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## Zymographic analysis of equine laminitis

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**Abstract** To investigate the role of matrix metalloproteinase (MMP) activity in the pathophysiology of equine laminitis, the techniques of *in situ* zymography and quantitative SDS-PAGE zymography were used to analyse the lamellae and plasma and serum of horses with carbohydrate overload-induced laminitis. The gelatinase activity localised within the epidermal lamellae of laminitic hooves did not differ significantly from normal hooves. In laminitis sections there was an increase in vascular gelatinase activity, possibly associated with the perivascular cuffing of polymorphonucleocytes. Both plasma and serum samples from horses developing laminitis showed a rapid increase in the concentration of circulating latent MMP-9, while MMP-2 remained relatively constant. These results support the hypothesis that laminitis histopathology results from an inadequate regulation of gelatinase activity, resulting in selective degradation of basement membrane components, leading to laminitis due to failure of the basement membrane–epidermis attachment.

### Introduction

The characteristic pathological changes in the equine hoof during the onset of laminitis have been well described (Pollitt 1996; Pollitt and Daradka 1998) although their cause is still poorly understood. Currently two theories attempt to explain the observed histopathology. The idea that local ischaemia results from either local shunting via dilated arteriovenous anastomoses or distal limb vasoconstriction (Hood et al. 1993) has been disputed (Trout et al. 1990) and to a large extent disproved by the work of Pollitt and Davies (1998). The hypothesis of Pollitt and Davies (1998) that increased digital blood

flow exposes the lamellar tissues to blood-borne trigger factors has fueled our investigations into the roles of matrix metalloproteinases (MMPs) in equine laminitis (Pass et al. 1998; Pollitt and Daradka 1998; Pollitt et al. 1998).

MMPs are a family of zinc-containing, calcium-dependent, proteolytic enzymes involved in normal physiological remodelling of the extracellular matrix (Birkedal-Hansen 1993). A subgroup of this family, the gelatinases (Matrisian 1992), gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane-bound metalloproteinase-1 (MT1-MMP), have been shown to be capable of degradation of basement membrane (BM) components (Woessner 1991; d'Ortho et al. 1998). Recently, Pollitt and Daradka (1998) reported progressive destruction of BM laminin, collagen IV and collagen VII after the development of laminitis. The BM degradation was attributed to the action of local gelatinases although numerous polymorphonucleocytes (PMNs) surrounded the areas of BM disintegration.

Gelatinases are capable of degrading critical BM components including type IV (Olson et al. 1998) and type V (Morodomi et al. 1992) non-fibrillar collagens, type VII collagen (Seltzer et al. 1989), fibronectin (Collier et al. 1988), type X collagen (Welgus et al. 1990) and elastin (Senior et al. 1991).

To clarify whether the gelatinase activity responsible for hoof BM destruction resulted when PMNs infiltrated the lamellar tissues or was due to the activation of constitutive gelatinases located within the lamellae, we examined the cellular location of gelatinases using *in situ* zymography (Mungall et al. 1998). Additionally, we analysed the plasma and serum gelatinase profiles of normal horses and of horses during the development of carbohydrate-induced laminitis.

### Materials and methods

Hooves from normal horses were obtained from a commercial knackery (Meramist, Caboolture, Australia) and transported on ice to the dissection room within 60 min, where they were dissected to obtain pieces of tissue approximately 6 by 6 mm extending

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from the inner hoof wall through the lamellar junction to the dermal connective tissue. Tissues were rapidly frozen by immersion in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until sectioned. Laminitis hoof tissue, graded using the scoring system of Pollitt (1996), was obtained from laminitis induction experiments utilising a carbohydrate overload model (Pollitt and Davies 1998) and stored at  $-70^{\circ}\text{C}$  until sectioned. Induction experiments were conducted according to the guidelines approved by the University of Queensland Animal Experimentation Ethics Committee. The Animal Welfare Officer inspected all horses under experimentation.

#### In situ zymography

In situ zymography was performed as described previously (Mungall et al. 1998). Briefly, 10- $\mu\text{m}$  serial frozen sections were mounted on untreated glass slides, equilibrated to room temperature and dipped in autoradiography emulsion as per the manufacturer's instructions (EM-1, Amersham International, Buckinghamshire, UK). Slides were dipped in emulsion containing 10 mM calcium chloride in addition to either: (1) 0.7 mM *p*-aminophenylmercuric acetate (APMA, an organomercurial MMP activator; Sigma, St. Louis, Mo., USA) dissolved in dimethyl sulphoxide (DMSO; Sigma), (2) 0.7 mM APMA and 100  $\mu\text{M}$  batimastat (a specific MMP inhibitor; British Biotech, Oxford, UK) dissolved in DMSO, (3) 5% DMSO and 100  $\mu\text{M}$  batimastat or (4) 1 mM EDTA. Coated slides were incubated in a humidity chamber at  $37^{\circ}\text{C}$  for up to 18 h then developed as per control slides. Control slides were developed immediately using D-19 developer (Eastman Kodak, Rochester, USA) and fixed with a 30% w/v solution of sodium thiosulphate (Sigma) in distilled water, as per the manufacturer's instructions. All sections were counterstained with Mayer's haematoxylin (Sigma), dehydrated using ascending alcohol solutions, cleared with xylene and coverslipped using Depex mounting media (Gurr, Poole, UK). Slides were examined with an Olympus BX50 microscope and images captured and stored on a Pentium II PC (Scientific Instruments and Optical Supplies, Normanby, Australia) via a JVC 3-CCD digital camera (JVC, Yokohama, Japan) utilizing a Flashbus MV Pro frame grabber card (Integral Technologies, Indianapolis, Ind., USA) and analysed using ImagePro software (Media Cybernetics, Silver Spring, USA). Serial sections (10  $\mu\text{m}$ ) were mounted on untreated glass slides and postfixed in 1.6% formaldehyde for 10 min. BM was demonstrated histochemically using the periodic acid-Schiff (PAS) method, while cell morphology was visualised by haematoxylin and eosin (Sigma) staining.

#### White blood cell zymography

Whole blood was collected from normal horses in EDTA tubes and centrifuged at 2000 rpm for 10 min. The buffy coat containing white blood cells was removed and smeared onto untreated glass slides. Control slides were air dried and stained with an Accustain Automated Wright Stain kit (Sigma) using an Ames Hema-Tek I 1000 series automated slide stainer (Ames, Elkhart, Ind., USA). Slides for zymography were air dried and dipped in autoradiography emulsion as per the manufacturer's instructions (EM-1; Amersham International) containing 10 mM calcium chloride alone (control slides) or in addition to APMA (treatment slides). Coated slides were incubated as for frozen sections for up to 18 h then developed as per control slides (as for frozen sections, see above). All sections were counterstained with Mayer's haematoxylin, dehydrated, cleared and mounted with Depex as for frozen sections (see above). Slides were examined as for in situ zymography sections.

#### SDS-PAGE zymography

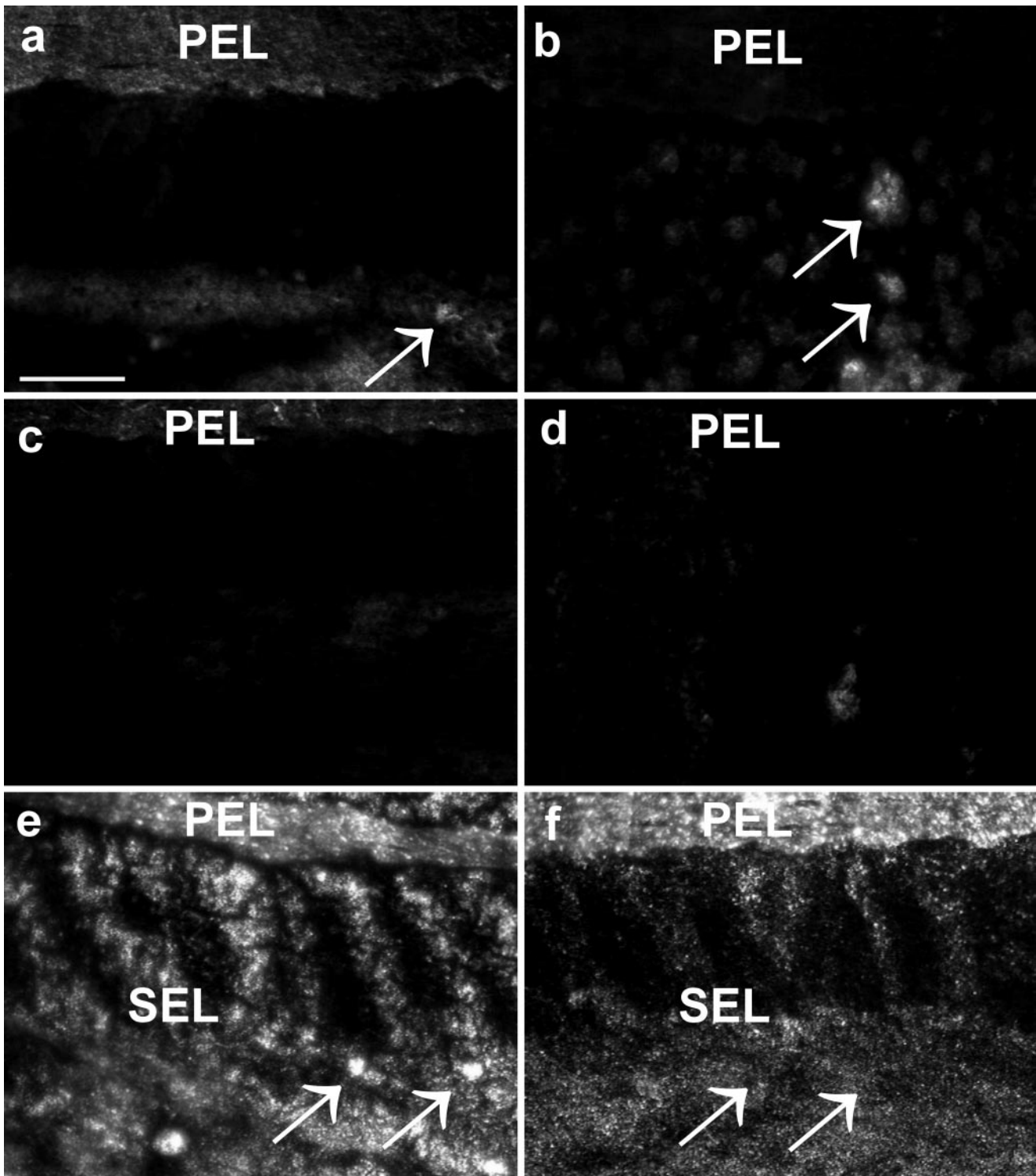
Plasma and serum samples were obtained at 8-h intervals during the induction of laminitis using the carbohydrate overload model

(Pollitt and Davies 1998). Samples were stored at  $-70^{\circ}\text{C}$  until assayed ( $n=4$ ). Control plasma and serum samples were obtained from physiologically normal horses and stored at  $-70^{\circ}\text{C}$  until assayed ( $n=5$ ). Samples were diluted 1:25 in electrophoresis buffer (25 mM TRIS-HCL, 200 mM glycine, 0.1% SDS), mixed with an equal volume of sample buffer (50 mM TRIS-HCL, 2% SDS, 20% glycerol, 0.01% bromophenol blue) and 5- $\mu\text{l}$  aliquots were electrophoresed for 90 min with 40 mA current at  $10^{\circ}\text{C}$  on non-reducing 7.5–15% gradient polyacrylamide gels containing 0.1% gelatin as described previously (Pollitt et al. 1998). After 90 min gels were washed in 2.5% Triton X-100 (Sigma) for  $2\times 30$  min then incubated for 18 h at  $37^{\circ}\text{C}$  in incubation buffer: 50 mM TRIS-HCL, 5 mM calcium chloride and 0.02% sodium azide. Gels were then stained for 30 min with Coomassie blue G-250 (LKB, Villeneuve-la-Garenne, France), destained with 5% acetic acid and 2% glycerol in water (24 h), dried using a Biorad (Hercules, Calif., USA) gel dryer and scanned using an HP Scanjet 3 C (Hewlett Packard) into densitometry analysis software (GelPro; Media Cybernetics). Standard curves were constructed using recombinant human MMP-2 and MMP-9 standards (Oncogene Research Products, Cambridge, Mass., USA). Measurements were averaged from four experiments and statistical analysis performed using a repeated measures analysis of variance procedure (Dunnett's test).

## Results

#### In situ zymography

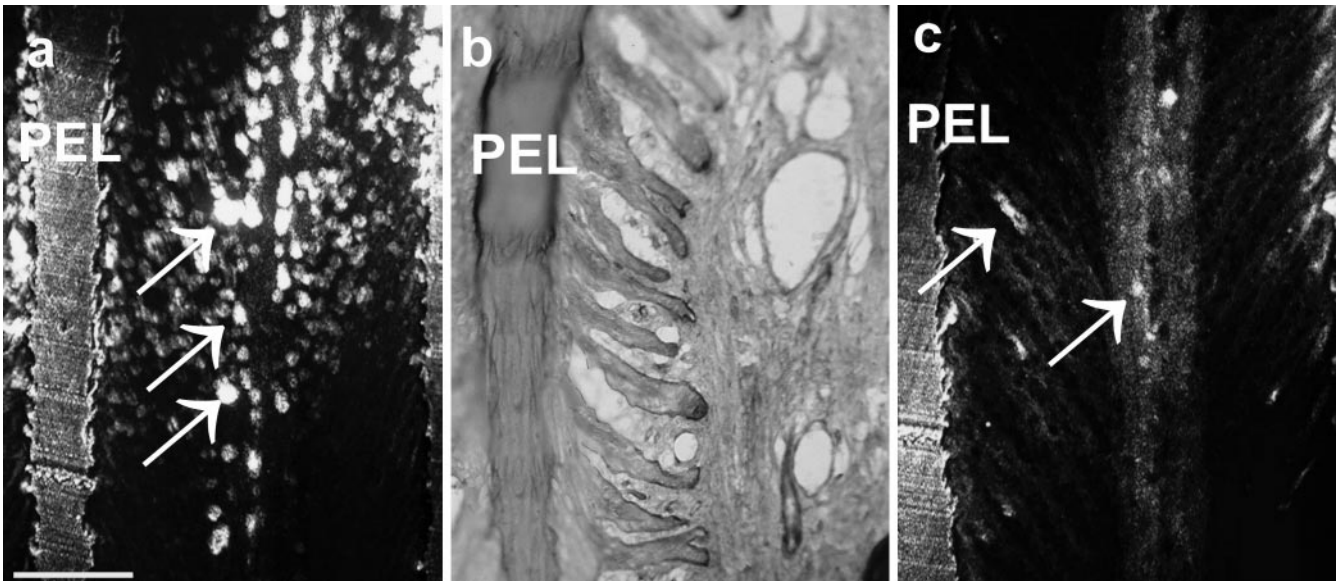
Clear areas of the emulsion overlay were interpreted to represent areas of gelatinase activity while dark areas represent areas where gelatinase activity was absent. Control sections from both normal (Fig. 1a) and laminitis-positive horses (Fig. 1b) incubated with emulsion containing only calcium chloride produced minimal digestion of the emulsion overlay after 18 h incubation (Fig. 1a,b). This digestion was completely abolished with the addition of batimastat to the emulsion indicating specific MMP activity (Fig. 1c,d; normal and laminitis-positive, respectively). Addition of the MMP activator, APMA, to the emulsion resulted in the normal hoof sections digesting the emulsion predominantly throughout the primary epidermal lamellae (PEL) and secondary epidermal lamellae, although circumscribed areas of digestion were observed overlying the vasculature in the dermis (Fig. 1e). Addition of batimastat to the APMA-treated emulsion inhibited the majority of emulsion digestion, although there was still some digestion over the PEL in addition to a low level of digestion throughout the dermis (Fig. 1f). This residual digestion was completely abolished by the addition of 1 mM EDTA (data not shown). Addition of APMA to the emulsion overlying laminitis hoof tissue revealed a similar digestion pattern to the normal hoof with the notable exception of significantly more digestion associated with dermal areas (Fig. 2a). For structural reference, a corresponding PAS section of the laminitis hoof is included (Fig. 2b). Batimastat again inhibited the majority of the digestion observed by APMA activation in both the dermis and epidermis, although some dermal areas still actively degraded the emulsion overlay (Fig. 2c). This remaining digestion was inhibited by the addition of 1 mM EDTA (data not shown).



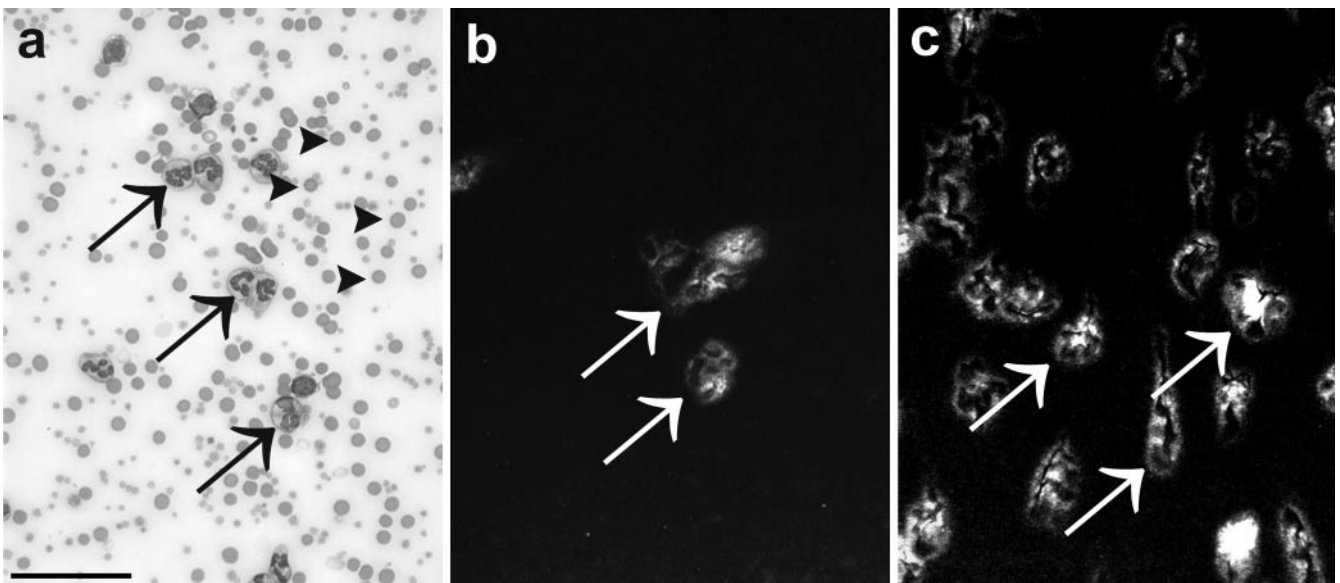
**Fig. 1** In situ zymography of normal (a,c,e,f) and laminitis (b,d) sections incubated in emulsion containing calcium only (a,b) and emulsion plus batimastat (c,d). Normal sections incubated with emulsion containing *p*-aminophenylmercuric acetate (APMA; e) and emulsion containing APMA and batimastat (f). Bar 50  $\mu$ m

#### White blood cell zymography

Control slides revealed multiple PMNs amongst a background of erythrocytes (Fig. 3a). Zymographic incubation for 6 h in the presence of calcium revealed discrete digestion of the emulsion over the PMN cell body (Fig. 3b), while the addition of APMA to the emulsion produced enhanced digestion of the emulsion overlying the PMN cell bodies (Fig. 3c).



**Fig. 2a-c** In situ zymography of laminitis sections. **a** Section incubated in emulsion containing APMA. **b** Corresponding section stained using the periodic acid-Schiff method. **c** Laminitis section incubated with emulsion containing both APMA and batimastat. Bar 100  $\mu$ m



**Fig. 3a-c** In situ zymography of white blood cell smears. **a** Control white blood cell smear, Wright stained. **b** Smear incubated in emulsion containing only calcium. **c** Smear incubated in emulsion containing APMA. Bar 20  $\mu$ m

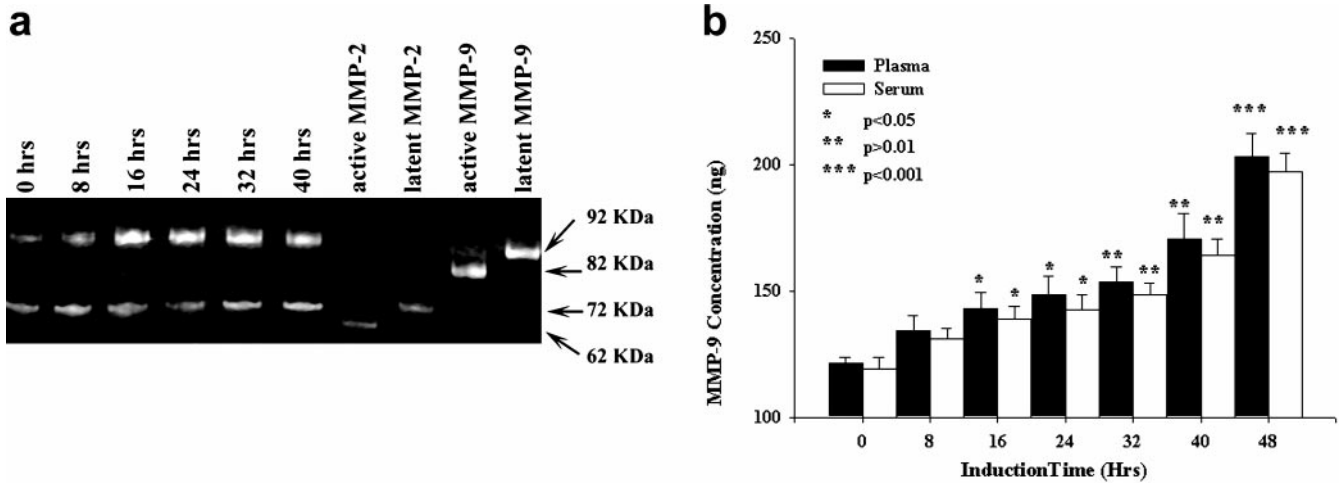
centration of MMP-9 rose immediately (within the first 8 h) and increased continually over the 48-h induction period where levels approached double the normal physiological concentration in horses (Fig. 4b).

#### SDS-PAGE zymography

A representative zymogram with serum samples from a laminitis induction experiment showed the rapid increase in latent MMP-9 (92 kDa) while latent MMP-2 remained relatively stable (72 kDa; Fig. 4a). Both plasma and serum concentrations of MMP-2 and MMP-9 followed a similar trend with MMP-2 remaining relatively stable throughout the 48-h induction period with no significant variation from normal values (data not shown). The con-

#### Discussion

While a broad range of clinical conditions have been linked to the pathophysiology of laminitis (Field and Jeffcott 1989; Baxter 1994) leading to a characteristic histopathological appearance (Pollitt 1996), no specific aetiological mechanism has been elucidated. Currently our working hypothesis is that MMPs, specifically gelatinases, play a key role in the selective degradation of



**Fig. 4a,b** SDS-PAGE zymography of plasma and serum samples over the course of laminitis induction. **a** Representative zymogram of serum samples obtained preinduction (0 h), then at 8-h intervals during the induction phase. Pro- and active-MMP standards are on the *right* with their apparent molecular weight indicated for each. **b** Plasma and serum MMP-9 changes during laminitis induction, quantitatively determined densitometrically from zymograms ( $n=4$ ). Each column represents the mean MMP-9 concentration ( $\pm$ SEM). Statistical significance is indicated as shown

BM proteins, possibly within hemidesmosome structural elements, leading to a failure of the BM-epidermal attachment apparatus. Recently, Pollitt and Daradka (1998) showed that progressive degradation of collagens IV and VII in addition to laminin, all major structural components of the BM (Leblond and Inoue 1989; Aumailley 1995) occurred in laminitic hooves. Additionally, Pollitt et al. (1998) and Johnson et al. (1998) have reported increases in gelatinase activity in hoof lamellar homogenates from laminitic horses. Recently, we demonstrated the presence of gelatinases within the hoof dermal-epidermal structure of normal horses (Mungall et al. 1998), although their state of activation appeared to be relatively low under normal physiological conditions. While MMP-9 has been localised to epidermal lamellar cells, definitive identification of which gelatinases are present is still under investigation.

In the current study, we examined sections from laminitic hooves using in situ zymography to localise the gelatinase activity present within the hoof lamellae. There was little difference in the quantity of gelatinase present between these and normal hooves. These findings confirm our initial hypothesis that, rather than a local increase in the quantity of gelatinase in the lamellae, the most likely scenario involves a gelatinase or gelatinase activator arriving at the lamellae via the circulation (Pollitt 1996; Mungall et al. 1998; Pollitt et al. 1998). The observation of Pollitt et al. (1998) that MMP-9 is increased in hoof lamellar homogenates may be explained by an influx of MMP-9 via the circulation, either as free enzyme or carried by PMNs. As was seen in our previous study (Mungall et al. 1998), the level of gelatinase activity in untreated tissue is very low, making detection

by in situ zymography difficult. Tissue levels of gelatinase are probably tightly regulated by endogenous inhibitors such that visualisation demands prior enzyme activation (this is achieved in the current study by pretreatment with APMA). Definitive quantitation of absolute levels of gelatinase activation occurring during the development of laminitis are beyond the limitations of in situ zymography.

The observation of emulsion digestion overlying the dermis was compared to white blood cell zymography in an attempt to identify possible sources of this activity. The volumetric area of emulsion digestion attributable to PMNs observed with zymography of white blood cell smears (Fig. 3c), correlates well with many of the digestion "spots" overlying the dermis with in situ zymography of laminitis sections (Fig. 2a). This suggests that much of this dermal gelatinase activity may be directly attributable to an influx of PMNs in laminitis. The parallel assay of plasma and serum from horses during the development of carbohydrate-induced laminitis in the current study provided confirmation of this phenomena, in that circulating gelatinase-B (MMP-9) levels rose as early as 8 h following ingestion of the carbohydrate.

In summary, we have demonstrated subtle differences in the gelatinase activity between normal and laminitic equine hooves, utilising the techniques of in situ zymography and SDS-PAGE zymography. We show for the first time, a rapid and sustained increase in the circulating concentration of latent MMP-9 as a result of carbohydrate-induced laminitis. The results presented here add support to the contention that laminitis histopathology is a result of inadequate control of local gelatinase activation within the hoof lamellae. Latent gelatinase may be derived locally or arrive systemically, possibly via PMNs, and after local activation, we postulate that this gelatinase activity degrades specific BM components, probably within the hemidesmosomal structure, resulting in detachment of the BM from the epidermis.

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