

Batimastat (BB-94) inhibits matrix metalloproteinases of equine laminitis

C. C. POLLITT, M. A. PASS* and S. POLLITT

*Companion Animal Clinical Sciences, School of Veterinary Science and Animal Production and *Department of Physiology and Pharmacology, The University of Queensland, Brisbane, Queensland 4072, Australia.*

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Abbreviations

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| APMA | = aminophenylmercuric acetate |
| BB-94 | = batimastat |
| BM | = basement membrane |
| D-MEM | = Dulbecco's modified Eagle medium |
| DMSO | = dimethyl sulphoxide |
| ECM | = extracellular matrix |
| H&E | = haematoxylin and eosin |
| KDa | = kilo Dalton |
| MMPs | = matrix metalloproteinases |
| PDL | = primary dermal lamella |
| PEL | = primary epidermal lamella |
| PMNs | = polymorphonuclear leucocytes |
| PAS | = periodic acid Schiff |
| SDS | = sodium dodecyl sulphate |
| SEL | = secondary epidermal lamellae |

• Summary

A method for culturing explants of lamellar hoof was developed to investigate the process of lamellar separation that occurs in laminitis. Explants, consisting of hoof wall, dermal and epidermal lamellae and the adjacent sub-lamellar connective tissue remained intact when cultured in tissue culture medium for 2 days. However, when cultured in the presence of the matrix metalloproteinase (MMP) activator aminophenylmercuric acetate (APMA), the lamellae separated when tension was applied by pulling the hoof wall in an opposite direction to the connective tissue. The separation occurred between the epidermal basal cells and the basement membrane therefore mimicking the lesion of laminitis. Electrophoresis of culture medium from control hoof explants into gradient polyacrylamide gels co-polymerised with gelatin revealed that the explants had produced 2 gelatinases of molecular weight 92 and 72 kDa corresponding to EqMMP-9 and EqMMP-2 respectively. Minor bands of lower molecular weight were the active forms of these enzymes. The zymograms of culture medium from APMA treated explants revealed an increase in the amount of active MMPs. Equine polymorphs cultured for 2 days produced only EqMMP-9. Lamellar explant medium from horses with acute laminitis contained increased amounts of zymogen and active EqMMP-2 and EqMMP-9 particularly in explants from the fore hooves.

Zymography of homogenates of normal lamellar hoof tissue revealed only EqMMP-2 and a minor active band. However, homogenates of lamellar tissue from horses with laminitis showed that EqMMP-9 was present as well as increased EqMMP-2 in both zymogen and active forms. Addition of the MMP inhibitor batimastat (BB-94) to the culture medium of APMA treated explants prevented lamellar separation. BB-94 incubated with polyacrylamide strips containing the MMPs from laminitis affected lamellar explants inhibited enzymatic activity at a concentration of 1 mmol/l. It is concluded that activation of MMPs may be responsible for the lamellar separation seen in laminitis and that MMP inhibitors may be useful clinically for preventing this process.

• Introduction

The extracellular matrix (ECM) can no longer be regarded as an inert and stable structure since it is clear that synthesis and degradation of matrix components is in a dynamic state (Matrisian 1992). Therefore, remodelling of the ECM is a normal biological process occurring constantly as tissues respond to changes in their environment. A convincing body of evidence supports the contention that an homologous family of zinc containing, calcium dependent enzymes, matrix metalloproteinases (MMPs) cleave macromolecules of the ECM during remodelling and this process is inhibited by tissue inhibitors of metalloproteinases (Birkedal-Hansen et al. 1993). More than 11 MMPs have so far been identified. They are expressed at low levels in normal mature tissues but are upregulated during normal and pathological remodelling processes such as embryonic development (Kinoh et al. 1996), tissue repair, inflammation, tumour invasion and metastasis (Birkedal-Hansen 1995).

A role for MMPs in the production of lamellar basement membrane pathology, a feature of acute equine laminitis, has been proposed by Pollitt (1996). The laminitis lesion is characterised by separation and disintegration of the lamellar basement membrane from the epidermal basal cells resulting in failure of the anatomy of the inner hoof wall. In severe cases the laminitis process may cause the distal phalanx to detach from the hoof, penetrate the sole (Pollitt 1995) and cripple the horse.

In this paper we report experiments that test the hypothesis that activation of MMPs is responsible for the separation of lamellar epidermal basal cells from their BM as seen in laminitis and that this process can be inhibited by MMP inhibitors.

• Materials and methods

D-MEM (glucose 25 mmol/l), gentamycin¹, APMA and DMSO² were obtained. APMA and batimastat being, sparingly soluble in water, were dissolved in DMSO (5 mg/ml) before addition to DMEM. Batimastat (BB-94) was a gift from British Biotech Pharmaceuticals Ltd, Oxford, UK. All other reagents were reagent grade chemicals purchased from chemical manufacturers.

Explants of horse's hooves consisted of 2 mm of the hard inner hoof wall, 6-8 intact epidermal lamellae, the corresponding dermal lamellae and 3-4 mm sublamellar connective tissue (Fig 1A) and were prepared from normal horses slaughtered at a knackery exporting meat for human consumption. The feet were collected within •6 min of the horses being killed by a shot to the head. The legs were disarticulated at the metacarpophalangeal joint and placed in crushed ice for transportation to the laboratory which took 1.5-2 h. The feet were trimmed with a band saw as described by Pollitt (1996). The explants were harvested from a 2 x 4 cm strip of the dorsal hoof wall of fore feet only and were trimmed with a number 11 scalpel blade. Potentially contaminated material at the edge of the strip was discarded and trimming of the explants was performed aseptically with instruments sterilised in 70% alcohol. Each explant was washed through 3 changes of sterile 0.9% sodium chloride. Up to 80 explants could be harvested from each strip of dorsal hoof wall. Explants from the fore and hind hooves of 2 horses with acute laminitis were also prepared.

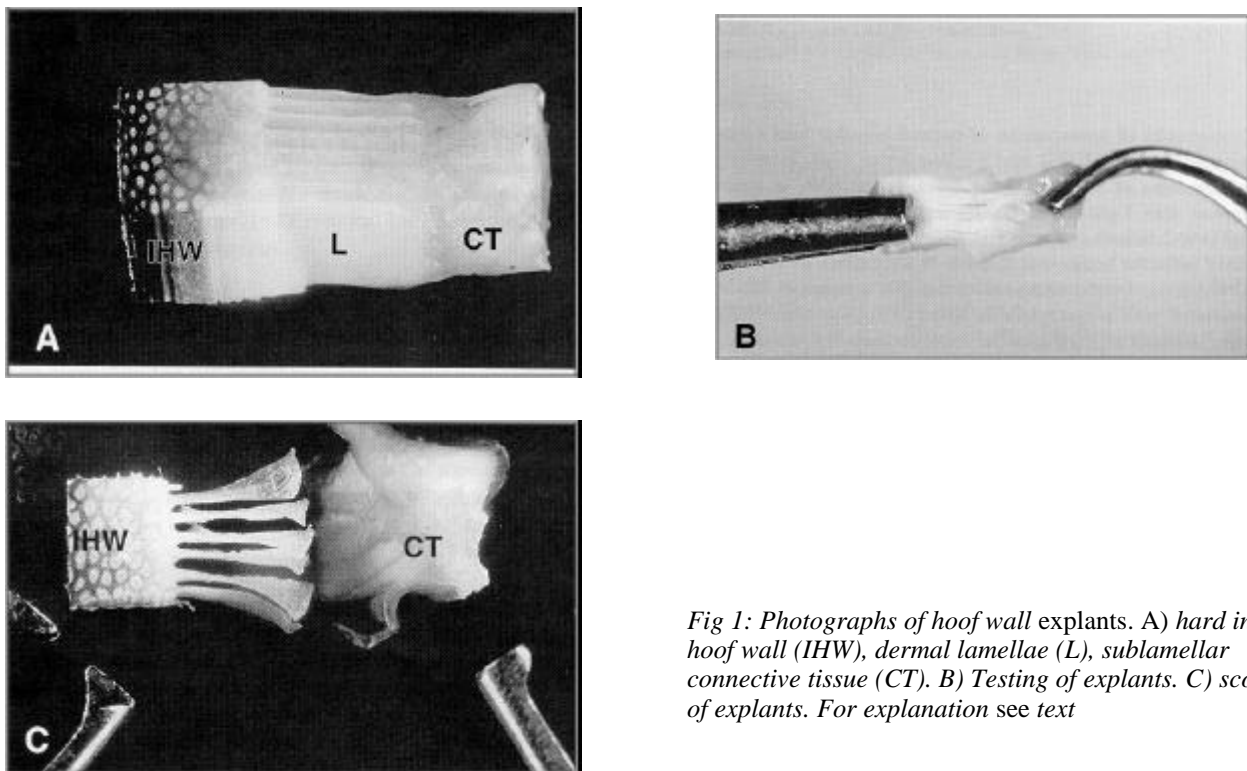


Fig 1: Photographs of hoof wall explants. A) hard inner hoof wall (IHW), dermal lamellae (L), sublamellar connective tissue (CT). B) Testing of explants. C) scoring of explants. For explanation see text

Explants were cultured in 24 well culture plates in 1 ml culture medium containing gentamycin (0.1 mg/ml) at 37°C in an atmosphere of 5% CO₂ in air. The integrity of the explants was tested, after the period of culture, by grasping the hoof wall in one pair of rat tooth forceps and the connective tissue in another pair and pulling first in a side to side direction and then in an up and down direction (Fig 1B). The result was scored as 'separated' if lamellar separation occurred and scored 'intact' if it did not (Fig 1C). For consistency, testing of explants for separation was always done by the same operator. Cultured explants and hoof lamellar samples collected at the time of explant preparation were fixed in 10% buffered formalin and histological sections prepared from selected samples. They were stained with H&E and PAS, and examined with an Olympus BXS0 light microscope..

Electrophoresis of culture media containing APMA showed the same gelatinase banding pattern but there was a change in the relative intensities of the bands (Fig 5). The 72 kDa band was much lower in intensity and the strongest band was that intermediate between the 72 and 62 kDa bands and was assumed to be an active form of EqMMP-2. EqMMP-9 was also present and it was accompanied by a gelatinase of 82 kDa, possibly the active form of the 92 kDa EqMMP-9 (Fig 5). The medium from explants cultured with BB-94 and APMA showed a similar pattern of gelatinase activity to those cultured with APMA alone.

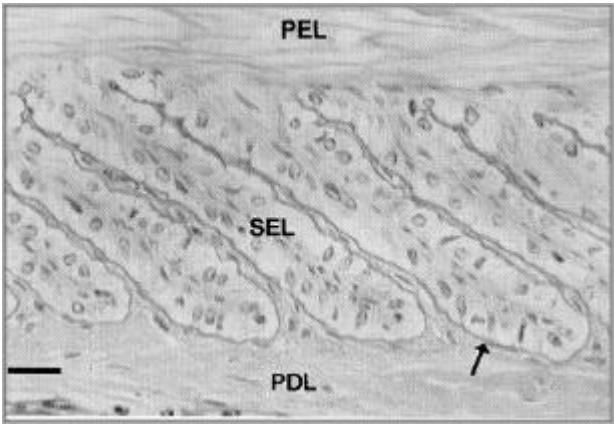


Fig 2: A section of normal hoof explant cultured for 2 days in D-MEM. The explant could not be separated when placed under tension. Basement membrane (arrowed) is attached to the basal cells of the secondary epidermal lamellae (SEL) which show normal anatomy. PAS stain. Bar = 25 μ m. PEL = primary epidermal lamella, PDL = primary dermal lamella.

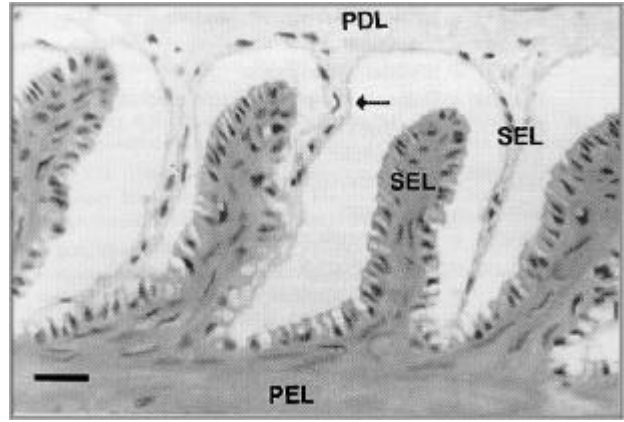


Fig3: Section of hoof explant cultured for 2 days in D-MEM with APMA. Similar explants separated easily when placed under tension. A space separates the basement membrane (BM:arrowed) from the basal cells of the secondary epidermal lamellae (SEL) which show normal anatomy. H&E stain. Bar = 25 μ m. PDL = primary dermal lamella, PEL = primary epidermal lamella.

Fig 4: Section of hoof explant cultured for 2 days in D-MEM, APMA and BB-94. Similar explants did not separate when placed under tension. For the most part the basement membrane (BM) is attached to the basal cells of the secondary epidermal lamellae (SEL). However, many SELs show Zones of BM separation (arrows). H&E stain. Bar = 25 μ m. PEL = primary epidermal lamella, PDL = primary dermal lamella.

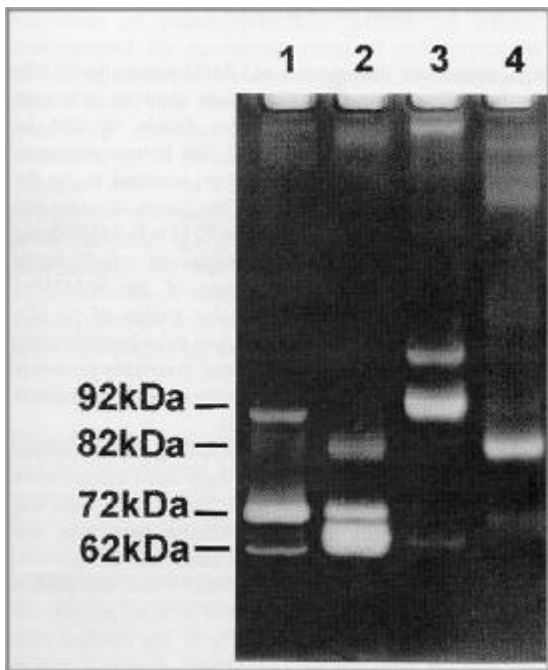
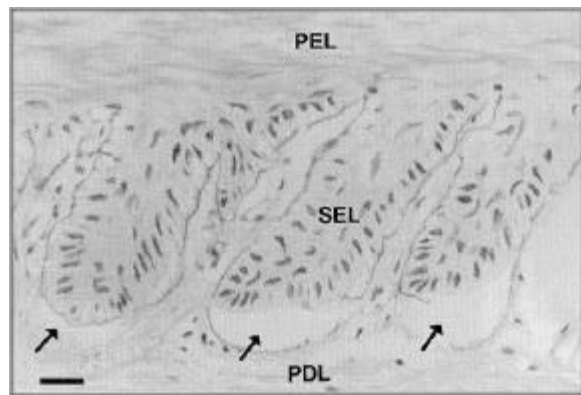


Fig 5: Zymography of normal lamellar explants and white bloodcells. Gradient (7-15%) polyacrylamide gel containing 0.1 % gelatin. Lane 1 = normal hoof explant in D-MEM with DMSO; Lane 2 = normal hoof explant in D-MEM, DMSO with APMA; Lane 3 = equine PMNs in DMEM with DMSO; Lane 4 = equine PMNs in D-MEM, DMSO and APMA. (Molecular weight standards are described in the text but not shown).

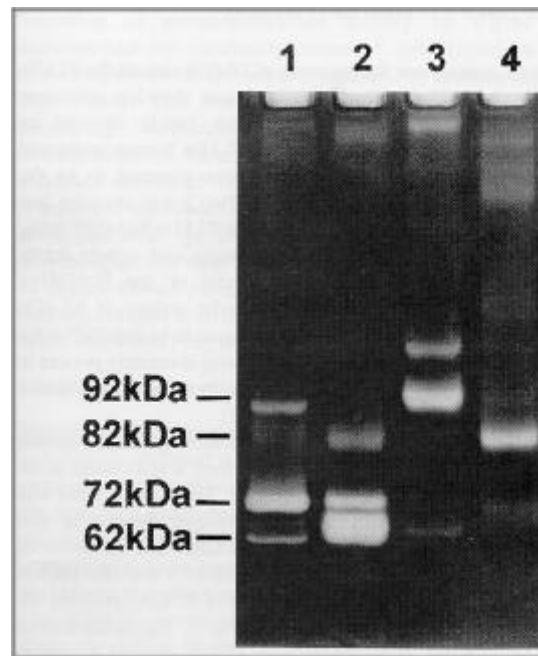


Fig 6: Zymography of lamellar explants from a horse with laminitis. Gradient (7-15%) polyacrylamide gel containing 0.1 % gelatin. Lane 1 = normal hoof explant supernatant; Lane 2 = laminitis fore hoof explant supernatant; Lane 3 = laminitis fore hoof explant supernatant; Lane 4 = laminitis hind hoof explant supernatant. (Molecular weight standard are described in the text but not shown).

Zymograms from cultured normal PMNs

The zymogram of the supernatant of equine PMNs cultured in D-MEM for 2 days showed that the 92 kDa EqMMP was expressed as well as a larger molecular weight species of unknown identity (Fig 5). When APMA was present the 92 kDa proenzyme was converted to the 82 kDa active form (Fig 5).

Zymograms from cultured laminitis hoof explants

The zymogram of the D-MEM supernatants of lamellar explants from horses with laminitis showed a dramatic increase in the presence EqMMP-2 and EqMMP-9 (Fig 6). There was also an increase in the presence of the lower molecular active forms of these enzymes particularly in both fore hooves of an individual horse with acute laminitis killed 48 h after carbohydrate overload (Pollitt 1996).

Some explants from all hooves were cultured routinely for 2 days in D-MEM, D-MEM with 5% DMSO and D-MEM with 5% DMSO and 0.7 mmol/l APMA to test the suitability of the hooves for the experiment. The tissue was judged suitable if the explants cultured in D-MEM/DMSO tested intact and those in D-MEM/DMSO/APMA separated. The remaining explants from each hoof were cultured in D-MEM with 15% DMSO; D-MEM with 15% DMSO and 0.7 mmol/l APMA; D-MEM with 15% DMSO and 0.7 mmol/l APMA and 500 μ mol/l BB-94.

Hoof tissue homogenates were prepared from 3 normal horses and 2 horses with acute laminitis. The homogenates were made by preparing explants from the hooves and then trimming off the hoof wall. The remaining lamellar and connective tissue were then homogenised in a motorised Potter-Elvehjem³ glass homogeniser containing 1 ml cold D-MEM. The homogenates were centrifuged at 10,000 g for 10 min and the supernatants stored at -20°C.

Gradient polyacrylamide gels containing 0.1 % gelatin and 0.1% SDS were prepared (Kleiner and Stetler-Stevenson 1994). The acrylamide concentration in the gradient was 7.5-15%. Gels (0.75 mm thickness) were run for 2.5 h at 40 mA in a Hoefer Mighty Small vertical electrophoresis apparatus⁴. They were then washed twice in 2.5% Triton X-100 for 30 min to remove SDS, incubated at 37°C overnight in zymogram buffer (50 mmol/l TrisHCl, pH7.4, containing 0.2 mmol/l NaCl, 5 mmol/l CaCl₂, 1% Triton X-100 and 0.02% NaN₃). They were stained with Coomassie Blue G-250. Clear areas in the blue stained gel indicated areas of MMP gelatinase activity. The culture medium from human malignant melanoma cells cultured in D-MEM was electrophoresed on the same gel as the hoof explant sample as a standard of known molecular weight and MMP activity. Equine white blood cells, collected from the buffy coat of blood samples from clinically normal horses, were washed in 0.9% sodium chloride and incubated for 48 h in either D-MEM and 0.5% DMSO; or D-MEM, 0.5% DMSO and 0.7 mmol/l APMA to provide a source of neutrophil gelatinase which was used as an electrophoresis standard.

Gels of cultured laminitis explant supernatants were cut into strips and incubated in zymogram buffer to which varying concentrations of BB-94 in DMSO had been added (Brown et al. 1993). Control strips were incubated in zymogram buffer and DMSO.

• Results

Lamellar separation

Explants cultured for 2 days in D-MEM or in D-MEM with DMSO did not separate (n = 26) whereas all explants cultured with D-MEM containing APMA separated (n = 26). The separation occurred along the dermo-epidermal junction. Histological examination of intact explants cultured in D-MEM or D-MEM/DMSO had an appearance resembling normal hoof tissue before culture. Sections of intact cultured explants, stained with PAS, resembled normal hoof lamellae (Pollitt 1994) and had an intact BM in close proximity to the basal cell layer of the secondary epidermal lamellae (Fig 2). Histological examination of explants cultured in the presence of APMA and not subjected to tension showed a clear zone (mean \pm s.d. 17.53 \pm 4.58 mm) between the lamellar BM and the epidermal cells of the SELs (Fig 3). This was confirmed with PAS stained sections that showed that the clear zone was between the BM and the epidermis.

Explants remained intact when BB-94 was added to the APMA containing D-MEM culture medium (n = 13) whereas the corresponding explants cultured without BB-94 separated. The histological appearance of BB-94 treated explants resembled explants intermediate between those cultured in DMEM with DMSO or D-MEM alone and those with APMA in that there were zones of dermal-epidermal separation interspersed with fully attached zones (Fig 4).

Zymograms from cultured normal hoof explants

Zymograms of culture media from normal hoof explants cultured in D-MEM or D-MEM with DMSO gave similar results although, sometimes, the presence of DMSO caused the 92 kDa band to fade. There were 2 major bands showing gelatinase activity (Fig 5). One of the major bands showed an electrophoretic mobility identical to 72 kDa human malignant melanoma gelatinase (MMP-2) and was assumed to be the equine equivalent EqMMP-2 (Fig 7). Two bands showing less gelatinase activity migrated ahead of the 72 kDa EqMMP-2 and these were assumed to be the lower molecular weight active forms of EqMMP-2. The most anodal of the EqMMP-2 gelatinases appeared to have a molecular weight of 62 kDa (Fig 5). The 92 kDa gelatinase was assumed to be EqMMP-9 the equine equivalent of human MMP-9. It was invariably present in the inactive proenzyme 92 kDa form without additional active forms of lesser molecular weight (Fig 5).

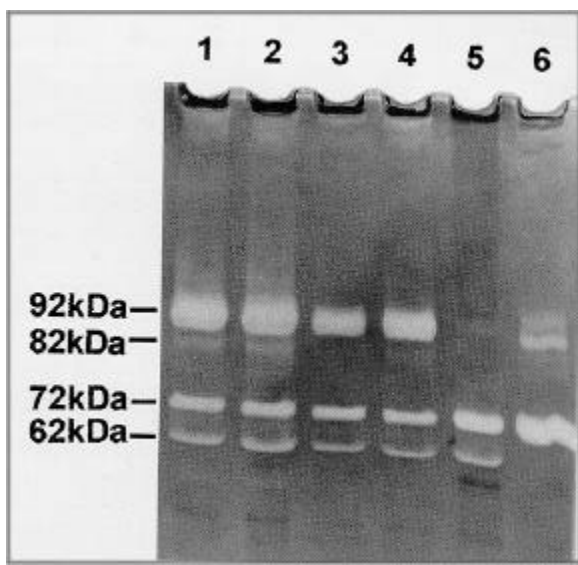


Fig 7: Zymography of lamellar homogenates from a normal horse and a horse with acute laminitis. Gradient (7-15%) polyacrylamide gel containing 0.1 % gelatin. Lane 1 = laminitis fore hoof homogenate; Lane 2 = laminitis fore hoof homogenate; Lane 3 = laminitis hind hoof homogenate; Lane 4 = laminitis hind hoof homogenate; Lane 5 = normal hoof homogenate; Lane 6 = human malignant melanoma cell culture supernatant

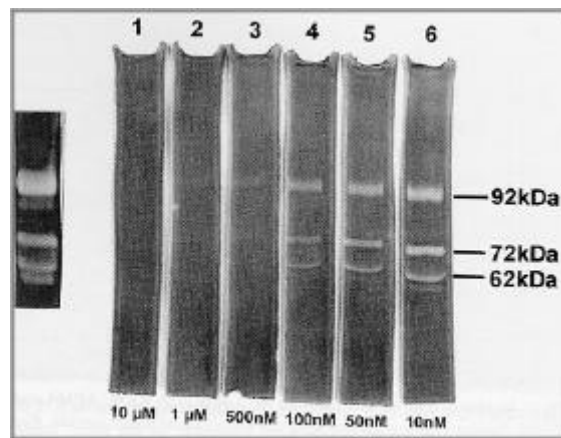


Fig 8: Batimastat inhibition of EqMMPs. Gradient (7-15%) polyacrylamide gel containing 0.1 % gelatin. Laminitis hoof explant in D-MEM incubated with: Lane 1 = 10 μM BB-94 in DMSO; Lane 2 = 1 μM BB-94 in DMSO; Lane 3 = 500 nM BB-94 in DMSO; Lane 4 = 100 nM BB-94 in DMSO; Lane 5 = 50 nM BB-94 in DMSO; Lane 6 = 10 nM BB-94 in DMSO

Zymograms from homogenates of normal hoof lamellae

The zymogram of homogenates from normal hoof lamellae showed that only the 72 kDa EqMMP-2 and a lesser amount of its 62 kDa active form was present. The 92 kDa EqMMP-9 which was present in the supernatant of cultured normal explants was invariably absent in the homogenates of normal lamellae (Fig 7).

Zymograms from homogenates of hoof lamellae with laminitis

Homogenates of lamellar tissues with acute laminitis showed a relatively large amount of 92 kDa EqMMP-9 as well as EqMMP-2. Interestingly, in the horse with laminitis killed 48 h after alimentary carbohydrate overload, homogenates of the fore hoof lamellae contained more EqMMP-9 and more EqMMP-2 than hind hoof lamellae. In addition, the fore hoof lamellae contained more lower molecular active EqMMP-2 and EqMMP-9 (Fig 7).

Metalloproteinase activity inhibited by BB-94

Inhibition of metalloproteinase activity by BB-94 was demonstrated by incubating strips of polyacrylamide gel, containing the metalloproteinases produced by lamellar explants from a horse with acute laminitis, in zymogram buffer with decreasing concentrations of BB-94 (Fig 8). BB-94 concentrations of 10 and 1 μM completely inhibited the gelatinase activity of the 92 and 72 kDa EqMMP enzymes and proenzymes expressed by lamellar explants from a horse with acute laminitis. At BB-94 concentrations of 500 nM gelatinase activity was just detectable and concentrations of 10 nM showed virtually no inhibition as the gel strip was indistinguishable from the control strip incubated in zymogram buffer and DMSO but without BB-94.

• Discussion

Explants of the equine lamellar hoof wall and dermis, cultured in D-MEM, appeared to be a suitable system for the study of some aspects of hoof biochemistry and physiology. In particular, the separation of the basal epidermal cells from their basement membrane mimicked the pathology of laminitis and cultured explants seem to be a useful model for studying this process. In the present experiments we have concentrated on the possible role of MMPs in this process as these enzymes are known to affect the ECM. Much information about MMPs has been generated from experiments using explants of tissues and monocultures of cells (Jones et al. 1994). Human foreskins (Windsor et al. 1993), equine joint cartilage (Morris and Treadwell 1994) and rat parietal bones (Chambers et al. 1985) have been successfully cultured in tissue culture medium. Various cytokines and hormones (Busiek et al. 1994), growth factors (Salo et al. 1991) and synthetic enzyme blocking agents (Brown and Giavazzi 1995) have been added to the medium to gauge their effect on the tissues and MMP production.

Matrix metalloproteinases MMPs are secreted as inactive proenzymes (zymogens) which at least *in vitro* can be activated by various proteinases including plasmin, trypsin, kallikrein, neutrophil elastase and cathepsin G (Ries and Petrides 1995). Also the organomercurial aminophenylmercuric acetate (APMA) readily converts an inactive MMP proenzyme to lower molecular weight active forms (Ries and Petrides 1995).

Much of the interest surrounding MMP research has focussed on their now undisputed role in the malignant transformation of tumours (Goldfarb and Liotta 1986). Indeed, increased expression of MMP-9 has been detected in brain tumours, bladder cancer, uveal melanoma, basal cell and squamous cell cancers of the skin, squamous cell carcinomas of the lung, colon adenocarcinomas, malignant lymphomas and breast cancer (Himmelstein et al. 1995). By electrophoresis of homogenates of micro-dissections of frozen sections of bladder tumours, Davies et al. (1993) were able to quantitate levels of MMP activity and found a close correlation between MMP activation and tumour grade and invasiveness. Many studies have shown the requirement of malignant tumours for MMP generation to enable them to invade adjacent tissue. Interestingly, although tumour derived MMPs lyse stroma in the path of the invading tumour they are also capable of inducing MMP production in normal stroma in the immediate vicinity of the tumour (Crawford and Matrisian 1995; Stetler-Stevenson et al. 1993) and tumour cell invasion may be viewed as a dysregulation of a physiological process. The key MMPs involved in tumour progression and metastasis are the gelatinases MMP-2 and MMP-9 and their target is the basement membrane.

The present study identified MMPs with gelatinase activity in culture media which had been incubated with hoof explants. The 72 kDa and 92 kDa proteins corresponded with the proenzyme forms of EqMMP-2 and EqMMP-9. Other proteins

corresponding to lower molecular weight forms of EqMMP-2 and EqMMP-9 and which had gelatinase activity were also present. Based on studies in other species (Brown *et al.* 1993; Davies *et al.* 1993; Cornelius *et al.* 1995), it would appear that the lower molecular weight molecules are likely to be active forms of EqMMP-2 and EqMMP-9. Incubation of explants with APMA resulted in lamellar separation and in a relative increase in the amount of active enzyme, as shown by zymography. Conversion of proenzyme metalloproteinase to a lower molecular weight active species by treatment with APMA is a characteristic of MMPs (Jones *et al.* 1994). Lower molecular weight active MMPs identical to those induced by treatment with APMA were also present in homogenates of lamellar tissue from horses with acute laminitis implying that the separation of the lamellar BM from the epidermal basal cells, which is a characteristic of acute laminitis, (Pollitt 1996) could be due to MMP activity. In addition the zymograms of 48 h culture medium of lamellar explants from horses with acute laminitis showed a dramatic relative increase in MMP production. Lamellar explants from the fore hooves showed a particularly large increase in MMP production especially of the lower molecular weight active forms. Overproduction of MMP in the fore hooves of horses with acute laminitis is a surprising result but may partially explain why the fore hooves of horses with laminitis are usually more severely affected than the hind (Pollitt 1995).

An exciting development in both cancer and arthritis research is the demonstration that synthetic low molecular weight MMP inhibitors such as batimastat (BB-94) can dramatically block both the growth and metastatic spread of malignant tumours (Brown and Giavazzi 1995; Watson *et al.* 1995). The MMP inhibitor MI-1 inhibited the removal of collagen and proteoglycan in a rat arthritis model (Karran *et al.* 1995). If inhibitors of MMP activity ameliorate tumour progression and inhibit cartilage and bone destruction in arthritis this strongly suggests that MMPs play a major role in tumour metastasis and joint destruction respectively.

The substrate of the MMPs is mimicked by BB-94 (Mr477), specifically the peptide residues on one side of a principal cleavage site of collagen. This allows it to fit tightly in the active site of the enzyme. The hydroxamate group (-CONHOH) of the BB-94 molecule binds to the zinc atom in the active site resulting in potent but reversible inhibition of the MMP (Brown and Giavazzi 1995).

Lamellar separation was inhibited by BB-94 but it did not decrease the activation of MMPs in the APMA treated explants. The protection afforded by BB-94 was not complete as shown by histological examination of the tissues but it was sufficient to prevent physical separation of lamellae even when considerable force was applied to the explants. Furthermore the gelatinase activity of the MMPs expressed in zymograms by lamellar explants from horses with acute laminitis was completely inhibited by nanomolar concentrations of BB-94. These observations, using a specific metalloproteinase inhibitor, confirm the identity of the equine gelatinolytic enzymes as MMPs and indicate that an MMP inhibitor such as batimastat (BB-94) could be useful in reducing the extent of lamellar separation in acute laminitis. The newly developed, orally active, BB-2516 has an IC_{50} of 6 nmol/l against MMP-2 and pilot phase II clinical studies have commenced in man with a variety of tumour types (Brown and Giavazzi 1995).

The gelatin zymogram of lamellar homogenates from horses with acute laminitis contained the basement membrane degrading gelatinases 92 kDa EqMMP-9 and 72 kDa EqMMP-2 in both their zymogen and active forms. EqMMP-9 was never present in homogenates from normal hooves and its appearance in homogenates of lamellae affected by laminitis could be due to the influx of PMNs into lamellar tissues previously reported by Pollitt (1996). During the menstrual cycle, an influx of extravascular PMNs into the endometrium of women produces most of the MMP-9 responsible for the degradation of extracellular matrix which characterises menstruation (Jesiorska *et al.* 1996). Furthermore cultured lamellar explants from hooves affected by laminitis showed increased MMP activity compared to normal explants. The data provide evidence that the induction of active EqMMPs during the developmental phase of laminitis could be responsible for the dermal-epidermal separation which characterises the *in vivo* laminitis lesion. Inhibition of metalloproteinase activity in lamellar tissue during the developmental phase of laminitis may prevent lamellar separation and deserves clinical investigation.

Acknowledgements

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Manufacturers' addresses

¹Gibco BRL, Australia.

²Sigma, Australia.

³Glas-Col, Indiana, USA.

⁴Hoefler Pharmacia Biotech Inc. California, USA.

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