

# Equine laminitis: cleavage of laminin 5 associated with basement membrane dysadhesion

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## Abbreviations

AF	Anchoring filament
BM	Basement membrane
EBC	Epidermal basal cell
HDs	Hemidesmosomes
IFM	Immunofluorescence microscopy
L5	Lamin 5
LM	Light microscopy
OF	Oligofructose
PL	Plasmalemma
SELS	Secondary epidermal lamellae
TEM	Transmission electron microscopy

## Summary

**Reasons for performing study:** The key lesion of laminitis is separation at the hoof lamellar dermal-epidermal interface. For this to happen the structural and adhesion proteins of the basement membrane zone must be altered. Which proteins and how damage to them leads to the lamellar separation of laminitis is unknown.

**Objectives:** To investigate lamellar hemidesmosome and cytoskeleton damage and basement membrane dysadhesion using light microscopy (LM) and immunofluorescence microscopy (IFM).

**Methods:** Cryostat sections of lamellar tissues from 2 control and 6 Standardbred horses with oligofructose induced laminitis were studied using LM and IFM. Plectin, integrin  $\alpha_6$  and BP230 antibody was used to label hemidesmosome intracellular plaque proteins and anti-BP180 and anti-laminin 5 (L5) was used to label anchoring filament (AF) proteins. Cytoskeleton intermediate filaments were labelled using anti-cytokeratin 14. The primary antibodies of selected sections were double labelled to show protein co-localisation.

**Results:** Laminitis caused reduction of transmembrane integrin  $\alpha_6$ , the AF proteins BP180 and L5, and failure of co-localisation of BP180 and L5. Proteins of the inner hemidesmosomal plaque, plectin and BP230, were unaffected.

**Conclusions:** Loss of co-localisation of L5 and BP180 suggests

that, during the acute phase of laminitis, L5 is cleaved and therefore, the AFs connecting the epidermis to the dermis, fail. Without a full complement of AFs separation at the lamellar dermo-epidermal junction occurs.

**Potential relevance:** Suppressing or inhibiting metalloproteinase activity may prevent L5 cleavage and therefore the lamellar dermo-epidermal separation of laminitis.

## Introduction

The key lesion of laminitis is failure of the attachment between the inner hoof wall and the distal phalanx of the foot at the lamellar dermal-epidermal junction (Pollitt 1996). Loss of lamellar laminin and collagen immunostaining occurs early in the acute phase of the disease (Pollitt and Daradka 1998) as well as disorganisation of onychogenic, acidic, fibrillar structures in the secondary epidermal lamellae (SELS) corresponding to the cellular cytoskeleton (Obel 1948; Wattle 2000). Investigation of 2 *in vitro* models of lamellar separation and carbohydrate overload induced laminitis using transmission electron microscopy (TEM), also showed changes in the epidermal basal cell (EBC) cytoskeleton, loss of hemidesmosomes (HDs) and dysadhesion of EBCs to the adjacent basement membrane (French and Pollitt 2004a,b).

In the normal epidermis, the cellular cytoskeleton is formed by an extensive array of filaments that give shape to the cell and convey the ability to move; they are responsible for the arrangement and internal motions of cellular organelles (Fuchs 1995). The cytoskeleton of EBCs consists of keratin intermediate filaments, composed of cytokeratin 5 and 14. The 3-dimensional web of intermediate filaments stretches across the cell, from nuclear envelope to desmosomes in cell-cell junctions and to HDs in the plasmalemma (PI), mediating cell-basement membrane-matrix interactions (Voet *et al.* 1999).

Attachment of EBCs to the basement membrane (BM) is reliant on HDs, which connect the cellular cytoskeleton through the PI to the *lamina densa* of the adjacent BM. HDs are composed of 6 key proteins. The intracellular plaque proteins plectin and BP230 aid cytoskeleton insertion into the PI. The transmembrane integrin  $\alpha_6\beta_4$  heterodimer serves as a cell receptor and interacts with the HD intracellular plaque proteins. Finally, BP180 and laminin 5 (L5), the anchoring filament (AF) proteins, bridge the *lamina lucida* and anchor the PI to the BM (Jones *et al.* 1998; Borradori and Sonnenberg 1999).

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Ultrastructural studies of OF induced laminitis demonstrated HD and cytoskeleton damage and BM dysadhesion (French and Pollitt 2004b). Identifying how this altered arrangement of proteins at the lamellar dermo-epidermal interface leads to the lamellar separation of laminitis has to be understood if the disease is to be prevented and treated effectively. The aim of this study was to investigate further these pathological changes using immunofluorescence microscopy (IFM).

## Materials and methods

Oligofructose (OF) overload laminitis was induced in 6 Standardbred horses involved in a study testing the efficacy of continuous cryotherapy applied to the left forelimb of each horse (van Eps and Pollitt 2004). The horses were dosed with 10 g/kg OF and the laminitis affected tissue of the right forelimbs was harvested for this study. Control tissue came from 2 normal horses killed for teaching purposes. Experiments on horses were conducted according to University of Queensland Animal Ethics Committee guidelines.

At 48 h, horses were subjected to euthanasia, by a shot to the head, and the forefeet disarticulated at the metacarpophalangeal joint. The feet were trimmed initially with a bandsaw (Pollitt 1996) and dissected to produce lamellar samples that were snap frozen in cryoprotectant (propan-2-ol) cooled in liquid N<sub>2</sub> and mounted in Tissue-Tek O.C.T. Compound<sup>1</sup>. Cryostat sections, 8 µm thick, mounted on poly-lysine coated slides were fixed in acetone for 2 mins at -20°C and then air-dried. Sections to be labelled with rat monoclonal antibody GoH3<sup>2</sup> were fixed in 3.7% formaldehyde for 5 mins, followed by acetone for 2 mins at -20°C, according to the manufacturer's directions.

To label the HD intracellular plaque 3 antibodies were used. These were human monoclonal antibody 5E-HY-4B directed against BP230, mouse monoclonal antibody 417D1 directed against plectin (both gifts of Dr J. Jones, Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago Illinois, USA) and rat monoclonal antibody GoH3<sup>2</sup> directed against integrin  $\alpha_6$ . To label the first AF protein, BP180, the mouse monoclonal antibody 233 (a gift of Dr K. Owaribe, Department of Molecular Biology, School of Science, Nagoya University, Chikusa-Ku, Nagoya, Japan) was used. To label the second AF protein the rabbit polyclonal antibody J18 directed against laminin 5 (also a gift of Dr Jones) was used. The intermediate filaments of the cytoskeleton were labelled using the mouse monoclonal antibody LL-0023 directed against cytokeratin 14.

Sections for single and double labelling were incubated in primary antibody overnight, at 4°C, followed by the appropriate secondary antibody<sup>4</sup> for 1 h at 37°C. Each incubation was followed by three, 5 min washes in phosphate buffered saline. The sections underwent a final 5 min wash in 20 mmol/l Tris-saline before being mounted in gelvatol containing 0.1% diazabicyclo (2,2,2) octane and coverslipped. Sections incubated in the absence of the primary antibodies and with irrelevant immunoglobulin were used as controls to determine nonspecific binding of secondary antibodies.

Tissue for light microscopy was fixed overnight in 10% formalin, sectioned at 5 µm and stained with haematoxylin and eosin (H&E) and periodic acid Schiff (PAS) reagent.

Sections were viewed with an Olympus BX50 microscope<sup>5</sup> equipped with an Olympus BX-FLA reflected light fluorescence attachment<sup>5</sup> and specific filters for fluorescein (U-MF) and

rhodamine (U-MWG). They were photographed with an Olympus SC35 camera<sup>5</sup> on Fujicolor Press CZ135 800 ISO colour film<sup>6</sup>. Negatives were scanned to generate a digital image. Where sections had been double labelled, identical fields labelled with fluorescein and rhodamine were overlaid digitally using Adobe PhotoShop 5<sup>7</sup>. Red and green areas of co-localised primary antibodies appeared yellow.

## Results

Control transverse sections stained with H&E and PAS showed SELs arranged symmetrically on either side of primary epidermal lamellae (Figs 1a,c). SELs were of uniform length with rounded tips. In PAS stained sections the BM stained magenta and clearly outlined each SEL perimeter (Fig 1e). In laminitis tissue, SELs were elongated with sharp instead of rounded tips (Figs 1b,d). SELs were abnormally thin and strands of basement membrane, no longer attached to SEL tips, were isolated in the adjacent lamellar dermis (Fig 1f).

### *Plectin*

Localisation and distribution of plectin was unaffected by OF induced laminitis. In control and experimental tissue plectin was located in EBCs only, concentrated in the basal cytoplasm and the PI.

### *BP230*

BP230 immunofluorescence was localised to the dermo-epidermal junction of the control lamellae. It clearly outlined each SEL and penetrated between adjacent SEL bases. The pattern of immunofluorescence associated with BP230 in laminitic tissue showed intermittent loss around the SEL perimeter, with a dashed line appearance. Despite the laminitis process and BM separation from the tips of SELs the BP230 label remained localised in the PI of EBCs.

### *Integrin $\alpha_6$*

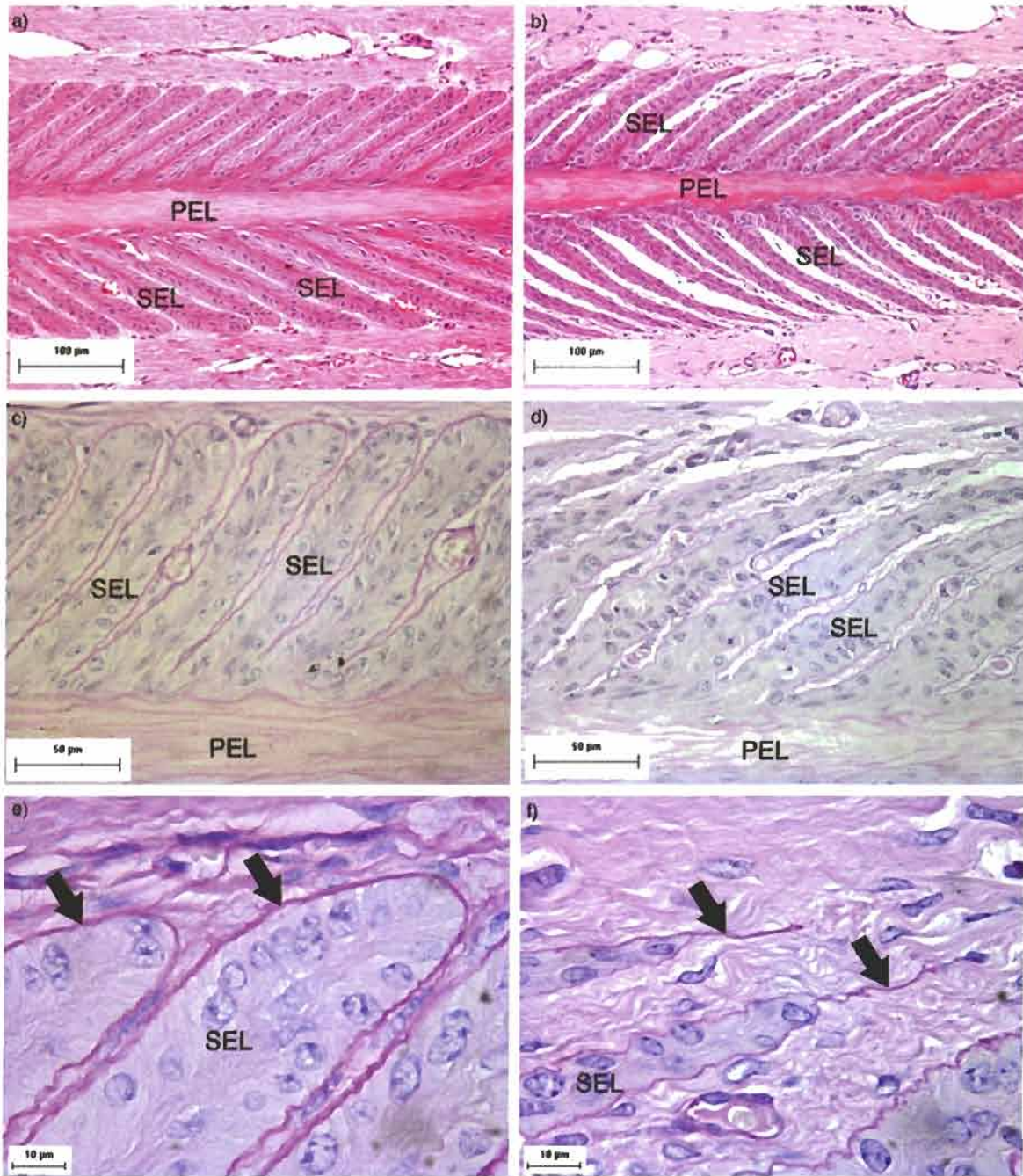
Immunofluorescence labelling of integrin  $\alpha_6$  outlined each EBC of the SELs in control tissue. The fluorescence was slightly brighter in the basal area of the PI adjacent to the BM. In laminitic tissue there was weak immunofluorescence labelling for integrin  $\alpha_6$ . The residual label localised to the EBC in the same pattern as control tissue, regardless of whether there was dermo-epidermal separation.

### *BP180*

In control tissue BP180 was present in SEL basal cells but was more concentrated adjacent to the BM (Fig 2a). In laminitis tissue the fluorescence associated with BP180 was reduced (Fig 2b). Residual BP180 fluorescence was localised, as it was in control tissue, to EBCs, concentrated in the basal cytoplasm and PI. At the SEL tips, where the BM had separated from the EBC, BP180 was associated only with epidermal tissue, not the BM.

### *Laminin 5*

L5 immunofluorescence was present in SEL basal cells, but was more concentrated close to the dermo-epidermal junction, outlining the round SEL tips in control tissue (Fig 2c). In the



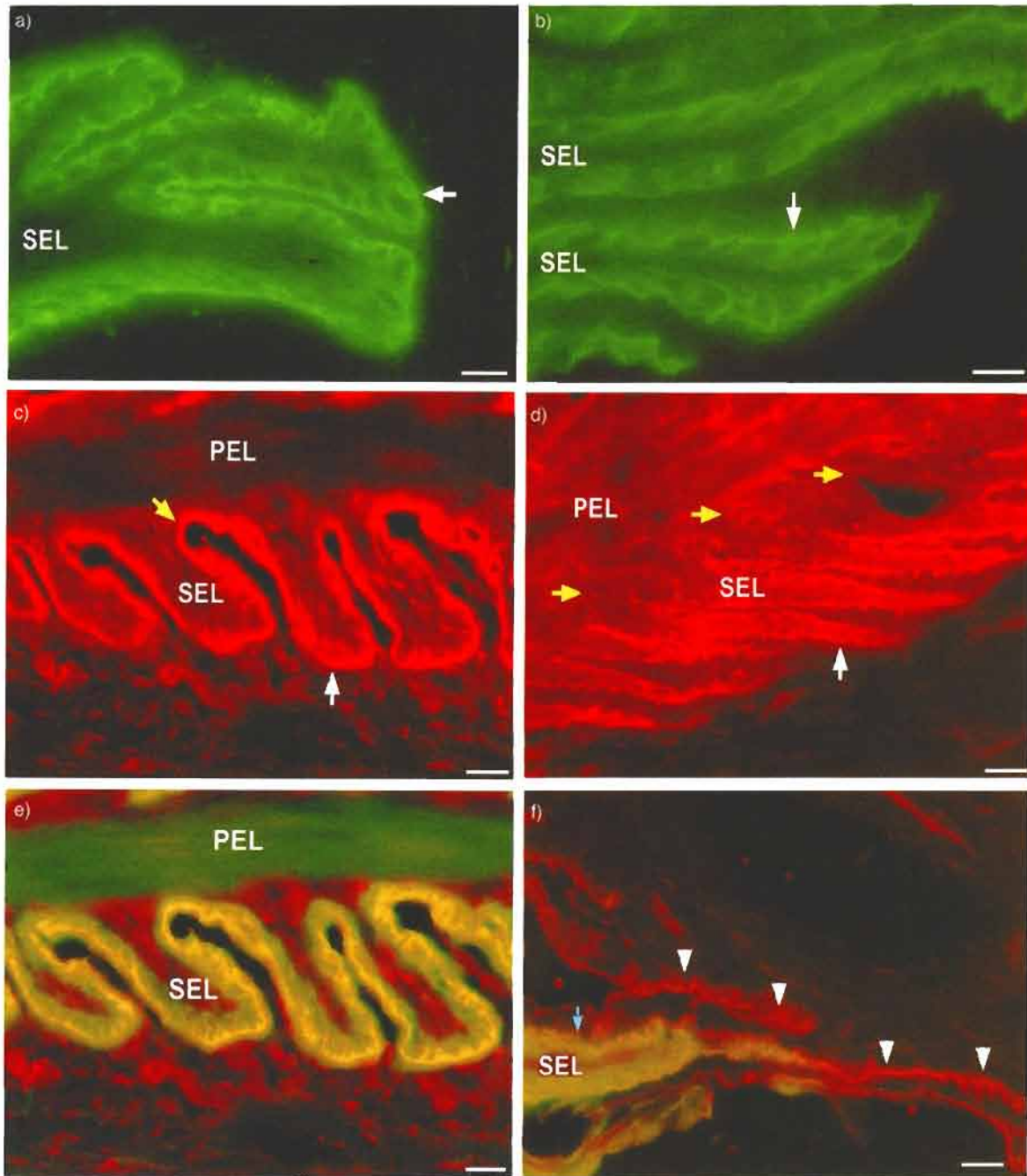
*Fig 1: Photomicrographs of transverse sections of control (left column a, c and e) and laminitic lamellae (right column b, d and f). Laminitis was induced by alimentary overload with 10 g/kg oligofructose (n = 6). Control tissue stained with haematoxylin and eosin (a) and periodic acid Schiff (c) show secondary epidermal lamellae (SELs) of normal shape and length arranged on either side of primary epidermal lamellae (PEL). In equivalent sections of laminitic tissue the SELs are elongated, with tapered instead of rounded tips (b) and (d). In control tissue, at high magnification, the tip of each normal SEL is outlined by basement membrane stained dark magenta (arrowed) by periodic acid Schiff stain (e). In laminitic tissue (f) SELs are abnormally thin and strands of basement membrane (arrowed), no longer attached to SEL tips, lie free in the adjacent lamellar dermis.*

laminitic tissue L5 fluorescence was diminished and at SEL tips, L5 was associated with the strands of BM that had separated from the EBCs (Fig 2d).

#### *BP180 and laminin 5 co-localisation*

Control sections, double-labelled for BP180 and L5, showed strong yellow co-localisation in EBCs of the SELs when identical fields

of view were overlaid (Fig 2e). Notably, the zone adjacent to the dermo-epidermal junction was bright yellow. Co-localisation of BP180 and L5 was disrupted in laminitic hoof lamellar tissue (Fig 2f). BP180 and L5 co-localised to the EBC of the SELs. However, the BM that had separated to form isolated strands in the dermis labelled only for L5. Therefore, the distribution of L5 immunofluorescence was split; L5 fluorescence was localised with the separated BM, but L5 fluorescence also remained with the



*Fig 2: Immunofluorescence microscopy, at the same magnification as Figures 1e and f, of control (left column a, c and d) and laminitic lamellae (right column b, d and f). Control SELs in cryostat sections of hoof lamellar tissue immunolabelled for BP180 (a) show green (fluorescein) fluorescence concentrated in the cytoplasm (arrowed) of epidermal basal cells. Laminitic tissue (b) shows decreased BP180 immunofluorescence however, localisation remained within epidermal basal cells (arrow) and was not associated with BM that had separated. Laminin (L5) immunofluorescence (labelled red with rhodamine) in control tissue (c) localises strongly to the dermo-epidermal junction, outlining SEL tips (white arrow). Laminitic tissue (d) shows decreased L5 immunofluorescence. However, immunolabelled L5 was associated with BM that had separated from SEL tips (white arrowheads). In double labelled, control lamellar tissue (e) green BP180 and red L5 fluoresce yellow indicating co-localisation. In laminitic tissue (f) yellow co-localised BP180 and L5 was present in SEL basal cells (blue arrow). However, only red L5 label was associated with BM that had separated (white arrowheads). Therefore, during laminitis development, L5 was cleaved, leaving red in SEL basal cells (co-localised with BP180 to give yellow) as well as in the separated BM.*

lamellar EBCs, giving the basal cells with co-localised BP180 and L5 a yellow-green appearance.

#### *Cytokeratin 14*

Immunofluorescence label for cytokeratin 14 localised strongly to

the EBC in both control and laminitic tissue. Cytokeratin 14 immunofluorescence label showed clearly the normal arrangement of the cytoskeleton, stretching from the nucleus to the cell perimeter in control tissue. Most EBCs contained a dark oval shape (with the long axis perpendicular to the BM) which corresponded to the nonstained nucleus. Cytokeratin 14 showed

an altered pattern of fluorescence in the laminitic tissue when compared to normal tissue. However, the intensity of the fluorescence appeared unaffected. In the laminitic tissue, the orientation of the oval shaped shadow corresponding to the nucleus was changed: it was rounded and its long axis parallel to the BM. It was also difficult to differentiate the strands of the cytoskeleton stretching from the nucleus to the EBC perimeter. The cytokeratin 14 label appeared flattened and dense around the nucleus in the EBC cytoplasm.

## Discussion

Tissue from horses with OF induced laminitis was similar in all respects to tissue affected by laminitis induced with wheat starch (Pollitt 1996). Laminitis caused reduction of the transmembrane integrin  $\alpha_6$  and AF proteins BP180 and L5 and failure of co-localisation of BP180 and L5. However, the proteins of the inner hemidesmosomal plaque, plectin and BP230, were relatively unaffected by the disease process. TEM of laminitis tissue (induced by an OF dose of 10 g/kg bwt) showed a decrease in HD size and number (French and Pollitt 2004b).

Immunofluorescence microscopy (IFM) showed that none of the components of the HD were physically destroyed. Studies of autoimmune and inherited skin blistering diseases show that loss or disruption of integrin  $\alpha_6$ , BP180 and L5 also causes separation at the dermo-epidermal junction (Green and Jones 1996; Hirako and Owaribe 1998; Borradori and Sonnenberg 1999). It is possible to have a significant decrease in the numbers of HDs and failure of HD attachment without loss of antigenic determinants (epitope). Disruption of cellular metabolism by lack of glucose could adversely affect the normal activity and interactions of the HD components and compromise their signalling to support molecules (Jones *et al.* 1998). HD disassembly, triggered by glucose starvation, could alter the rate of phosphorylation within HDs, affecting integrin  $\alpha_6\beta_4$  and also the recruitment of the cytoskeleton into the PI (Borradori and Sonnenberg 1996). It is possible that the decreased immunofluorescence seen in the laminitic tissues parallels the lamellar changes induced by the absence of glucose *in vitro* (French and Pollitt 2004a) and results from alterations in whole body glucose metabolism, causing glucose starvation in peripheral tissues, therefore triggering dermo-epidermal separation in the hoof lamellae.

There was no loss of cytokeratin 14 staining confirming Wattle's (2000) conclusion that the primary disturbance in laminitis does not affect the cytokeratins. However, there was a change in the pattern of cytokeratin 14 staining (dense around the nucleus) and in the shape and orientation of the EBC nucleus (rounded with its long axis parallel to the BM) when laminitis had developed. Plectin and BP230 connect the EBC cytoskeleton to the HD and disassembly of HDs would release intermediate filaments from the PI and alter cell and nuclear shape in the pattern characteristic of laminitis (Pollitt 1996; French and Pollitt 2004b).

The loss of co-localisation of BP180 and L5, that was coupled with separation of the EBCs from their underlying BM, during the acute phase of laminitis is interesting. These 2 transmembrane proteins form the AFs that anchor HDs of EBCs to the *lamina densa* of the BM. Loss of co-localisation could, in part, be due to the generalised loss of these proteins throughout the lamellae. Of particular interest is the fate of each protein after co-localisation was lost. BP180 remained localised to EBCs, concentrated in the basal cytoplasm and PI. Much of the L5 label remained with the

BM as it separated from the EBCs and was visible as a thick fluorescent line far removed from the SEL tips. However, there was still L5 fluorescence associated with EBCs. This dual localisation of L5 in tissue where the BM has separated from the epidermal cells suggests that L5 is cleaved during the process of separation, leaving part of the molecule in the epidermal cell PI and part embedded in the detached BM.

During laminitis there is up-regulation of transcription and increased enzymatic activity of matrix metalloproteinase-2 (MMP-2) (Pollitt *et al.* 1998; Kyaw-Tanner and Pollitt 2004). L5 is a known substrate for MMP-2 (Gianneli *et al.* 1997). Exogenous addition of activated MMP-2 stimulates breast epithelial cells to migrate over L5 via cleavage of the gamma 2 subunit of L5. MMP cleavage of L5 may be a widespread mechanism that triggers migration in EBCs in contact with a BM (Koshikawa *et al.* 2000). During laminitis development cleavage of L5 by MMP-2 would destroy the AFs that connect HDs to the BM and would contribute to movement of EBCs away from the BM. The localisation of MMP-2 in the basal and parabasal cells of the SEL (Kyaw-Tanner and Pollitt 2004), conveniently close to L5, provides further support for the role of MMP-2 in the development of laminitis.

Progressive loss in HD size and number correlates with the severity of laminitis in ultrastructural studies (French and Pollitt 2004b). IFM further enhances knowledge of the damage done to HDs by pinpointing reduction of 3 key components (integrin  $\alpha_6$ , and AF proteins BP180 and L5) during developmental and acute laminitis, possibly triggered by glucose starvation and MMP activation within hoof tissues. Loss of co-localisation of L5 and BP180 in BM that has separated from SEL tips, and dual localisation of L5 and BP180 in the remainder of the SEL suggests that during the acute phase of laminitis L5 is cleaved causing failure of the AFs that connect epidermis to dermis. Without a full complement of anchoring filaments separation at the lamellar dermo-epidermal junction, the signature lesion of laminitis, inevitably occurs.

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## Manufacturers' addresses

- <sup>1</sup>Sakura Finetechnical Co., Tokyo, Japan.
- <sup>2</sup>Immunotech, Marseilles, France.
- <sup>3</sup>Novocastra Laboratories, Newcastle upon Tyne, UK.
- <sup>4</sup>Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA.
- <sup>5</sup>Olympus Optical Co., Tokyo, Japan.
- <sup>6</sup>Fuji Photo Film Co., Tokyo, Japan.
- <sup>7</sup>Adobe Systems Inc., Australia.

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