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## Localisation of gelatinase activity in epidermal hoof lamellae by in situ zymography

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**Abstract** In situ gelatin zymography is a technique, which utilises a gelatin-based emulsion overlay to detect and, more importantly, localise the gelatinase activity in underlying tissue. Gelatinase A [matrix metalloproteinase-2 (MMP-2)] and gelatinase B [matrix metalloproteinase-9 (MMP-9)] are present in equine hoof homogenates and supernatants from cultured hoof explants by SDS-PAGE gelatin zymography, and it has been assumed that the enzymes are derived solely from matrix and epithelia and not from other sources such as leucocytes. Using in situ zymography, gelatinases are shown to be localised within the equine epidermal hoof lamellae and, more specifically, are apparently produced by epidermal basal and/or parabasal cells. The pattern of expression correlates with that expected based on the progression of pathological changes observed during the onset of laminitis, thus providing further evidence that laminitis pathology probably arises as a result of inadequate local MMP regulation.

### Introduction

SDS-PAGE zymography is an established research technique for the routine detection of gelatinase activity even at low picogram concentrations (Kleiner and Stetler-Stevenson 1994; Leber and Balkwill 1997). Zymographic techniques were considered to be qualitative only, providing reliable identification of gelatinases based on the molecular mass of precursor matrix metalloproteinase (pro-MMP) and active forms (Adler et al. 1990). More

recently, attention has focused on standardising the technique to allow direct quantitation of samples, initially using conditioned media (Kato et al. 1992; Brown et al. 1993; Davies et al. 1993) and more recently utilising pure matrix metalloproteinase (MMP) preparations (Kleiner and Stetler-Stevenson 1994; Leber and Balkwill 1997). While electrophoresis-based zymography provides useful information regarding quantitation of gelatinase activity, it provides little information regarding the cellular source of these enzymes.

Immunocytochemistry has been widely used to localise both MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in many tissues but it is time consuming and uses expensive antisera. A major problem with immunolabelling techniques is that antisera often have limited species cross-reactivity requiring the production of species-specific antisera. In situ gelatin zymography is a relatively low-cost technique requiring only a gelatin-based emulsion available commercially for autoradiographic studies (Galis et al. 1994, 1995; Pardo et al. 1996). While this technique has limitations regarding quantitation, it can be utilised effectively for localisation of gelatinase activity at a cellular level. Specifically, in situ zymography enables localisation of gelatinase activity, whereas immunohistochemical techniques only localise MMP proteins and thus both their active and inactive forms (pro-forms and molecules bound to inhibitors). Reported here, for the first time, is the distribution pattern of gelatinase activity in equine hoof lamellae.

### Materials and methods

#### Histochemistry

All hoof tissue was obtained from a commercial knacker (Meramist, Caboolture, Australia) and transported on ice to the dissection room within 60 min, where hooves were dissected to obtain pieces of tissue approximately 6 by 6 mm extending 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. Serial sections, 10  $\mu\text{m}$  thick, were mounted on untreated glass slides and postfixed in

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1.6% formaldehyde for 10 min. Basement membrane (BM) was demonstrated histochemically using the periodic acid Schiff (PAS) method while MMP-9 was detected immuno-histochemically as follows. Endogenous peroxidase was blocked by incubating in 1% hydrogen peroxide in methanol for 10 min. Sections were washed with 0.1 M phosphate-buffered saline (PBS, Sigma, St. Louis, USA) twice for 5 min each then blocked with 10% sheep serum in PBS followed by incubation with primary antisera for 4 h at room temperature. Rabbit anti-MMP-9 (Biogenesis, Poole, UK) was diluted 1:100 in 0.1 M PBS. Tissues were washed twice for 5 min each in PBS and then biotinylated sheep anti-rabbit secondary antisera (Dako, Glostrup, Denmark), diluted 1:200 with PBS, was applied for 30 min at room temperature. After washing again with PBS (2×5 min) a streptavidin-linked horseradish peroxidase conjugate (Dako) was applied for 30 min at room temperature then developed using 3,3'-diaminobenzidine substrate (Sigma). All sections were counterstained with Mayer's haematoxylin (Sigma), dehydrated using ascending alcohol solutions, cleared with xylene and coverslipped using Depex mounting media (Gurr, BDH, Poole, UK). Negative control sections were incubated with antisera (1:100 in 0.1 M PBS) which had been preincubated with 1 ng/ml pure MMP-9 (Oncogene Research Products, Cambridge, USA) for 30 min at room temperature.

#### In situ gelatin zymography

Serial sections, 10 µm thick, were mounted on untreated glass slides, equilibrated to room temperature and dipped in autoradiography emulsion as per the manufacturers instructions (EM-1; Amersham International, Buckinghamshire, UK). All slides were dipped in emulsion containing 10 mM calcium chloride in addition to one of four additives: 0.7 mM of the organomercurial MMP activator *p*-aminophenyl mercuric acetate (APMA; Sigma) dissolved in dimethyl sulphoxide (DMSO; Sigma), 0.7 mM APMA and the specific MMP inhibitor Batimastat (100 µM, British Biotechnology, Oxford, UK) dissolved in DMSO, 5% DMSO or 100 µM Batimastat. Once dipped, the emulsion-coated slides were allowed to gel at 4°C for 60 min prior to incubation in a humidity chamber at 37°C for up to 18 h. 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 manufacturers instructions. Treatment slides were developed after 4, 6, 8, 10, 12 or 18 h incubation. Once developed, slides were counterstained, dehydrated, cleared and coverslipped as for histochemistry preparations. 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) utilising a Flashbus MV Pro frame grabber card (Integral Technologies, Indianapolis, USA) and analysed using ImagePro software (Media Cybernetics, Silver Spring, USA).

## Results

### Histochemistry

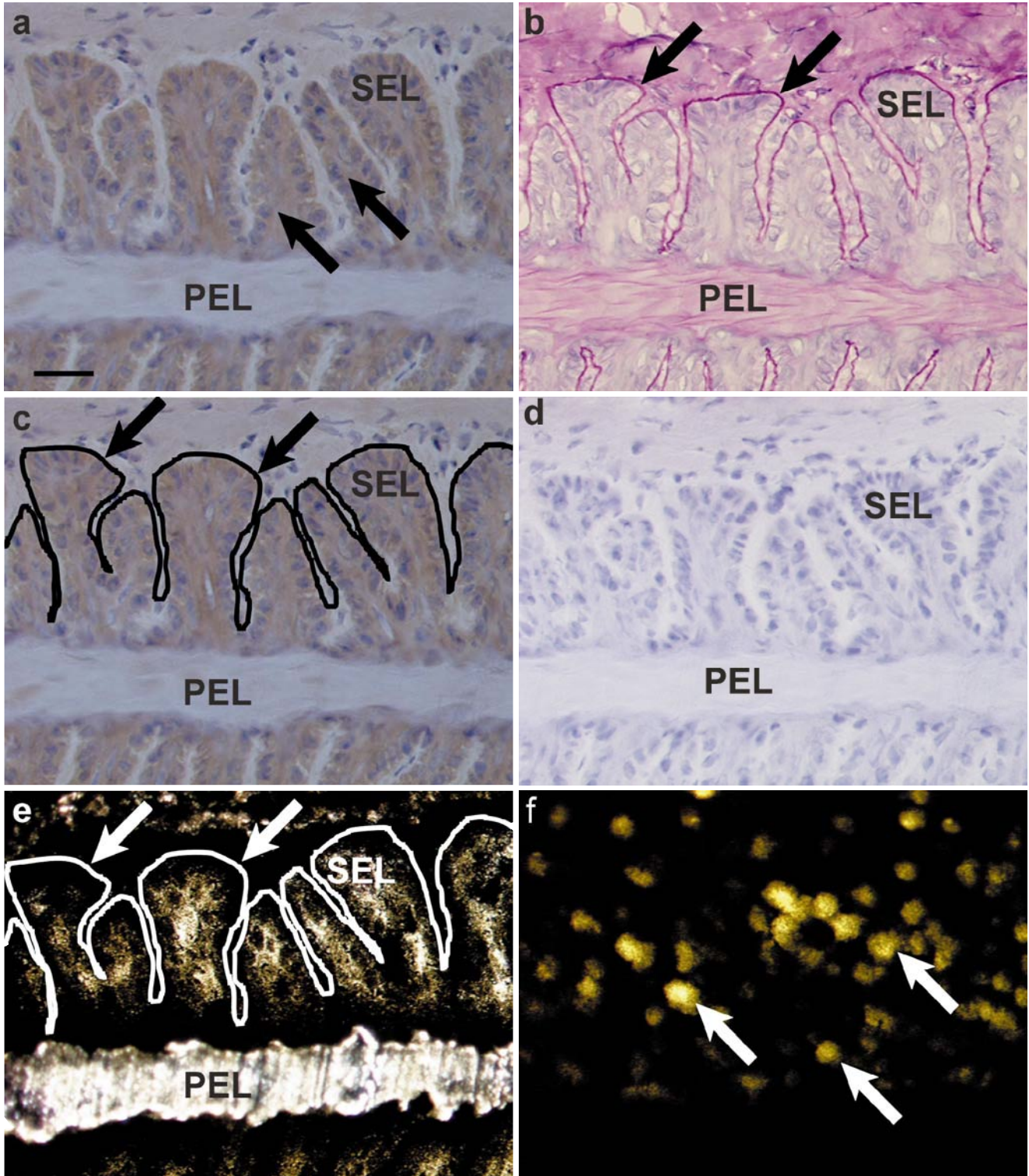
Sections stained immunohistochemically for MMP-9 showed a clear distinction between dermal and epidermal tissues, with the enzyme being distributed exclusively throughout the secondary epidermal lamellae (SEL; Fig. 1a). A computer-generated image of the lamellar BM (derived from PAS staining; Fig. 1b), superimposed onto the image of the MMP-9-stained serial section confirmed that MMP-9 immunoreactivity was confined to SEL (Fig. 1c). Control sections incubated with preab-

sorbed MMP-9 antisera showed no immunoreactivity (Fig. 1d).

### In situ zymography

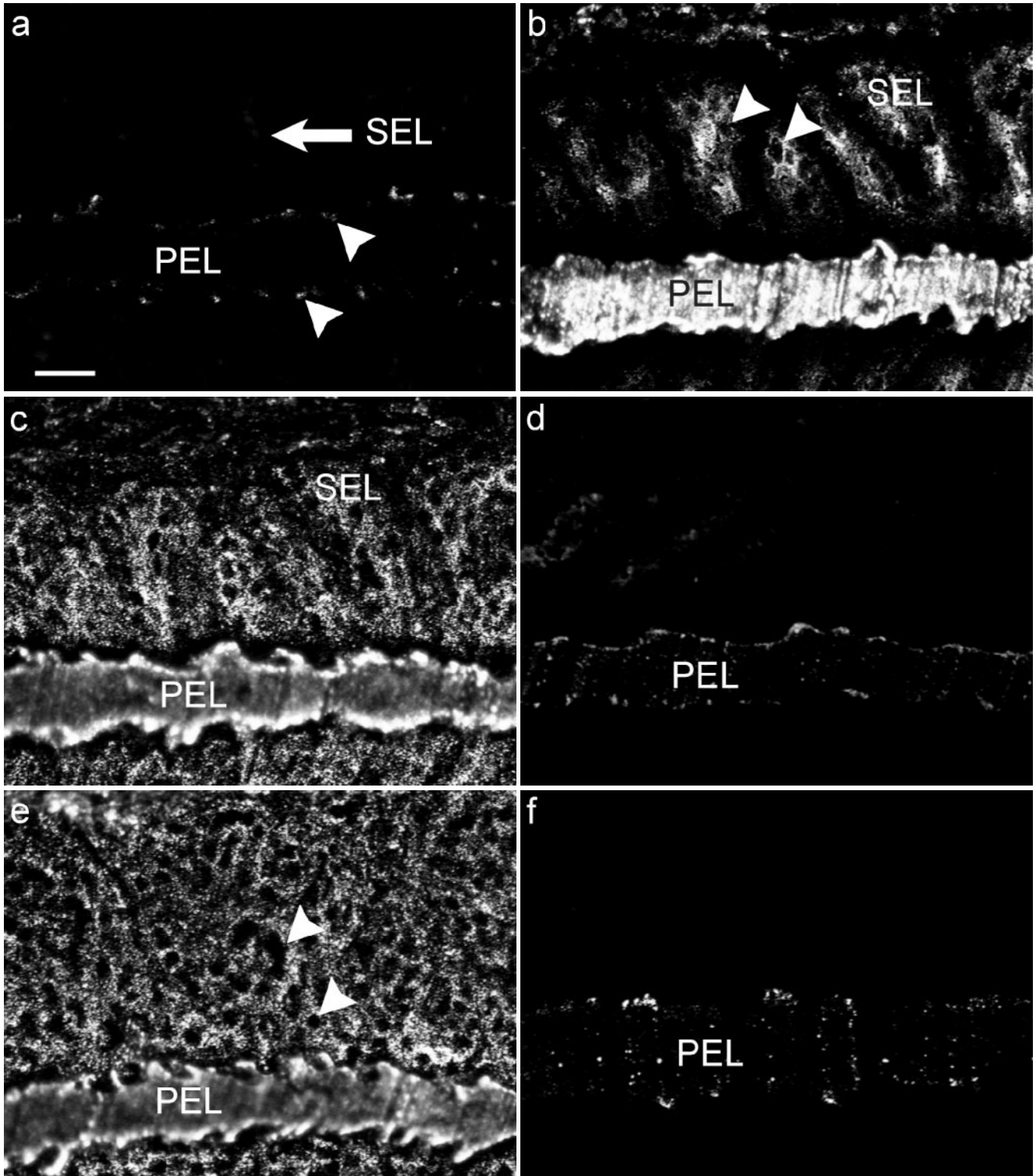
Clear areas of the emulsion overlay were interpreted as representing areas of gelatinase activity while dark areas represent areas where gelatinase activity is absent. Control sections incubated with emulsion containing only calcium chloride produced minimal digestion of the emulsion overlay after 6–8 h incubation and displayed only diffuse, non-specific degradation when developed after 18 h incubation (Fig. 1f). Sections incubated with emulsion to which APMA had been added showed a rapid and continuous pattern of emulsion digestion. During the first 6 h of incubation, points of emulsion digestion first appeared directly above the primary epidermal lamellae (PEL), with faint digestion apparent over SEL (Fig. 2a). After 8 h incubation, the nuclei of the epidermal basal cells (Fig. 2b) were still covered by dark areas of undigested gelatin overlay, contrasting against the lighter cytosolic portions of the SEL where digestion of the emulsion was already underway (Fig. 2b). The PEL had completely digested the overlying emulsion. For comparison, this same section is shown with the computer-generated BM superimposed (Fig. 1e), indicating well-localised digestion over the SEL. With prolonged incubation, emulsion digestion became less specific, showing degradation over both epidermal and dermal tissues. After 10 h incubation, the SEL were barely discernible as lighter segments amongst a background of darker nuclei (both epidermal and dermal) showing little difference in the extent of emulsion digestion between dermal and epidermal tissues (Fig. 2c).

The addition of Batimastat, a potent inhibitor of MMP-2 and MMP-9, to the emulsion effectively prevented the specific epidermal digestion pattern seen with APMA-treated sections. A 10-h incubation with emulsion containing both APMA and Batimastat (Fig. 2d) revealed less emulsion digestion than a 6-h incubation in the presence of APMA alone (Fig. 2a). Continued incubation to 18 h revealed a relatively non-specific digestion pattern in which SEL cannot be distinguished from the surrounding dermal tissue (Fig. 2e). Cell nuclei were visible as darker shadows amidst a partially digested, and thus, lighter emulsion overlay (Fig. 2e). Additionally, sections incubated for 18 h with emulsion containing Batimastat without APMA (Fig. 2f), produced a digestion pattern similar to a 6-h incubation in the presence of APMA (Fig. 2a), or a 10-h incubation with emulsion containing both APMA and Batimastat (Fig. 2d). Note also, that incubating for 18 h with emulsion containing Batimastat (Fig. 2f) revealed less digestion than control sections incubated with emulsion containing only calcium chloride (Fig. 1f).



**Fig. 1a-f** Serial sections of the dermal-epidermal junction of equine hoof. **a** Gelatinase B [matrix metalloproteinase-9 (MMP-9)] immunoreactivity (*arrows*) exclusively in secondary epidermal lamellae (*SEL*) with no immunoreactivity observed in the primary epidermal lamellae (*PEL*). **b** Basement membrane (BM; *arrows*) identified by periodic acid Schiff staining. **c** BM superimposed (*arrows*) on MMP-9 immunoreactivity confirming MMP-9 local-

isation is confined to SEL. **d** Control serial section incubated with pre-absorbed MMP-9 antisera. **e** Serial in situ zymography section with the BM superimposed (*arrows*) reveals discrete localisation of gelatinase activity within SEL. **f** In situ zymography incubated for 18 h without activation by *p*-aminophenyl mercuric acetate (APMA) revealing non-specific digestion (*arrows*). Bar 50  $\mu$ m



**Fig. 2a** In situ zymography of serial sections of equine hoof tissue incubated with emulsion containing 0.7 mM APMA for 6 h reveals points of digestion (*arrowheads*) over the *PEL* and faint digestion over the *SEL* (*arrow*). **b** Serial section after 8 h incubation revealing discrete digestion over epidermal basal cells, recognisable by the pattern of their dark nuclei (*arrowheads*). **c** Ten-hour incubation shows a less specific digestion pattern, with dark nuclei in both dermal and epidermal tissues. **d** Incubation with emulsion

containing 0.7 mM APMA and 100  $\mu$ M Batimastat revealed less digestion after 10 h than after 6 h digestion with APMA (**a**). **e** Continued incubation in the presence of APMA and Batimastat up to 18 h revealed a non-specific digestion pattern where *SEL* could not be distinguished from the surrounding dermal tissue, despite the shadowing effect of cell nuclei (*arrowheads*). **f** Incubation in the presence of 100  $\mu$ M Batimastat alone for 18 h prevented most of the digestion seen in APMA-treated sections. *Bar* 50  $\mu$ m

## Discussion

The gelatinases are a subgroup of the family of MMPs characterised by their substrate specificity (Matrisian 1992). These enzymes, named for their ability to degrade denatured collagens (gelatin), are capable of degrading type IV collagen, a major structural component of basement membranes. At present there are two MMPs identified as gelatinases, gelatinase A (MMP-2) and gelatinase B (MMP-9), while a third MMP, membrane-type 1 MMP (MT1-MMP) has recently been shown to focally degrade gelatin films (d'Ortho et al. 1998). Under normal physiological conditions, these enzymes are tightly regulated by tissue inhibitors of metalloproteinases (TIMPs; Woessner 1991). MMPs are becoming increasingly implicated in normal tissue remodelling processes (Matrisian 1992; Salo et al. 1994; Birkedal-Hansen 1995; Kahari and Saarialho-Kere 1997) in addition to a rapidly growing repertoire of pathological conditions, particularly cancer metastasis (Stetler-Stevenson 1990, 1996; Stetler-Stevenson et al. 1996) and bullous skin diseases (for review see Kahari and Saarialho-Kere 1997). MMPs are secreted as inactive proenzymes but can be readily activated by organomercurial compounds such as APMA (Ries and Petrides 1995). The lack of gelatin digestion by sections incubated with emulsion containing only calcium chloride, indicates that the gelatinases are relatively inactive in the normal hoof. Activation of these enzymes, by inclusion of APMA in the emulsion overlay, provides direct evidence that gelatinases are present in significant quantities in equine lamellar hoof.

While expression of MMP-2 has been demonstrated in several cell types *in vitro* (Kahari and Saarialho-Kere 1997) and *in vivo* (Matrisian 1992) including endothelial cells (Kalebic et al. 1983), MMP-9 appears to be more common to epithelial cells (Salo et al. 1991), neutrophils (Hibbs et al. 1985) and eosinophils (Kahari and Saarialho-Kere 1997). The observation here of MMP-9 selectively immunostaining epidermal tissue is consistent with a large body of evidence supporting MMP-9 production by keratinocytes (Salo et al. 1994; Putnins et al. 1995; Borchers et al. 1997). Additionally, the pattern of MMP-9 immunoreactivity is completely consistent with the observed pattern of emulsion digestion in the current study, with the obvious exception of the PEL which were devoid of MMP-9 immunoreactivity. Unfortunately, the technique of *in situ* gelatin zymography is unable to distinguish between different gelatinases, and without subsequent immunolocalisation and identification of each individual gelatinase, their identity remains speculative. MMP-9, MMP-2 and MT1-MMP may all play some part in the emulsion digestion observed here but the physiological roles of each remain to be elucidated. Since the PEL are the first and the most efficient tissue to degrade the emulsion overlay, the presence of PEL gelatinase is not in dispute. The lack of detectable MMP-9 immunoreactivity in the PEL may indicate that the cornified nature of the PEL and the adjacent inner hoof wall prevents immunolocalisation. MMP-9 may be present in the PEL

and inner hoof wall but, due to keratinisation, may be antigenically unavailable for immunodetection. Alternatively, MMP-9 may be exclusively produced by basal and/or parabasal cells of the equine hoof lamellae, as has been shown in the lower epidermis of the skin (Oikarinen et al. 1993) by mRNA expression studies. MMPs in skin are not constitutively expressed, with the exception of neutrophil collagenase (Hasty et al. 1990), MMP-9 in neutrophils (Stahle-Backdahl and Parks 1993) and MMP-7 in sweat glands (Saarialho-Kere et al. 1995). Most MMPs in skin appear to be induced temporarily in response to some exogenous event (Saarialho-Kere et al. 1993; Heino 1996; Kahari and Saarialho-Kere 1997). The pattern of MMP production in hoof lamellae has yet to be elucidated.

The extensive presence of MMP-9 in the SEL of normal equine hoof tissue may represent a vastly different regulatory system from that seen in normal skin. In contrast to the predominant pattern of minimal MMP secretion under normal physiological conditions in other species (Woessner 1991), there may be a need for high levels of gelatinase production at the dermal/epidermal junction in the hoof to allow for the extensive extracellular matrix remodelling necessary for continued hoof growth and weight bearing. The current observations of a relatively rapid digestion pattern in the presence of APMA and its subsequent inhibition by Batimastat indicates a potentially high basal rate of gelatinase production. It should be noted however, that although Batimastat is not a specific gelatinase inhibitor, it is unlikely that the high rate and extent of digestion seen here is attributable to other MMPs, such as the collagenases, with less specific gelatinase activities. Additionally, while the parallel localisation of MMP-9 presented here appears to account for most of the observed phenomena, the possibility of involvement by other gelatinases cannot be discounted.

Upregulation of gelatinase activity in the hoof lamellar epidermis appears to account for the anatomical separation of epidermis from dermis, which is a feature of the histopathology of equine laminitis (Pollitt 1996; Johnson et al. 1998; Pollitt and Daradka 1998; Pollitt et al. 1998). This upregulation may simply be a result of the balance between gelatinase and TIMP production being tipped in favour of gelatinases. The localisation of high levels of gelatinase activity to within the SEL compartment, adjacent to the lamellar BM in normal, healthy equine hoof tissue, adds support to this contention.

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