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Equine laminitis: glucose deprivation and MMP activation induce dermo-epidermal separation *in vitro*

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Summary

Reasons for performing study: **Acute laminitis is characterised by hoof lamellar dermal-epidermal separation at the basement membrane (BM) zone. Hoof lamellar explants cultured *in vitro* can also be made to separate at the basement membrane zone and investigating how this occurs may give insight into the poorly understood pathophysiology of laminitis.**

Objectives: **To investigate why glucose deprivation and metalloproteinase (MMP) activation in cultured lamellar explants leads to dermo-epidermal separation.**

Methods: **Explants, cultured without glucose or with the MMP activator *p*-amino-phenol-mercuric acetate (APMA), were subjected to tension and processed for transmission electron microscopy (TEM).**

Results: **Without glucose, or with APMA, explants under tension separated at the dermo-epidermal junction. This *in vitro* separation occurred via 2 different ultrastructural processes. Lack of glucose reduced hemidesmosomes (HDs) numbers until they disappeared and the basal cell cytoskeleton collapsed. Anchoring filaments (AFs), connecting the basal cell plasmalemma to the BM, were unaffected although they failed under tension. APMA activation of constituent lamellar MMPs did not affect HDs but caused AFs to disappear, also leading to dermo-epidermal separation under tension.**

Conclusions: **Natural laminitis may occur in situations where glucose uptake by lamellar basal cells is compromised (e.g. equine Cushing's disease, obesity, hyperlipaemia, ischaemia and septicaemia) or when lamellar MMPs are activated (alimentary carbohydrate overload).**

Potential relevance: **Therapies designed to facilitate peripheral glucose uptake and inhibit lamellar MMP activation may prevent or ameliorate laminitis.**

Introduction

The distal phalanx is suspended from the inner hoof wall by a resilient lamellar attachment apparatus that not only supports the weight of the horse but plays a vital role in ameliorating ground impact and rotational forces (Dyhre-Poulsen *et al.* 1994). The basement membrane (BM) of the inner hoof wall lamellae, divides the epidermal compartment of the hoof from the dermal connective tissue that is attached to the periosteum of the distal phalanx (Pollitt 1992). On one side of this dermo-epidermal junction is a layer of epidermal basal cells firmly attached to the BM, while the other (dermal) side consists of dense, collagenous connective tissue woven into the structure of the BM. The lamellar BM is the site of the primary lesion of laminitis, a condition where the attachment between dermis and epidermis fails and the distal phalanx separates from the inner hoof wall (Pollitt 1996; Pollitt and Daradka 1998).

Viewed with the transmission electron microscope the BM has a 3 layered appearance, dominated by the *lamina densa*, that follows the contours of the epidermal basal cells (Pollitt 1994). Electron dense adhesion plaques, or hemidesmosomes (HDs), attach basal cells to the *lamina densa* of the BM and consist of the proteins plectin, BP230, BP180 and integrin $\alpha_6\beta_4$. Plectin directly links the basal cell cytoskeleton with integrin $\alpha_6\beta_4$, therefore inserting keratin filaments of the cytoskeleton directly into the basal cell plasmalemma via the HD (Langhofer *et al.* 1994; Niessen *et al.* 1996; Burgeson and Christiano 1997). Glucose consuming HD phosphorylation is crucial for continuing HD maintenance and assembly (Borradori and Sonnenberg 1996; Jones *et al.* 1998).

Lamellar hoof explants, cultured with the matrix metalloproteinases (MMP) activator, *p*-amino-phenyl-mercuric acetate (APMA) develop a laminitis-like separation at the dermo-epidermal junction (Pollitt *et al.* 1998; Mungall *et al.* 2001). MMP activity is likewise increased in hoof tissue taken from horses with laminitis (Johnson *et al.* 1998; Pollitt *et al.* 1998) and laminitis is associated with increased transcription of lamellar MMP-2 (Kyaw-Tanner and Pollitt 2004). Glucose is essential for hoof explants in culture, and culture without glucose or inhibition of glycolysis also causes BM zone separation under tension (Pass *et al.* 1998).

Here we used transmission electron microscopy (TEM) and tension testing to study the separation process of the 2 *in vitro* models of basement membrane zone separation to gain insight into the lesion of natural laminitis.

Materials and methods

Explant preparation and culture

Explants of two sizes were prepared and tested, as described previously, (Pollitt *et al.* 1998; Mungall *et al.* 2001). Normal feet

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TABLE 1: Explants cultured in DMEM, DMEM without glucose and DMEM with APMA were subjected to tension up to 700 g and the weight causing separation recorded. Lack of glucose and MMP activation by APMA significantly reduced explant integrity

Time (h)	DMEM mean (g) ± s.e.	DMEM no glucose mean (g) ± s.e.	DMEM with APMA mean (g) ± s.e
0	700	700	700
1	700	700	700
2	700	700	700
4	700	700	700
6	700	700	700
12	700	482.86 ± 65.31[a]	700
24	700	297.14 ± 66.28[a]	604.29 ± 47.99
48	700	314.29 ± 71.34[a]	481.43 ± 55.01[b]

n = 10 (number of explants tested per time point): [a]DMEM vs. DMEM no glucose ($P<0.001$); [b]DMEM vs DMEM with APMA ($P<0.001$).

TABLE 2: Explants cultured in DMEM, DMEM without glucose and DMEM with APMA were fixed and analysed by TEM. The mean distance (μm) from the basal cell plasmalemma to the centre of the *lamina densa* of the BM was measured. Lack of glucose caused HD shrinkage and disappearance but did not cause BM separation. In contrast MMP activation by APMA caused the BM plasmalemma distance to significantly increase

Time (h)	DMEM mean distance (μm) ± s.e.	Number of HDs measured/ treatment	DMEM no glucose mean distance (μm) ± s.e.	Number of HDs measured/ treatment	DMEM with APMA mean distance (μm) ± s.e.	Number of HDs measured/ treatment
0	0.061 ± 0.001	30				
1	0.064 ± 0.001	18	0.074 ± 0.002	14	0.124 ± 0.031	16
2	0.057 ± 0.001	14	0.077 ± 0.005	14	0.069 ± 0.003	15
4	0.054 ± 0.002	15	0.073 ± 0.004	14	0.11 ± 0.015[a]	14
6	0.053 ± 0.002	14	0.095 ± 0.009	14	0.622 ± 0.454[b]	14
12	0.061 ± 0.002	18	0.078 ± 0.007	20	0.343 ± 0.118[a]	17
24	0.060 ± 0.002	17			0.15 ± 0.011[c]	14
48	0.062 ± 0.003	15			0.355 ± 0.069[c]	18

n = 6 (number of explants in pooled data per time point); DMEM vs. DMEM + APMA [a]($P<0.05$), [b]($P<0.01$), [c]($P<0.001$).

TABLE 3: Explants cultured in DMEM, DMEM without glucose and DMEM with APMA were fixed and analysed by TEM. The mean percentage surface area occupied by HDs in BM contact was derived from the measured length of HDs. As lack of glucose caused HD shrinkage and disappearance the surface area occupied by in contact HDs decreased. As MMP activation by APMA caused BM separation the area occupied by in contact HDs also decreased

Time (h)	DMEM mean % of BM surface occupied by in contact HDs ± s.e.	Number of HDs measured/ treatment	DMEM no glucose mean % of BM surface occupied by in contact HDs ± s.e.	Number of HDs measured/ treatment	DMEM with APMA mean % of BM surface occupied by in contact HDs ± s.e.	Number of HDs measured/ treatment
0	33.246 ± 0.93	30				
1	27.012 ± 1.821	18	26.737 ± 0.981	14	21.091 ± 2.759	16
2	31.842 ± 1.841	14	16.853 ± 2.997[b]	14	35.334 ± 1.947	15
4	32.225 ± 1.331	15	20.407 ± 2.339[a]	14	14.347 ± 2.123	14
6	29.907 ± 1.653	14	7.869 ± 4.326[c]	14	9.36 ± 2.513	14
12	25.391 ± 1.093	18	4.41 ± 0.78[c]	20	11.271 ± 2.388	17
24	24.096 ± 1.154	17			12.318 ± 1.487	14
48	27.83 ± 1.808	15			4.15 ± 1.228	18

n = 6 (number of explants in pooled data per time point): DMEM vs. DMEM no glucose [a]($P<0.05$), [b]($P<0.01$), [c]($P<0.001$); DMEM vs. DMEM + APMA [d]($P<0.001$).

from 10 horses, obtained from a commercial abattoir, were transported on ice and dissected within 60 mins of humane slaughter. Structural integrity was assessed by anchoring one end of the explant and attaching the other to a calibrated force transducer. Explants that did not separate were stretched to a maximum force of 700 *g*. When explants did separate the force required to cause separation, at the dermo-epidermal junction, was recorded and expressed as a percentage of the maximum. Small explants, for TEM, consisted of 1–2 mm of inner hoof wall, 2–3 intact epidermal and dermal lamellae and 2–3 mm of connective tissue (Pollitt 1994). The second size, used to test explant structural integrity, were approximately 3 times larger.

The effect of time on explants cultured with and without glucose and with APMA

Explants of both sizes were cultured at 37°C in Dulbecco's modified Eagle's medium[1] (DMEM) containing 0.1 mg/ml gentamycin[2], the equivalent DMEM containing no glucose[1], DMEM + APMA[2] (0.25 mg/ml in 0.001% dimethylsulphoxide[2]), and DMEM + 0.001% dimethylsulphoxide for 1, 2, 4, 6, 12, 24 and 48 h. Some explants were tensioned before fixation to determine the force required to separate them. Small explants were fixed, stained and examined by TEM (Pollitt 1994).

TABLE 4: Explants cultured in DMEM, DMEM without glucose and DMEM with APMA were fixed and analysed by TEM. The numbers of HDs in BM contact were counted from mosaics of digital TEM images and expressed as the mean density of in contact HDs per micrometer of plasmalemma. As lack of glucose caused HD shrinkage and disappearance the number of HDs in BM contact decreased. As MMP activation by APMA caused BM separation the number of in contact HDs also decreased

Time (h)	DMEM mean HDs/µm plasmalemma ± s.e.	Number of HDs measured/ treatment	DMEM no glucose mean HDs/µm plasmalemma ± s.e	Number of HDs measured/ treatment	DMEM with APMA mean HDs/µm plasmalemma ± s.e	Number of HDs measured/ treatment
0	4.118 ± 0.1201	30				
1	3.459 ± 0.2247	18	3.528 ± 0.1004	14	2.59 ± 0.3844	16
2	4.105 ± 0.2317	14	2.372 ± 0.4228	14	5.016 ± 0.3019	15
4	4.215 ± 0.1689	15	2.984 ± 0.2727	14	2.179 ± 0.3186	14
6	4.486 ± 0.2925	14	1.349 ± 0.7233 ±[a]	14	1.354 ± 0.407[c]	14
12	3.654 ± 0.1276	18	0.8304 ± 0.1457[b]	20	1.582 ± 0.3323[d]	17
24	3.314 ± 0.1939	17			1.794 ± 0.2344[e]	14
48	4.144 ± 0.3199	15			0.6403 ± 0.1942[e]	18

n = 6 (number of explants in pooled data per time point); DMEM vs. DMEM no glucose [a](P<0.05), [b](P<0.01); DMEM vs. DMEM + APMA [c](P<0.05), [d](P<0.01), [e](P<0.001).

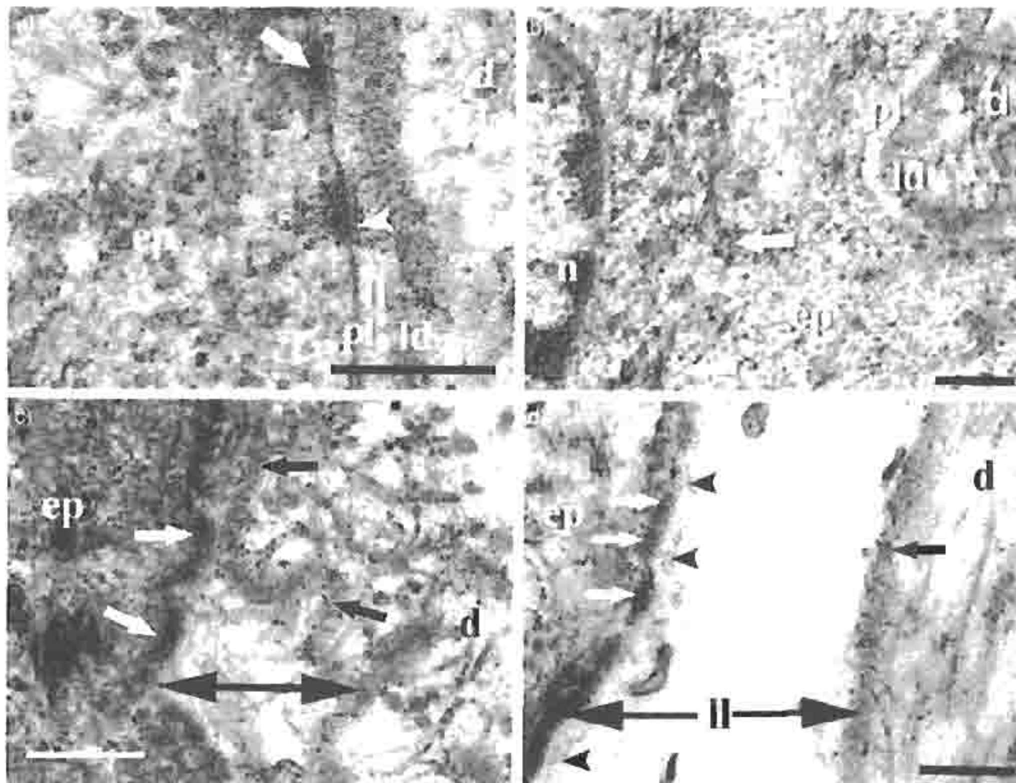

Fig 1: TEMs of the BM zone of equine hoof lamellar explants. In explants cultured in DMEM with glucose for 48 h (a) the BM zone is dominated by the lamina densa *(ld). Between the* lamina densa *and the basal cell plasmalemma (pl) is the* lamina lucida *(ll). Many anchoring filaments (AFs) traverse the* lamina lucida *and are especially dense adjacent to HDs (white arrow). The sub-basal dense plate (arrowhead) lies parallel to the plasmalemma and the* lamina densa. *The tonofilament cytoskeleton is uniform within the cytoplasm of the basal cell (ep) and merges into the dense intracellular plaque of the HDs. Dermis = d. bar = 200 nm. In explants cultured in the absence of glucose (b) for 6 h the* lamina densa *(ld) is still clearly visible. The tonofilament cytoskeleton (white arrows) is detached from the HD intracellular plaque and has condensed around the nucleus (n) of the epidermal cell. There are no HDs present in the epidermal cell plasmalemma (pl). Bar = 250 nm. In explants cultured with APMA for 4 h (c) portions of the* lamina densa *(black arrows) have detached from the basal cell (ep) and the width of the* lamina lucida *has increased (double headed arrows), however HDs (white arrows) appear intact. Bar = 250 nm. In explants cultured with APMA for 24 h (d) the distance between the* lamina densa *of the BM (black arrow) and the basal cell plasmalemma has increased and the width of the* lamina lucida *(ll) is greater than normal (double headed arrow). HDs are still present in the basal cell plasmalemma (white arrows) but with only remnants of their sub-basal dense plates (black arrowheads). AFs are absent. Bar = 250 nm.*

HD and BM zone analysis by TEM

Small explant sections were examined and photographed using a JEOL 1010 TEM. A length of approximately 200 µm of BM, in each section, was photographed at 15,000 magnification. A digital image mosaic of continuous BM spanning 3 or 4 cells (34 fields) was generated from the photographs using digital image editing software (Adobe Photoshop 6.0)[3]. Using image analysis software (ImagePro Plus)[4] the number of HDs per micrometer of basal cell plasmalemma (Pl), in *lamina densa*, contact, was counted (N_B). The length of each HDs was measured and assuming HDs to be thin flat discs, HD length

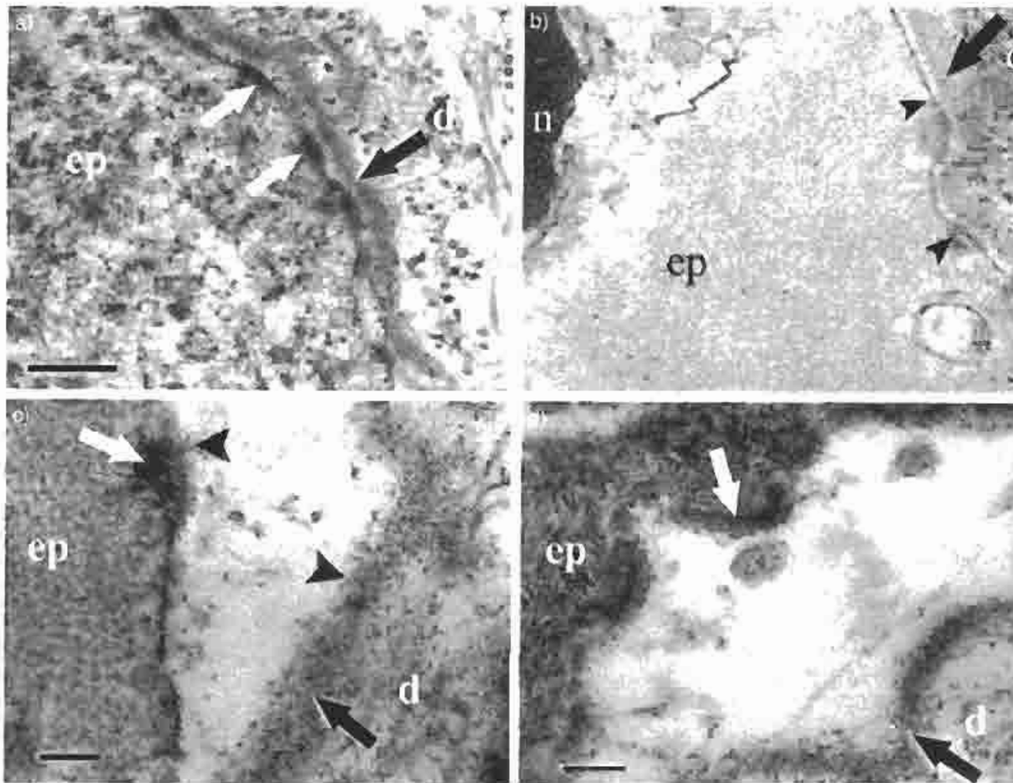

Fig 2: TEMs of the BM zone of lamellar explants before and after tension testing. In explants cultured in DMEM with glucose for 24 h and subjected to tension (a) the lamina densa *(black arrow) retains a normal appearance, as do the HDs (white arrows). The cytoskeleton is uniform within the basal cell (ep) and merges into the dense intracellular plaque of the HDs Bar = 250 nm. In explants cultured without glucose (b) for 24 h the* lamina densa *(black arrow) is visible but the cytoskeleton has disappeared from the amorphous, basal cell cytoplasm, except for a few remnants (white arrow) adjacent to the nucleus (n). HDs in the epidermal cell plasmalemma (black arrowheads) are small and faded. In explants cultured without glucose for 24 h and tensioned (c) the basal cell cytoplasm is without a cytoskeleton and HDs appear disorganised (white arrow). Remnants of AFs (black arrowheads) are present on both the basal cell plasmalemma and the* lamina densa *of the BM. Bar = 100 nm. In explants cultured with APMA and tensioned (d) the basal cell cytoskeleton is unaffected and is connected to HDs (white arrow). In contrast to the explant shown in c), there are no AFs between plasmalemma and the* lamina densa *(black arrow). Bar = 100 nm, dermis = d.*

(B_{HD}) was converted to true HD diameter (HD_T) (Madigan and Holden 1992) by the formula: $HD_T = 4/\pi \times B_{HD}$. The numeric density of HDs per unit surface of basal cell plasmalemma (N_S) was derived by the formula: $N_S = N_B/HD_T + (4/\pi t)$ where t = section thickness = 0.07 μm). The approximate percentage area of *lamina densa* surface in contact with HDs was derived by the formula: $\%\ area_{HD} = N_S \times HD\ area/\mu m^2 \times 100$ where the area of each $HD = \pi(HD_T/2)^2$. The distance from the epidermal cell plasmalemma to the centre of the *lamina densa* of the BM was also measured. The means of each parameter measured were compared between treatments using one way analysis of variance (ANOVA) and *post hoc* Tukey-Kramer Multiple Comparisons Test. Statistical analysis was conducted with computer software (Instat GraphPad 2.02)[5].

Results

Explants cultured in DMEM

Explants cultured in DMEM could not be separated by tension throughout the 48 h experimental period (Table 1) and, when examined by TEM, had normal BM zones, HDs, cytoskeleton and AFs (Fig 1a). The range of distances between the epidermal cell plasmalemma and centre of the *lamina densa* are shown in Table 2. The percentage surface area of HDs in *lamina densa* contact and the number of HDs per micrometer of PI in *lamina densa* contact are shown in Tables 3 and 4, respectively. Explants cultured in normal media for 24 h and subjected to tension also had normal TEM ultrastructure (Fig 2a).

Explants cultured in DMEM without glucose

Explants cultured without glucose first separated under tension at 12 h and all explants could be separated at 24 h (Table 1). By TEM the percentage surface area of HDs in *lamina densa* contact had decreased significantly by 2 h of culture (Table 3). However, the number of HDs in *lamina densa* contact was not significantly decreased until 6 h of culture (Table 4). At 6 and 12 h, HDs had disappeared and the cytoskeleton had withdrawn from the plasmalemma and condensed close to the nucleus (Fig 1b, white arrows). At 24 h the cytoskeleton had disappeared except for a few remnants adjacent to the nucleus (Fig 2b, white arrow). If not tensioned, the distance between the plasmalemma and the *lamina densa* did not increase significantly (Table 2). Explants tensioned and examined by TEM had a normal *lamina densa* that was separated from the basal cell plasmalemma. Remnants of AFs were present around HDs and on the *lamina densa* of the BM (Fig 2c).

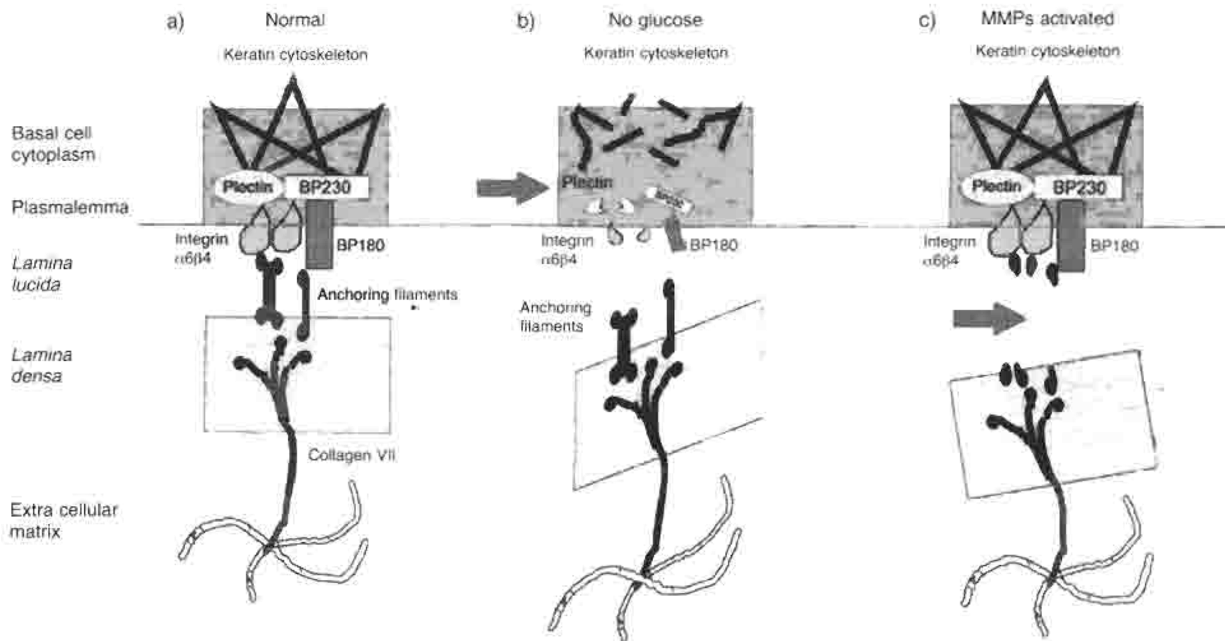

Fig 3. Diagram showing effects of no glucose and MMP activation on the hemidesmosomes of lamellar explants. The major components of the HD intracellular plaque (a) are plectin, integrin 64, BP180 and BP230. Intermediate filaments of the basal cell cytoskeleton insert into the HD thus connecting the cytoskeleton to the cell plasmalemma. Anchoring filaments connect the HD to the lamina densa *of the basement membrane. Without glucose the HD dysassembles (b) causing failure of cytoskeleton insertion into the HD, dysadhesion of anchoring filaments and BM separation under tension. The target for activated MMPs (c) is anchoring filaments and their loss allows wholesale movement of the BM away from the epidermal basal cell plasmalemma leaving HDs unaffected.*

Explants cultured in DMEM and dimethylsulphoxide

Explants were cultured in DMEM + 0.001% dimethylsulphoxide (solvent for APMA) as controls for explant culture with APMA. These explants did not differ significantly from those cultured in DMEM in any parameter (data not shown).

Explants cultured in DMEM, dimethylsulphoxide and APMA

Explants cultured with APMA first separated under tension at 24 h and all explants separated by 48 h (Table 2). However, by TEM, ultrastructural changes occurred by 4 h. The mean distance between the BM and plasmalemma had increased (Table 2) and the detached BM was folded (Fig 1c). By 4 h of culture, the number of HDs in contact with the BM had decreased significantly (Table 4). In-contact HDs occupied significantly less of the *lamina densa* percentage surface area (Table 3) and the distance between the plasmalemma and the center of the *lamina densa* had increased significantly (Table 2). As the period of culture increased, the *lamina densa* detached from the basal cell plasmalemma (Fig 1d) leaving an empty *lamina lucida*. HDs were present in the plasmalemma although their sub-basal dense plates had disintegrated and AFs were absent (Figs 1d and 2d).

Discussion

This TEM study shows that the mechanisms of the 2 *in vitro* models of dermo-epidermal separation differ: there are 2 processes operating on different time scales. When explants are cultured in media without glucose, they separate under tension after 12 h of culture. However, changes can be seen using TEM much earlier. Without glucose, lamellar explant HDs shrink, are in contact with less of the *lamina densa* surface area by 2 h, and decrease significantly in number by 6 h. As the time of culture without glucose increases cytoskeleton intermediate filaments lose their attachments to the intracellular plaque of the HD, collapse internally, and characteristically, condense around a rounded nucleus. The rounding of basal cell nuclei is also characteristic of carbohydrate overload laminitis histopathology (Pollitt 1996) suggesting that failure of glucose availability and cytoskeleton failure may also occur in laminitis induced this way. In fact shrinkage of the cytoskeleton as shown in the present TEM study may represent the 'loss of onchychogenic substance' (presumably cytokeratin) described in the cytoplasm of laminitis affected basal cells by Obel (1948) using light microscopy. Interestingly, immunohistochemical studies of laminitis tissue by Wattle (1998) showed no loss of basal cell cytokeratin suggesting its continued existence in the nonfunctional, weakened configuration shown here.

Glucose is important in maintaining lamellar integrity (Pass *et al.* 1998). Glucose deprivation could cause HD dysassembly directly by disrupting energy-dependent phosphorylation vital for HD assembly and maintenance (Borradori and Sonnenberg 1999). The BM remained in fairly close apposition to the epidermal cell Pl throughout the culture period but separated under tension, presumably because of weak anchoring filament insertions into disintegrating HDs. Defective $\alpha_6\beta_4$ expression, in human blistering skin disorders (Niessen *et al.* 1996; Nievers *et al.* 1999) and targeted removal of the β_4 gene in experimental mice also results in loss of HDs and widespread dermo-epidermal separation (McMillian *et al.* 1998).

MMPs are present in normal hoof tissue and have physiological roles (Mungall *et al.* 1998; Pollitt *et al.* 1998). Virtually the entire lamellar region is nonproliferative (Daradka

and Pollitt 2004) and remodeling of the various cells of the secondary and primary lamellae occurs via controlled MMP activity. Explants cultured with APMA first separated under tension at 24 h. However, ultrastructural changes had occurred by 4 h; the numbers and surface area of HDs in *lamina densa* contact had decreased yet HDs were always present in the plasmalemma, despite separation from the *lamina densa*. Therefore, in contrast to HDs without glucose, HDs resist MMP activation and do not disappear. What does disappear is the AF connection between plasmalemma and *lamina densa*. AF failure has been reported in the inherited disorder junctional epidermolysis bullosa (JEB) of Belgian foals (Spirito *et al.* 2002) and hooves and skin separate at the BM zone as in the hoof explant APMA model.

The results of the present study support the proposal that the *lamina densa* of the lamellar BM can be separated from lamellar basal cells by 2 different mechanisms (Fig 3). Glucose starvation weakens HDs, leads to their disappearance and causes the cytoskeleton of basal cells to collapse (Fig 3b). A similar mechanism may operate in natural cases of laminitis where basal cell uptake of glucose may be compromised e.g. equine Cushing's disease (Couetil 1996), obesity (Johnson 2002), ischaemia and hyperlipaemia (Jeffcott and Field 1985). APMA activation of the MMPs resident in lamellar basal cells appears to destroy AFs (Koshikawa *et al.* 2000) and separates the *lamina densa* without significantly altering HD structure (Fig 3c). Histopathology of carbohydrate overload laminitis (Pollitt 1996; Pollitt and Daradka 1998; French and Pollitt 2004a) shows wholesale dysadhesion of lamellar *lamina densa* from basal cells suggesting that AF destruction by activated MMPs, occurs during the developmental process. In addition, an ultrastructural study of oligofructose induced laminitis shows clear evidence of HD dysassembly, separation of the *lamina densa* and cytoskeleton failure amongst basal cells of the lamellar BM zone (French and Pollitt 2004b). These features of natural laminitis also occur *in vitro* suggesting that both glucose unavailability and MMP activation may be involved in the development of the dermo-epidermal separation that characterises the disease.

Manufacturers' addresses

[1]Gibco BRL, Invitrogen Australia Pty. Ltd., Mt. Waverly, Victoria, Australia.
[2]Sigma, Castle Hill, New South Wales Australia.
[3]Adobe Systems Inc., San Jose, California, USA.
[4]Media Cybernetics, Silver Spring, Maryland, USA.
[5]GraphPad Software Inc., San Diego, California, USA.

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