in the new BM on Day one, but the appearance of type VII collagen does not occur until Day 3 (Rigal *et al.* 1991).

In this study the BM pathology of laminitis was investigated further, by immunolabelling type IV collagen, type VII collagen and laminin in the lamellar BM of horses 48 h after the induction of laminitis, to determine the changes that occurred in these components of the BM.

Materials and methods

Lamellar tissues were harvested from 8 normal horses and 2 horses with acute laminitis 48 h after the administration of an

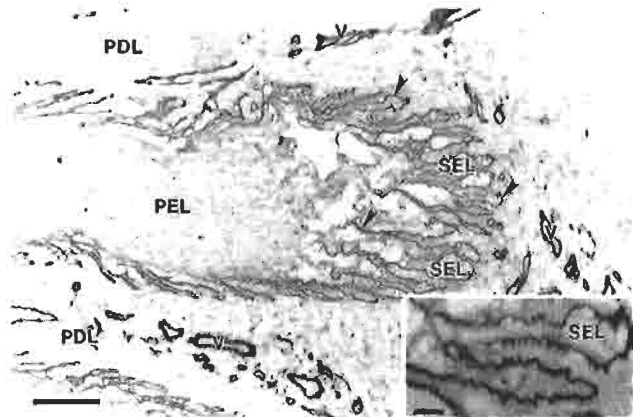


Fig 2: Laminitis: type IV collagen immunostaining. Section of the tip of a primary epidermal lamella (PEL) affected by grade 3 laminitis. At the tip of the PEL the epidermal basal cells are clumped together and are devoid of BM. The immunostained BM (arrowheads) from which they have separated has remained in its original position in the dermis and has retained the outline of the secondary epidermal lamellae (SEL). The BM of blood vessels (V) in the primary dermal lamellae (PDL) are also stained and serve as positive controls. Bar = 100 μ m. Inset shows laminin immunostained BM of SEL, empty of epidermal cells. Bar = 10 μ m.

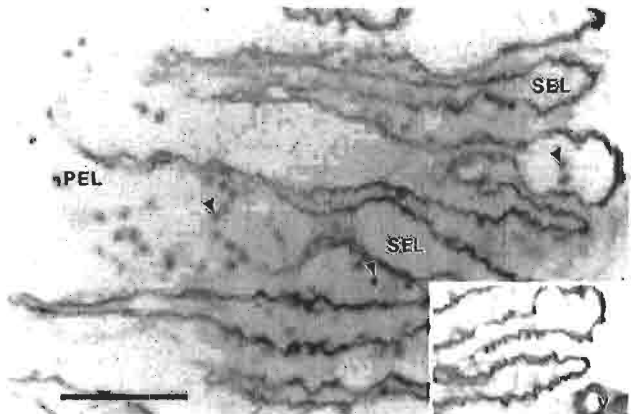


Fig 3: Laminitis: type VII collagen immunostaining. Section from the tip of a primary epidermal lamella (PEL) affected by grade 3 laminitis. The SELs are devoid of epidermal cells. The immunostained BM has remained in its original position in the dermis retaining the outline of the SELs. Numerous PMNs (arrowheads) have already crossed the lamellar BM and are within the epidermal compartments of the SELs. The BM of dermal blood vessels did not immunostain with type VII collagen antibody. Bar = 50 μ m. Inset shows, at the same magnification, laminin immunostained section cut from the same block. The BM of the blood vessels (V) as well as the lamellar BM is immunostained.

alimentary carbohydrate overload. The 2 horses with acute laminitis were from another study (Pollitt and Davies 1998) which was conducted according to guidelines approved by The University of Queensland Animal Experimentation Ethics Committee. All horses under experimentation were inspected by the Animal Welfare Officer. The mid-dorsal hoof wall lamellae from 16 normal forefeet and 6 laminitis affected feet (4 fore and 2 hind) were trimmed using the method of Pollitt (1996). In some lamellar tissue haemorrhage was present at the tips of PELs. The tissue was fixed in cold (4°C) 4% phosphate buffered formaldehyde (pH7.6) for 4 h, dehydrated, cleared and embedded in paraffin. Paraffin sections of 5 μ m thickness, mounted on silanised glass slides, were deparaffinised, blocked for endogenous peroxidase with 1% hydrogen peroxide and predigested with 0.1% trypsin¹ for 7 min using the method of

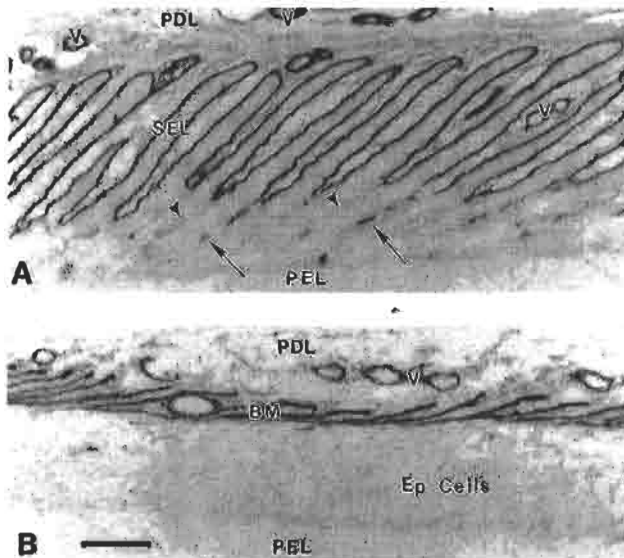


Fig 4: Laminitis: laminin immunostaining. Sections from mid-region of single lamellae affected by laminitis. The BM of blood vessels (V) in the primary dermal lamellae (PDL) are immunostained and serve as positive controls. A. At the bases of the SELs most of the BM did not immunostain (arrowheads) suggesting that disintegration of the BM had occurred. Fragments of immunostained lamellar BM are still present in this region (arrows) suggesting that BM lysis is incomplete at this stage. The SEL tips are attenuated (compare with Fig 1) but the BM has not separated from the basal cells. B. The BM has disappeared from most of the SEL epidermal cells which are now an amorphous column of epidermal cells (Ep Cells) on either side of the central keratinised axis of the PEL. The lamellar BM has detached from the SEL tips and immunostains as strands along the edges of the PDLs. We propose that A and B represent stages in the progression of the laminitis lesion. Bar = 100 μ m.

Quondamatteo *et al.* (1994). The sections were then incubated in 10% goat serum for 30 min prior to overnight incubation at 4°C, in a humidity chamber, with the specific antiserum. The following antisera, diluted appropriately, were used: monoclonal mouse anti-human type IV collagen² 1:500, monoclonal mouse anti-human type VII collagen¹ 1:200, polyclonal rabbit anti-human laminin³ 1:500. Biotinylated goat anti-mouse³ or sheep anti-rabbit sera¹ were diluted 1:200 and applied to the sections following overnight incubation for 30 min at room temperature. Immunolocalisation was shown by incubating the tissues in horseradish peroxidase and diaminobenzidine and lightly counterstaining with haematoxylin. Normal sheep serum was substituted for the primary antibody, secondary antibody and streptavidin-peroxidase complex, and diaminobenzidine was omitted from the reaction in control sections. Sections were examined with an Olympus BX-50 light microscope and photographed using Kodak T-Max 100 film.

Results

Mouse monoclonal antibody to human type IV collagen and rabbit polyclonal antibody to human laminin clearly immunostained the lamellar BM and the BM of all the blood vessels in the surrounding dermis as a fine dark brown line (Fig 1A, B). Mouse monoclonal antibody to human type VII collagen stained only the lamellar BM (Fig 1C). In the sections of lamellae from normal feet the lamellar BM was adhered to the basal cells of the SELs and there was no evidence of separation. Control sections did not stain when normal sheep serum was substituted for the primary antibody, secondary antibody and

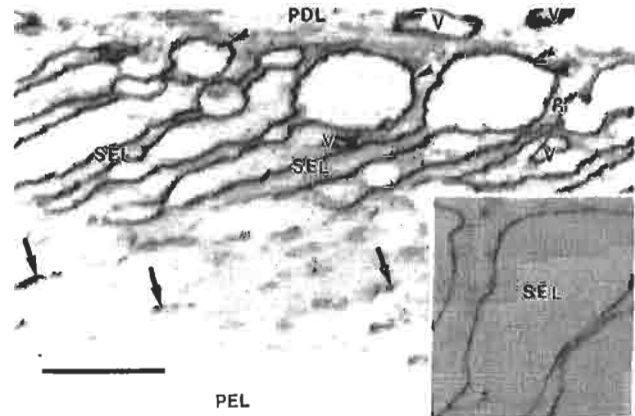


Fig 5: Laminitis: type IV collagen immunostaining. Section from mid-region of a single lamella affected by grade 3 laminitis. The SELs are attenuated and have tapered, stretched tips. A few distorted epidermal cells are still enclosed by BM. The BM of the SEL tips encloses dilatations (arrowheads). Fragments of immunostained BM (arrows) are occasionally present between SELs otherwise devoid of BM. The BM of blood vessels (V) is immunostained. Bar = 50 μ m. Inset at the same magnification shows occasional, very large, BM enclosed dilatations of the SEL tip, type VII collagen immunostaining.

streptavidin-peroxidase complex and when diaminobenzidine was omitted from the reaction mixture.

In some sections from feet affected by acute laminitis, there was widespread separation of the SELs from their BM without loss of BM immunostaining and, in others, there was extensive loss of BM immunostaining. The laminitis lesions were scored as *Grade 3* using the histopathological grading system of Pollitt (1996). In lesions characterised by lamellar separation, the epidermal basal cells at the tips of the primary epidermal lamellae were clumped together and were devoid of immunostained BM (Fig 2). The BM from which they had separate (mean separation distance 13.10 μ m s.e. = 2.55) appeared to have remained in its original position in the dermis and was clearly outlined by all 3 antibodies (Figs 2,3). The tubes of empty BM, derived from the tips of each SEL, had retained their normal shape and resembled the fingers of an empty glove described by Pollitt (1996). Neutrophils were numerous in the dermis surrounding the PEL tip and many had left the dermis and were located between layers of BM in what were once epidermal compartments (Fig 3).

Away from the PEL tip, in the mid-region of the lamellae, immunostaining of the BM of many SELs was absent. Loss of BM immunostaining was interpreted to mean that lysis of the structural elements of the BM had occurred. In some lamellae loss of BM immunostaining had occurred only at the bases of the SELs (Fig 4A). Fragments of immunostained BM were present in the zones of lysed BM suggesting that BM lysis was incomplete at the time of tissue fixation. In other lamellae, lysis of the BM was complete; there was no immunostained BM between SELs and the bulk of the epidermal cells of each PEL were an amorphous column of cells on either side of the central keratinised axis of the PEL (Fig 4B). Capillaries, normally present in the SDL, between most of the SELs (Fig 1) were no longer present at this stage. What lamellar BM remained immunostained as strands of BM along the edges of the PDLs (Fig 4B). The BM strands were, in fact, BM bilayers derived from BM originally lining the sides of each SEL tip. This was different to the separated BM from the PEL tips which retained its original shape (Fig 2). In SELs with the tapered, stretched tips, described by Pollitt (1996) a few attenuated epidermal cells

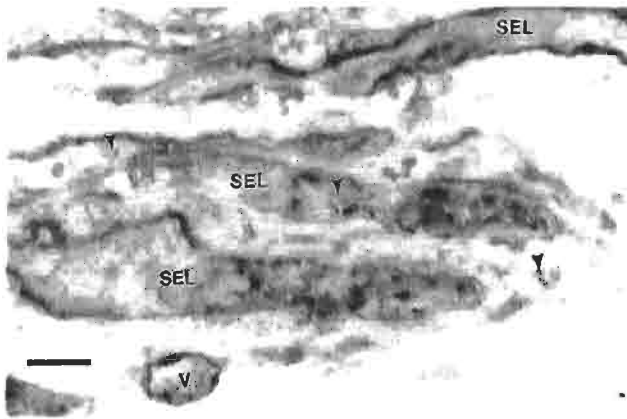


Fig 6: Laminitis: laminin immunostaining. Virtually all the immunoreactivity of the lamellar BM is absent. Only the narrow attenuated tip of an occasional SEL retains sufficient immunoreactivity to allow identification. The remainder of the BM appears to have been degraded into small fragments or has disappeared altogether. Numerous PMNs (arrowheads) either surround the disintegrating lamellar BM or are within the SEL epidermal compartments. Bar = 10 μ m.

were still enclosed by BM and often the BM of the SEL tip formed a dilatation (Fig 5).

Virtually all the BM immunoreactivity at the tips of some PELs was absent. Only the occasional distorted SEL tip and fragments of BM retained sufficient immunostaining to allow anatomical identification (Fig 6). Numerous polymorphonuclear leucocytes (PMNs) invariably surrounded the tips of lamellae showing large scale loss of immunoreactivity and many PMNs had penetrated the lamellar BM and were within the epidermal compartments (Figs 3, 6). PMNs were less frequent in the midlamellar region.

Many small veins and capillaries were located with no BM immunostaining for laminin and *type IV* collagen and many were surrounded by extravasated erythrocytes (Fig 7). Often the capillaries which did not stain, were beside capillaries which had normal staining BMs.

Discussion

Immunostaining with antibodies raised against human epitopes for *type IV* collagen, *type VII* collagen and laminin successfully stained the basement membranes of horse hoof lamellar tissues. Vascular tissue did not immunostain with *type VII* collagen antibodies. Normal basement membrane stained as a continuous dark brown line contrasting starkly against a light blue background of connective tissue and epidermis counterstained with haematoxylin. The method was superior to the histochemical methods of BM staining described by Pollitt (1996). The clear delineation of the lamellar BM in tissue sections affected by laminitis confirmed the findings of Pollitt (1996) that at least 2 changes to the lamellar BM occurred in developmental laminitis: loss of attachment of lamellar epidermal basal cells to their underlying BM and disintegration of the lamellar BM. Epidermal basal cells lost their attachment to the BM without undergoing obvious necrosis and when lamellar separation occurred the BM detached from the epidermal basal cells in intact sheets and remained attached to the dermal connective tissue. This implies that the substances causing BM lysis and separation emanate on the epidermal side of the BM and are products of the epidermal cells. Adhesion receptor molecules, called integrins, are responsible for the

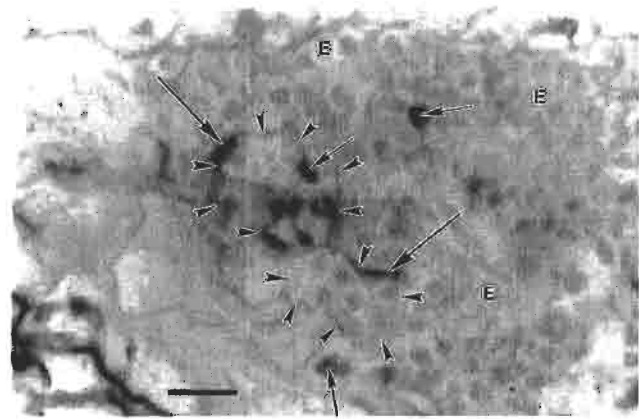


Fig 7: Laminitis: laminin immunostaining of dermal blood vessels. Many small veins and capillaries had lost BM immunostaining and numerous extravasated erythrocytes (E) were in the surrounding dermal connective tissue. The remains of the vessel walls (outlined with arrowheads) and fragments of immunostained vascular BM (large arrows) are still present. PMNs are in the lumen of one of the vessels and in the adjacent dermis (small arrows). Bar = 10 μ m.

adhesion of epidermal basal cells to the BM. Antibody raised against $\beta 1$ integrin receptors interferes with adhesion and stimulates a severalfold increase in production of 92 kDa matrix metalloproteinase (*type IV* collagenase) in cultures of human keratinocytes (Larjava *et al.* 1993). Keratinocyte MMP production leads to pericellular proteolysis which is the essential first step in the detachment of keratinocytes from the BM (Salo *et al.* 1991) during wound healing. Triggering of lamellar keratinocyte MMP production could initiate the BM lysis and detachment of lamellar epidermal cells from BM as shown here in laminitis.

The laminitis grading system described by Pollitt (1996) was based on differences, ranging from mild to severe, of the laminitis lesions between individual horses all killed 48 h after being dosed with a laminitis inducing diet. The assumption was made that the grades of severity represented stages in the temporal progression of the laminitis disease process. Horses with *Grade 3* lesions progressed through stages 1 and 2. A similar assumption, made here, enables recognition of stages of disease progression within individual horses in sections of tissues affected by laminitis. Lamellar separation and lysis of the BM appeared coincident in some sections but, in others, lysis of the BM appeared to be the first change to have occurred (Fig 4A). Lysis of the BM appears to commence between the bases of the SELs. *Type IV* collagen, *type VII* collagen and laminin, all known substrates of matrix metalloproteinases (Salamonsen 1994), disappear from the BM together. In addition to focal lysis, the BM also separates from the basal cells in intact sheets with the bulk of the *lamina densa* apparently intact. However, the *lamina densa* is attached to the plasmalemma of the basal cell by the anchoring filaments traversing the *lamina lucida* (Pollitt 1994) which are composed of laminin 5, a subtype of laminin. Lysis of anchoring filament laminin by MMP produced by lamellar keratinocytes could explain the wholesale detachment of lamellar BM in laminitis.

In this study, we used a panel of BM immunomarkers (laminin, *type IV* collagen and *type VII* collagen antibodies) to establish that the loss of lamellar BM immunostaining represented a genuine structural disintegration rather than the loss of individual epitopes. The loss of an epitope could result in loss of monoclonal antibody immunostaining without

necessarily implicating structural failure. Included in our panel was a polyclonal antibody to laminin and, since the BM lesions in the lamellar BM of the horses affected by laminitis were common to all 3 antibodies, we conclude that loss of immunostaining represents genuine anatomical disintegration of BM. Further, the antibodies located BM proteins of quite different structure and function: *type IV* collagen is the structural backbone of the BM *lamina densa*, laminin is a glycoprotein which coats *type IV* collagen and *type VII* collagen forms the anchoring fibrils and anchoring plaques which attach the BM to the adjacent dermis.

When the lamellar BM first separates from the lamellar basal cells it slides free of the SEL to form a dilatation at the SEL tip. When the separation process is extensive the separated BM from each SEL forms a BM bilayer with the appearance of a densely staining single strand. At intervals along the length of the single strand, and often at the tip, bifurcation of the strand reveals that the strand is in fact a bilayer. When the BM of many SELs separates and slides free, the bilayered strands form a layer of overlapping strands in the dermis on either side of the PEL. The PEL becomes an amorphous multi nucleate column of epidermal cells containing the original keratinised axis of the PEL and all the basal and suprabasal cells of the SELs, which are now without an attachment to BM. It can be assumed that the forces dragging the separated BM away from the SELs, in the direction of the distal phalanx, are those of weight bearing and locomotion (Pollitt 1996). The BM dilatations and the splits in the bilayered strands sometimes enlarge to form vacuole-like dilatations with a diameter of up to 50 μm and far exceed the diameter of neighbouring veins and capillaries. The membrane enclosing the dilatations was immunolabelled with all 3 BM antibodies used in this study. The reaction with antibodies to *type VII* collagen establishes unequivocally that the membrane of the dilatations is lamellar and not vascular in origin, as *type VII* collagen is not present in blood vessels and the lamellar vasculature never reacted with antibodies to *type VII* collagen. In the past, in sections stained by routine histochemical methods, the lamellar BM dilatations may have been confused with dilated blood vessels, giving rise to the concept that oedema was present at the lamellar dermoepidermal junction (Roberts *et al.* 1980). The diameter of the lumen of dermal blood vessels adjacent to the lamellar BM dilatations appeared normal and, although we can offer no explanation for the formation of the nonvascular lamellar BM dilatations, our results challenge the commonly held concept that oedema of vascular origin is involved in the pathogenesis of laminitis. Haemorrhage at the tips of PELs was noted in some tissue blocks prior to fixation. Many small veins and capillaries without BM immunostaining, surrounded by extravasated erythrocytes near the tips of the PELs, were present in sections of this tissue. The haemorrhage observed at the tips of PELs probably occurred because of damage to the BM of the veins and capillaries. The loss of BM immunostaining, and presumably BM lysis, in small veins and capillaries associated with the lamellar haemorrhage is evidence that BM damage did occur. Microvascular endothelial cells when exposed to cytokines such as TNF- α produce MMPs capable of destroying BM structural proteins (Cornelius *et al.* 1995) and this may have occurred in the lamellar tissues that haemorrhaged.

Virtually all the capillaries, normally present in the dermis between the SELs of each lamella were absent in the lamellar tissues affected by laminitis. Their disappearance coincided with the loss of immunostaining of the lamellar BM. There were never any capillaries with BM immunostaining in the

amorphous columns of SEL epidermal cells shown in Figure 5. The loss of these capillaries may explain why resistance to blood flow was increased 3.5 times in horses during early laminitis (Allen *et al.* 1990) and why blood was bypassing the capillary bed through dilated arteriovenous anastomoses in the horses with acute laminitis studied by Hood *et al.* (1978).

Care had to be taken with the interpretation of loss of BM immunostaining. If the BM had separated and migrated then the residual basal cells would appear to have lost BM immunostaining. Therefore, loss of immunostaining was ascribed to lamellar BM only when the loss occurred in sections of BM that were continuous with BM that had retained immunoreactivity. Fragmental disintegration of the BM was identified in every section of the 6 laminitis affected feet examined. It was never present in normal controls.

By what mechanism is the lamellar BM made to lyse and separate? Many examples of loss of BM immunostaining in epithelial tissues and detachment of basal cells from BM, similar to that shown here for laminitis, have been reported in the literature. In all cases BM loss and detachment is linked to the local production of matrix metalloproteinases (MMP) or *type IV* collagenase. Cancers arising from keratinocytes (basal cell and squamous cell carcinomas) penetrate their underlying BM after first lysing it by the production of *type IV* collagenase (Slade *et al.* 1995). As a prerequisite for wound healing keratinocytes detach from the BM and migrate to cover the exposed connective tissue. However, lysis of the BM precedes keratinocyte migration (Salo *et al.* 1991). Because of their ability to produce MMP, keratinocytes are considered major participants in the degradation of extracellular matrix during wound healing. A strong signal for 92kDa *type IV* collagenase (MMP-9) is found in the basal and suprabasal epithelial cell layer of normal human oral mucosa (Salo *et al.* 1994). Increased quantities of MMP-9 are found in migrating basal cells, wound fluid and granulation tissue after experimental wounding. Furthermore, cultures of oral mucosal keratinocytes respond to the addition of the cytokines tumour necrosis factor - α (TNF- α), interleukin-1 β (IL-1 β) and transforming growth factor- β 1 (TGF- β 1) by increasing production of MMP-9 (Salo *et al.* 1994).

The loss of immunostaining of the BM in lamellar tissues affected by laminitis resembled closely the pattern of BM destruction which occurs when the mouse blastocyst invades the uterine epithelium (Blankenship and Given 1995). On Day 5 of pregnancy the BM of the uterine epithelium still showed positive immunostaining for *type IV* collagen and laminin, but by Day 7 this had disappeared over the entire embryonic half of the uterine lining. The loss of BM immunostaining for *type IV* collagen and laminin closely followed its anatomical disintegration and occurred adjacent to trophoblast cells, known producers of matrix metalloproteinases (Shimonovitz *et al.* 1994).

Increased amounts of the active forms of BM degrading enzymes (EqMMP-2 and EqMMP-9) are present in equine lamellar tissues affected by laminitis 48 h after the alimentary administration of a laminitis inducing dose of carbohydrate (Pollitt *et al.* 1998). This increase in MMP production by lamellar keratinocytes, possibly triggered by the arrival of cytokines via a dilated lamellar vasculature (Pollitt and Davies 1998) or by changes in glucose metabolism in lamellar tissues (Pass *et al.* 1998), appears to be responsible for the detachment from the lamellar BM and loss of lamellar BM demonstrated in this study. A feature of acute laminitis is the large number of PMNs found within the BM enclosed but keratinocyte free epidermal compartments of the secondary epidermal lamellae of

the lamellar tips (Pollitt 1996). Equine PMNs are a potent source of Eq MMP-9 (Pollitt *et al.* 1998b) and the arrival and extravasation of PMNs into the lamellar tissues undoubtedly accelerates the process of lamellar BM destruction. PMNs probably gain access to the epidermal compartments of the lamellae by focal lysis of the lamellar BM.

This study contributes new information to our understanding of the mechanism causing laminitis. It demonstrates that early in acute laminitis lamellar BM is lysed and consequently disappears from many of the epidermal lamellae lining the inner hoof wall of the equine foot. It also shows that lamellar BM detaches from lamellar basal cells and forms loose strands of bilayered BM in the connective tissue adjoining the lamellae. 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 disorganisation of the lamellar BM demonstrated here would lead inexorably to the failure of lamellar anatomy so characteristic of equine laminitis.

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Manufacturers' addresses

¹Sigma Chemical Co. St Louis, Missouri, USA.

²Zymed Laboratories Inc, San Francisco, USA.

³Dako (Australia) Pty Ltd., Botany, NSW 2019, Australia.

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