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Basement membrane integrity and keratinization in healthy and ulcerated bovine hoof tissue

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Damage to, or deterioration of, the keratinized horn tissue of the bovine hoof claw culminates ultimately in the development of solear ulceration. We have observed abnormal keratin distribution at the site of solear ulceration in the bovine claw that may be due to alteration of the positional cues of the keratinocytes. In this study we have characterized key cell biological changes associated with ulceration in the claw that may precipitate abnormal keratinization. Loss of basement membrane at sites of ulceration was found by immunofluorescent detection of laminin and integrins. In other tissues, basement membrane breakdown results from degradation by matrix metalloproteinases (MMPs). Similarly, elevated levels of MMPs 2 and 9 were observed in ulcerated bovine claw tissue both by zymography and, quantitatively, by assay of enzyme activity. In the sole of claws that contained an ulcer, tissue distal to the ulcer site also had elevated MMP 2 when compared with healthy sole tissue from the same animals, as did sole tissue of claws recovering from ulceration. Tissue inhibitor of metalloproteinase 2 (TIMP 2) was detected by ELISA in healthy tissue. TIMP 2 tended to be lower in diseased tissue distal to ulcer sites, and was significantly lower in ulcerated tissue. MMP 2 was located by immunofluorescence in the dermal and basal epidermal region of sole tissue, in the region of the basement membrane. Increased punctate staining of material in the dermis was associated with ulcerated material. ELISA of TIMP 2 in tissue extracts enriched for dermis or epidermis confirmed that the inhibitor was located predominantly in the dermis. To investigate a possible causal relationship between basement membrane anchorage and epidermal keratinization, the effect of function-blocking antibodies to laminins and integrins was tested in tissue explant cultures prepared from healthy sole tissue. Anti-integrin antibody treatment had no effect on either protein or DNA synthesis. In contrast, in the presence of anti-laminin antibody, protein synthesis was decreased in a concentration-dependent manner, a significant effect being observed at the highest concentration after treatment for 24 h. At this concentration, DNA synthesis was also decreased after 48 h of culture, an effect that may be relevant to a hibernal reduction in claw cell turnover, and the associated seasonal vulnerability of cows to claw damage. The results provide evidence for basement membrane disruption at ulcer sites, and an increased potential for disruption in the diseased claw, and a causal link between this and abnormal epidermal keratinization. Basement membrane disruption is in turn associated with reciprocal changes in MMPs and their inhibitors, favouring extracellular proteolysis. Whether MMP activation is the primary cause of dermal–epidermal deterioration and, if so, how MMP activation is triggered, remains to be determined.

Keywords: Hoof, cow, lameness, ulceration, keratinocytes, MMPs, TIMPs.

Horn deterioration is a feature of lesions in the bovine claw, which in extreme circumstances are manifested in solear ulceration. Horn is composed primarily of keratin proteins, whose distribution is altered at ulcer sites in the claw sole: in an earlier study, we found basal cell keratins in supra-basal epidermal locations (Hendry et al. 2001). Aberrant keratin expression in epidermal tissues is, in several instances, related to changes in basement membrane signalling cues to the keratinizing cells (Suter et al. 1997), and keratin subtype expression is altered in bovine claw cells cultured in the absence of a basement membrane (Kitahara & Ogawa,

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1994). Although keratin subtype synthesis was generally unaffected by ulceration *in vivo* (Hendry et al. 2001), our analysis did not preclude the possibility of localized changes in keratin synthesis, and the possibility that such changes, and the aberrant location of some keratins in ulcerated tissue, were the result of basement membrane disruption. If so, then the loss of basement membrane integrity in the early stages of equine laminitis (Pollitt, 1996) and preceding claw horn disease in the cow (Kempson & Logue, 1993) may be an important determinant of impaired keratinization. Basement membrane disruption may in turn be the result of extracellular proteolysis caused by matrix metalloproteinase enzymes (MMPs) (Nagase & Woessner, 1999). Among the MMPs are gelatinases, notably MMP2 and MMP9, which are known to degrade extracellular collagen and disrupt cell anchorage to the basement membrane (Matrisan, 1992; Nagase & Woessner, 1999). Gelatinase activity is elevated in laminitic equine hoof tissue (Johnson et al. 1998; Pollitt et al. 1998) under conditions where basement membrane integrity would also be expected to be compromised. MMP activity may, therefore, act to promote, or may itself induce basement membrane disruption and so precipitate alterations in keratin depositions that predispose cows to solear lesions. In this study, we examined tissue from healthy and diseased bovine claws for evidence of basement membrane damage and alterations in MMP activity that would induce such damage. We also did experiments to determine whether basement membrane disruption would in turn be expected to inhibit keratin deposition.

A preliminary communication of this work was presented at the 11th International Symposium on Lameness in Ruminants (Hendry et al. 2000).

Materials and Methods

Animals and Tissue

Holstein-Friesian cows were from the Hannah Research Institute Herd, or the Acrehead Herd of the Scottish Agricultural College, Crichton Royal Farm, Dumfries, UK. Animals used in the study were as described previously (Hendry et al. 2001), and were culled for various reasons at ages ranging from six weeks to several years. They were killed by captive bolt and exsanguination, and tissue was taken immediately *post mortem* from the central sole of paired claws. When a sole ulcer was present, regardless of location, tissue was collected from the ulcer site. Tissue was cut from the sole using a power saw as previously described (Hendry et al. 2001) and immediately frozen in liquid nitrogen or processed for microscopy. Samples were designated 1) healthy: tissue from a claw having no evidence of claw disease, 2) diseased: sole tissue from a claw with ulceration at another location in that claw, 3) recovered: sole tissue from a claw with a healed ulcer, 4) ulcerated: tissue from an ulcer site. Ulcerated tissue included such tissue surrounding it as allowed collection of the ulcerated material.

Microscopy

Small ($\sim 3\ mm^3$) blocks of sole tissue containing all epithelial layers were fixed immediately in Methacarn after removal from the animal and processed conventionally for embedding in fibrowax. In other instances, unfixed material for cryosections was collected, placed in OTC embedding medium (BDH Merck Ltd., Poole, BH15 1TD, UK) and immediately frozen in liquid nitrogen. Sections (5 μm) were cut onto silanized slides as previously described (Hendry et al. 2001).

Immunofluorescence microscopy

Paraffin sections (5 μm) were dewaxed and rehydrated by conventional methods and treated with 1 mg/ml $NaBH_4$ in PBS (0·14 M-NaCl–9 mM-Na_2HPO_4–1·3 mM-NaH_2PO_4, pH 7·6) for 10 min. Sections were rinsed in PBS, then incubated for 10 min at 37 °C with 1 mg/ml Pronase (Sigma Chemical Co. Ltd., Poole, UK, BH12 4QH) in PBS. Cryosections were thawed at room temperature. All sections were incubated with non-immune serum from the same species as that used to raise the secondary antibody, diluted 1 : 3 in PBS containing 1 mg Tween 20/ml (PBST). Sections were then treated with primary antibody diluted in PBST. Antibodies used for immunofluorescence were anti-laminin antisera (Sigma) at a dilution of 1 : 3 in PBST, anti-integrin β_4 at 1 : 100 dilution (BioWhittaker UK Ltd, Wokingham, RG41 2PL, UK), anti-integrin α_6 diluted 1 : 100 (Chemicon International Ltd., Harrow, HA3 5UT, UK), anti-MMP 2 (gelatinase A hinge region, 1 : 100 dilution; Chemicon International), anti-MMP 2 (Ab 10) and anti-MMP 2 (Ab 3) (both used at 2·5 μg/ml; Oncogene Research Products at CN Biosciences UK, Nottingham, NG9 2JR, UK). After washing in PBST, sections were incubated in FITC labelled anti-species antibody (Diagnostics Scotland, Carluke, ML8 5QZ, UK) diluted in PBST. All incubations were carried out at room temperature in a humid chamber. Sections were mounted in Vectashield mounting media containing DAPI (Vector Laboratories, Peterborough, PE2 6XS, UK) and examined using a Lecia DMR microscope with epifluorescence optics viewed with a No 13 (UV) and A filter. Images were photographed onto Fujichrome Provia 400 ASA film.

Gelatin zymography

Homogenates enriched in epidermal or dermal tissue components were prepared as previously described (Hendry et al. 1995) and processed by standard methods for non-denaturing gel electrophoresis. Gelatinases were detected by zymography in non-reducing 8% SDS-PAGE gels containing 2·5 g gelatin/l. SDS was removed from the gels by incubation in 25 ml/l Triton-X 100. The enzymes were activated by incubation in re-folding buffer consisting of 50 mM-Tris, pH 7·4 containing 0·2 M-NaCl, 25 mM-$CaCl_2$ and 0·67 ml Brij 35/l (Sigma), pH 7·6 at 37 °C for 16 h. Gels were stained with Coomassie blue (10 g/l) and analysed

using a Personal Densitometer SI and ImageQuaNT software version 4.1 (Molecular Dynamics, Amersham-Pharmacia Biotech, Little Chalfont, HP7 9NA, UK). In some cases, homogenates were incubated with 10 mM-1,10 phenanthroline (Sigma) before preparation of gel samples.

MMP 2 Activity assay

A commercial assay was used to measure MMP 2 activity in claw tissue samples (Amersham Pharmacia Biotech). Homogenates of dermal and epidermal claw tissue (0·5 g/ml) were prepared in 50 mM-Tris-HCl buffer pH 7·4 containing 1 mM monothioglycerol and a particle-free supernatant was prepared by centrifugation (2000 ***g***). The assay was carried out according to the manufacturer's instructions on untreated homogenate supernatants to detect endogenous MMP 2 and, after chemical activation, with 0·25 mM *para*-aminophenylmercuric acetate (APMA) to measure total activity.

TIMP 2 ELISA

A commercial ELISA for detection of TIMP 2 (Amersham Pharmacia Biotech) was used. Homogenates of claw epidermis-enriched fractions were made at 0·5 g tissue/ml in 20 mM Tris-HCl–0·1 M-NaCl–1 mM-$CaCl_2$, pH 7·5 containing 0·05 ml Brij 35/l. The ELISA was performed according to the manufacturer's instructions.

Induction of basement membrane deterioration in vitro

Tissue explants from the sole region of bovine claws were prepared and cultured as previously described (Hendry et al. 1995, 1999). The tissue was cultured in defined medium for 24 or 48 h in the presence or absence of antibodies to laminin at dilutions of 1 : 100, 1 : 300, 1 : 1000, integrin β_4 (1 : 100) or integrin α_6 (1 : 100). Protein synthesis was measured by incorporation of [^{35}S]-labelled amino acids (20 µCi/ml; EasyTag EXPRE^{35}S protein labelling mix, NEN Life Science Products UK Ltd., Hounslow, TW5 9RT, UK) into explant protein during antibody treatment. Alternatively, DNA synthesis was measured by incorporation of [methyl-^{3}H]-thymidine (5 µCi/ml; ICN Pharmacuticals Ltd., Basingstoke, RG24 8WG, UK). Cultured explants were rinsed in non-radioactive medium, blotted, snap frozen in liquid nitrogen and stored at –70 °C. In some instances, explants cultured in the presence of the antibodies were prepared for microscopy by placing in OTC embedding medium and immediately frozen in liquid nitrogen.

Protein and DNA synthesis

Homogenates from the cultured claw tissue were prepared as previously described (Hendry et al. 1995, 1999). DNA content of the homogenate was measured by a fluorimetric method (Labarca & Paigen, 1980) using calf thymus DNA as a standard. Radiolabelled protein and DNA were extracted by precipitation with trichloroacetic acid (100 g/l; TCA) as previously described (Hendry et al. 1995) and processed for scintillation counting by standard methods.

Statistical analysis

Data were analysed by one-way ANOVA using Tukey's family error rate comparisons and least squares difference of analysis. Tests were performed on data or natural log-transformed data where appropriate using Minitab release 11 (Minitab Inc., PA, 16801-3008, USA).

Results

Basement membrane morphology in healthy and ulcerated claw sole

Healthy tissue, diseased tissue and ulcer sites were examined by immunofluorescent staining with anti-laminin antiserum. In the healthy tissue (Fig. 1A) a heavily interdigitating line of basement membrane was visible at the epidermal-dermal junction in the papillae of the sole and also in the blood vessel walls of the dermis. Tissue categorized as diseased (see Methods) appeared very similar to that of healthy samples (Fig. 1B). At ulcer sites, however, anti-laminin staining was either absent or fragmentary (Fig. 1C). This loss of continuity was pronounced in regions close to the necrotic area. Anti-laminin staining of ulcer tissue also showed a marked reduction in the degree of crenellation along the papillary surfaces in the epidermal-dermal junction, which appeared much smoother than in healthy tissue.

Immunofluorescence associated with anti-integrin α_6 antibodies was also localized at epidermal-dermal junctions in the papillary region of healthy claw tissue (Fig. 1D). Some basal membranes of epidermal cells were also stained, as were the dermal capillaries. Tissue taken from diseased claws distal to the ulcerated region again showed staining similar to that of healthy tissue (Fig. 1E). In ulcerated regions of the sole, the extent of anti-integrin α_6 staining was, like that for laminin, reduced or absent (Fig. 1F). Immunofluorescent detection of anti-integrin, β_4 using a second antibody was similarly low in dermal-epidermal junctions adjacent to sites of ulceration (results not shown).

Zymographic detection of gelatinases in sole tissue

Gelatin zymography under non-reducing conditions detected gelatinases in sole tissue. Strong bands were visible at 72 and 68 kDa at the migration position of the pro- and active forms of gelatinase A in the human standard. Less prominent bands at 88–98 kDa corresponded to gelatinase B. Gelatinases A and B are also known as matrix metalloproteinases (MMP) 2 and 9 respectively. Higher molecular mass bands were also seen in some tracks, probably owing to gelatinase dimerization. Gelatinase zymograms were compared by densitometry for samples loaded on an equal

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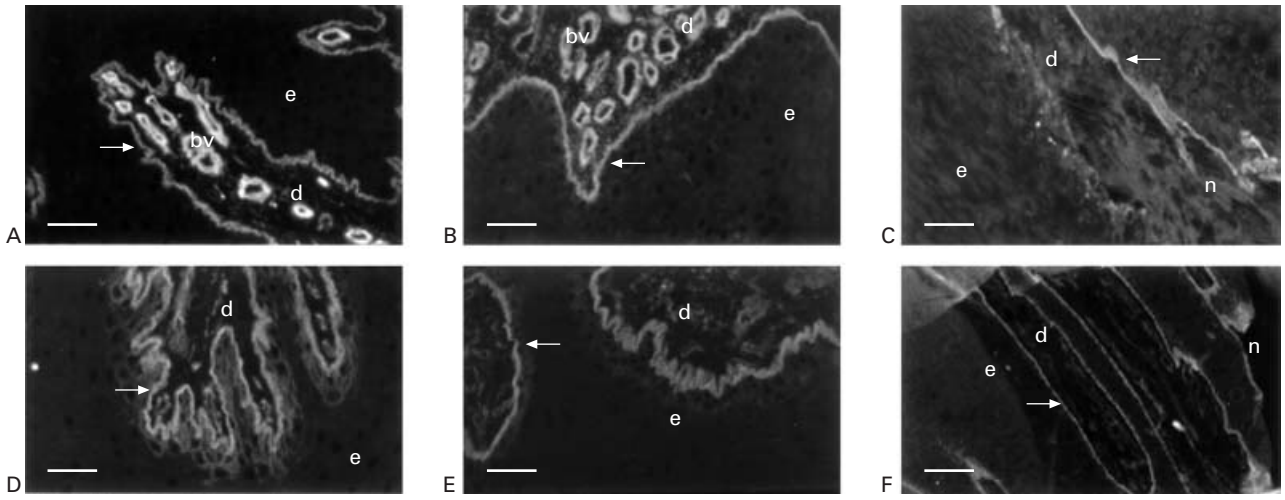

Fig. 1. Immunofluorescence detection of laminin and integrin in the basement membrane of cryosections of healthy and diseased and ulcerated bovine claw soles. A, B, C anti-laminin. D, E, F anti-integrin α_6. A, D tissue from healthy claw; B, E tissue from non-ulcerated area of ulcerated claw (diseased); C, F tissue from ulcerated claw at ulcer area. e – epidermis, d – dermis, arrow – basement membrane, n – necrosis, bv – capillary. Bar A–E 43 μm, F 185 μm.

protein basis (Fig. 2). Activities varied both between animals, and in different claws in the same animal, the degree of variation being unrelated to animal age, parity or lactational status (results not shown). Variability between animals obscured variation between experimental groups, such that differences were not statistically significant (Table 1). However, tissue from diseased claws, or those that had previously been diseased ('recovered'), tended to have higher potential activity in the pro-form bands of MMP2 and higher levels of endogenous, activated MMP 2 than healthy tissue (Table 1). Also, an increase in active MMP2 was evident at ulcer sites. In addition, MMP 9 activity tended to be higher in diseased and ulcerated tissues than in healthy tissue. Gelatinase activity measured by zymography was greatly reduced when homogenates were pre-incubated with the zinc ion chelator 1,10-phrenanthrolate (Fig. 2C), indicating that gelatinolytic activity in the extract was due to tissue matrix metalloproteinases.

MMP 2 activity assay

MMP 2 activity in claw tissue was measured using a commercial kit, latent activity in the pro-form of the enzyme being measured after activation by APMA (Table 2). Healthy sole tissue contained relatively low amounts of endogenous activity and significantly less chemically activated total activity than that of ulcerated tissue, which displayed the highest endogenous and total activity of the various categories of tissue examined (P=0·05, oneway ANOVA; Table 2). Diseased claws also contained a substantially higher endogenous and total activity than healthy tissue. Only one sample of recovered tissue was available, and its total MMP 2 activity (0·22 ng/g tissue) was similar to the mean value for healthy tissue.

TIMP 2 assay

TIMP 2 was assayed using a commercial ELISA. Concentration of inhibitor varied considerably between animals (5–416 ng/g tissue) but was consistently higher in healthy tissue than in diseased tissue when claws from the same animal were compared (Table 2). The lowest level of TIMP 2 was present in material taken from ulcer sites (P=0·002, compared with healthy tissue, oneway ANOVA on $\log_e$-transformed data). The single sample of recovered tissue contained 272 ng TIMP 2/g tissue, which is within the range detected in healthy tissue.

Localization of gelatinase activity in sole tissue

Zymography. Zymography of sole tissue homogenates made from tissue enriched for dermis or epidermis detected more gelatinase activity in dermal-enriched tissue (Fig. 2B). Densitometric analysis indicated enrichment by 46% of pro-MMP2 in the dermal preparation compared with the mixed dermal/epidermal sample. An increase of only 4% was observed in the epidermal-enriched preparation. The band corresponding to active MMP2 was of similar intensity in dermal and mixed samples, but was reduced by 40% in epidermal-enriched preparations. MMP 9 bands were enhanced by 13% in the dermal-enriched preparation and reduced by 80% in the epidermal-enriched preparation compared with the mixed sample.

Immunofluorescence detection of MMP2. Immunofluorescence microscopy detected MMP 2 in epidermal keratinocytes of healthy tissue, particularly in basal epidermal layers, and also in dermis where, in addition to a general light staining, brighter punctate staining was also observed (Fig. 3A, C). The same pattern was repeated in

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Table 1. Densitometry analysis of zymograph bands for MMP2 and MMP9. Values are the mean ± SEM for *n* claws expressed relative to the activity of a 1 ng standard of human gelatinase. Claws were derived from several animals, number of animals in brackets

All differences were not significant (one way ANOVA)

Gelatinase	Healthy claws	Diseased claws (non-ulcer site)	Recovered	Ulcer sites
MMP 2 pro form	0·417 ± 0·14	0·698 ± 0·15	0·675 ± 0·43	0·206 ± 0·24
	n = 11 (9 animals)	*n* = 8 (6 animals)	*n* = 4 (4 animals)	*n* = 4 (3 animals)
MMP 2 active form	0·184 ± 0·20	0·955 ± 0·31	0·973 ± 0·42	0·878 ± 0·29
	n = 10 (8 animals)	*n* = 8 (6 animals)	*n* = 4 (4 animals)	*n* = 4 (3 animals)
MMP 9 total	0·192 ± 0·09	0·300 ± 0·08	0·350 ± 0·20	0·290 ± 0·21
	n = 11 (9 animals)	*n* = 8 (6 animals)	*n* = 4 (4 animals)	*n* = 4 (3 animals)

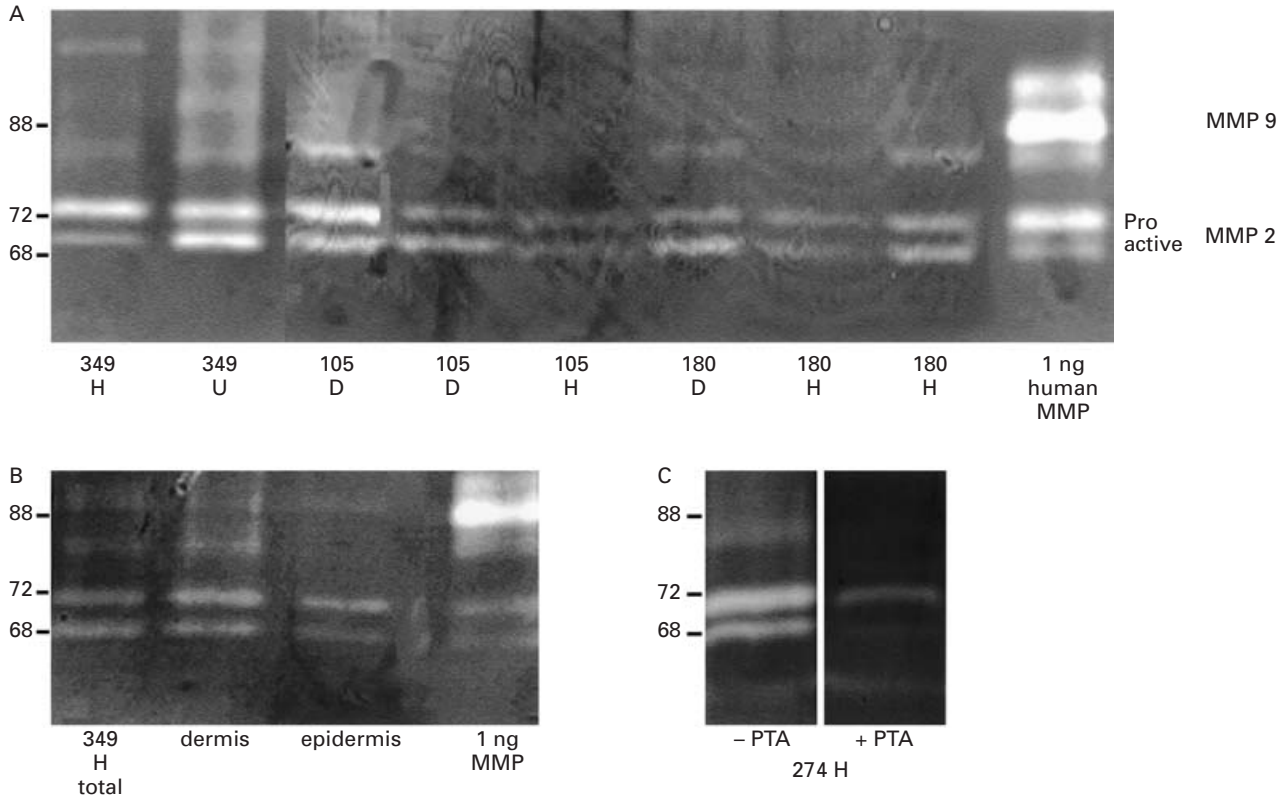

Fig. 2. Gelatin zymography of bovine claw homogenates. Tissue homogenates were resolved under non-reducing conditions in 8% acrylamide gels containing 2·5 g gelatin/l, and incubated to activate gelatinase activity. Bands denote gel regions digested by gelatinases. A. Composite zymograph for claws from several animals. Numbers identify animals, with claw status indentified as H – healthy claw, D – diseased claw, U – ulcer site. The migration position of pro- and active forms of MMP 2 and MMP 9 are indicated. B. Zymograph of homogenates from one animal in which the tissue was dissected to enrich the homogenate for epidermis or dermis. C. Gelatinase activity in homogenates incubated with 1,10 phenanthrolate (–/+ PTA).

tissue derived from non-ulcerated tissue from ulcerated claws (Fig. 3D). In ulcerated tissue, the staining was similar in pattern but punctate staining in the dermis was increased (Fig. 3E), and aggregates of brightly-stained material were associated particularly with ulcerated tissue and foreign material present at the ulcer site. The punctate staining appeared to reside in individual cells (insert Fig. 3A). All three antibodies tested showed the same staining pattern.

TIMP 2 ELISA. ELISA of tissue homogenates enriched for dermis or epidermis detected higher levels of TIMP 2 in dermal tissue than in epidermal enriched material (70 ng/g tissue and 44 ng/g tissue respectively).

Effect of impaired basement membrane signalling in vitro

Explants of healthy sole tissue were cultured for 24 h or 48 h in the presence of antibodies to basement membrane

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Table 2. MMP 2 activity assay and ELISA detection of tissue inhibitor of metalloproteinase 2 (TIMP 2) in tissue homogenates from healthy, diseased and ulcerated claw sole tissue. Total MMP 2 activity was measured after chemical activation of non-active MMP 2 with p-aminophenylmercuric acid (APMA)

Values are the mean ± SEM for *n* claws expressed as ng/g tissue, with the number of animals shown in brackets

Protein (ng/g tissue)	Healthy claws	Diseased claws (non-ulcer site)	Ulcer sites
Endogenous MMP2 activity	0·201 ± 0·06	0·552 ± 0·26	0·62 ± 0·28
	n=6 (6 animals)	*n*=5 (3 animals)	*n*=4 (3 animals)
Total activity MMP2	0·225 ± 0·09[a]	0·674 ± 0·26[a]	1·055 ± 0·32[b]
	n=6 (6 animals)	*n*=5 (3 animals)	*n*=4 (3 animals)
TIMP 2	69·3 ± 21·7[a]	18·5 ± 6·26[a]	1·32 ± 0·62[c]
	n=10 (8 animals)	*n*=7 (5 animals)	*n*=4 (3 animals)

[a, b, c] Superscripts differing horizontally denote significant difference (one way ANOVA, TIMP 2 on $\log_e$-transformed data)

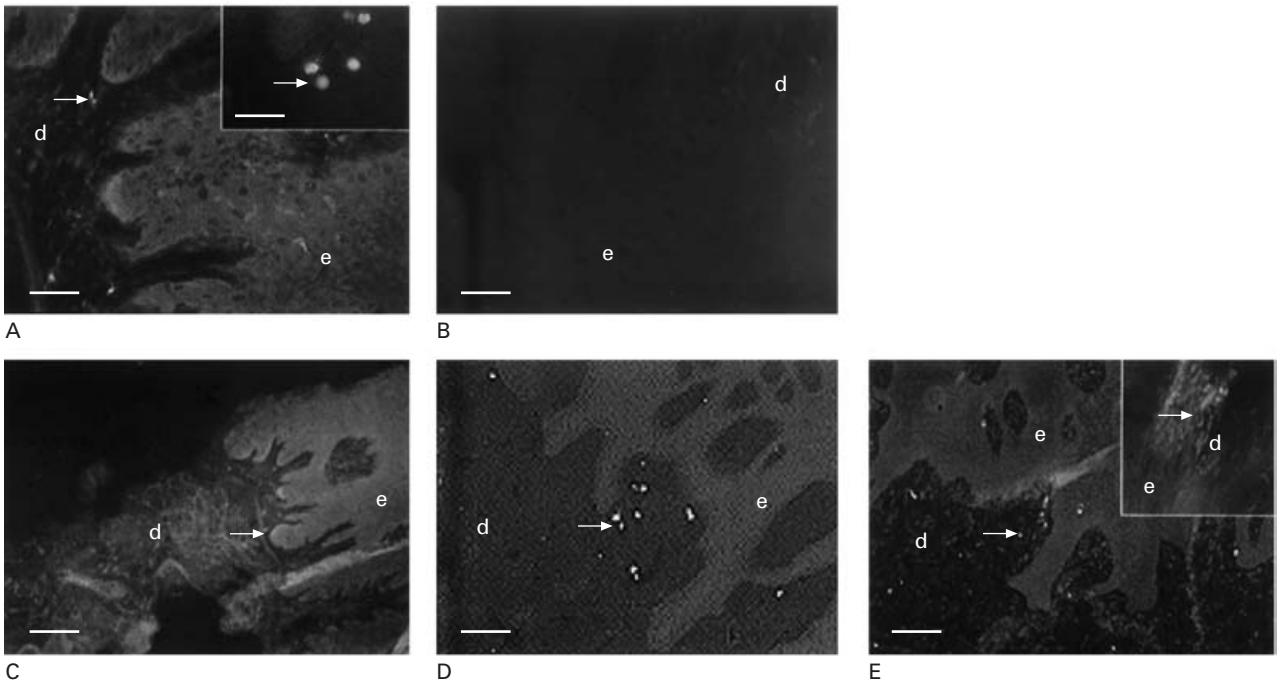

Fig. 3. Localization of MMP 2 in bovine claw sole healthy and diseased tissue by immunofluorescence. A, C Healthy tissue; B, D diseased tissue nonulcer site; E, ulcerated tissue. B – FITC labelled second anti body control. e – epidermis, d – dermis, arrow – punctate staining. Bar A, B 35 μm; A insert 25 μm; C–E 145 μm.

components. Immunofluorescence microscopy of explant tissue sections prepared after culture, using a fluorescent anti-species antibody only, confirmed that anti-integrin α_6 (Fig. 4C) or anti-laminin (not shown) antibodies were bound *in vitro*. In each case, the antibodies were bound to the basement membrane at the dermal-epidermal junction, and in the dermal capillaries.

In explant cultures treated with anti-laminin antibodies, protein synthesis decreased in a concentration-dependent manner (Fig. 4A), the effect being significant after 24 h in cultures containing the highest antibody concentration (P=0·015, Oneway ANOVA, $\log_e$-transformed data). DNA synthesis, measured by incorporation of ^{3}H-thymidine during the final 3 h of culture, also tended to be lower in anti-laminin treated cultures, but the effect was not statistically significant at any of the antibody concentrations. Culture in the presence of anti integrin α_6 (1 : 100 dilution, 24 h) did not alter protein or DNA synthesis rates (Fig. 4B). A similar result was obtained in a single experiment in which protein and DNA synthesis rates were unaffected by culture in the presence of anti-integrin β_4 antibodies (1 : 100 dilution, 48 h; data not shown).

Discussion

This study confirmed earlier histological evidence (Hendry et al. 2001) that, in the bovine claw sole, integrity of the epidermal basement membrane is lost at sites of ulceration.

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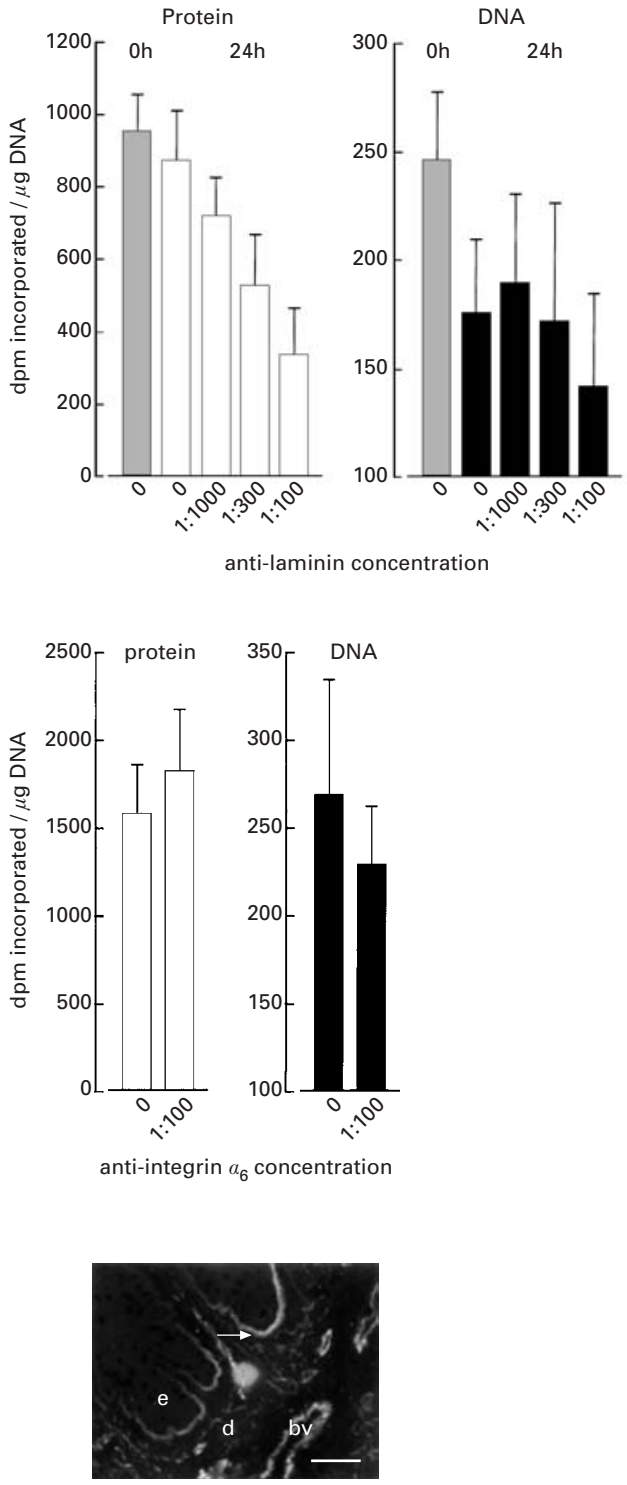

Fig. 4. Protein and DNA synthesis in bovine sole tissue cultured in the presence of function-blocking antibodies to laminin or integrin α_6. Protein (□) and DNA (■) synthesis were measured by incorporation of [^{35}S]-labelled amino acids and [^{3}H]-thymidine respectively in the final 3 h of the culture period. A. Healthy sole tissue explants cultured for 24 h and for 48 h in anti-laminin antisera (1 : 000, 1 : 300, 1 : 100). 0 h synthesis rates indicate synthesis in the tissue at the inititation of the experiments. Values are the mean ± SEM for 3 or 4 animals, 3 replicates per experiment. B. Healthy sole tissue explants cultured for 24 h in anti-integrin α_6 (1 : 100). C. Immunofluorescent detection of anti-integrin α_6 binding to claw sole explant tissue in culture. Bound anti-integrin α_6 was detected by FITC-labelled anti-species antibody in cryosections of cultured tissue. e – epidermis, d – dermis, arrow – basement membrane, bv – capillary. Bar 35 μm.

Deterioration of the tissue in such circumstances appears, therefore, to reflect that in the equine hoof, where disruption of the hoof basement membrane has been noted in the early stages of laminitis (Pollitt, 1996). Basement membrane deterioration in diseased or dysfunctional bovine claw might then explain the appearance of blood-derived material in epidermal intercellular spaces and horny tissue in such circumstances. However, basement membrane integrity observed in healthy tissue is in contrast to the intercellular infiltration observed in otherwise healthy claws (Kempson & Logue, 1993).

MMPs are a family of related zinc- or calcium-stabilized endopeptidases. They are secreted as inactive pro-forms and are subsequently activated by proteolytic cleavage. Their production can be induced by some growth factors, cytokines (Murphy, 1995; Matrisan, 1992) and hormones, e.g. relaxin (Qin et al. 1997; Song et al. 2001) and progesterone (Lee et al. 2001). Elevated levels of MMPs have been associated with a number of physiological and pathological conditions involving tissue remodelling (Alexander & Werb, 1989; Salo et al. 1994). MMP 2 and MMP 9 are known to be involved in breakdown of collagen, and each has the basement membrane collagens type IV and V amongst its substrates (Nagase & Woessner, 1999; Matrisan, 1992). They promote cell detachment from the basement membrane during damage or wound repair in epithelia, allowing keratinocytes to migrate (Agren, 1994) and, in so doing, remove keratinocytes from the basement membrane signals that determine their terminal differentiation. MMP 9 is also associated with infiltrating cell types including neutrophils in inflammation (Li et al. 1999), while MMP 2 is associated with connective tissue cells and epithelia amongst other cell types. Thus, MMPs expressed constitutively have a role in adaptive remodelling of extracellular matrix in response to physiological stimuli, and are involved in the processes of both degeneration of tissue and its subsequent repair. It is not, therefore, unexpected that MMPs should be detected in normal bovine claw tissue, nor that the activities of these enzymes should be elevated at sites of tissue damage.

Increased MMP activity at sites of bovine hoof claw damage reflects recent observations in equine hoof tissue, where MMP 2 and MMP 9 activity was elevated during laminitis (Johnson et al. 1998; Pollitt & Daradka, 1998). In the case of the bovine claw, both pro- and active forms of MMP were detected in all conditions, but it was apparent that, at the site of ulceration, the enzymes were present predominantly in their active forms. Conversely, in healthy tissue, MMP levels were relatively low, and present

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predominantly as inactive, pro-enzyme. Diseased tissue, and also tissue at sites recovered from a sole lesion, represented an intermediate state, with a relatively high level of pro-enzyme available in the event of disease progression or, in the case of recovered tissue, presumably to assist solear regeneration if required.

The predominantly dermal location of MMP 2, and the punctate immunofluorescence indicative of localized concentration in ulcerated tissue, may reflect the presence of MMP-containing polymorphic neutrophils (PMN), which are known to secrete gelatinases, particularly MMP 9. It may be that, in the present study, immunofluorescence detected the presence of MMP 9 in ulcerated tissue, in addition to MMP 2. Although the anti-human antibody used was raised against MMP 2, it is possible that it might cross react with bovine MMP 9. Immunoblotting of bovine sole homogenates using one of these antibodies showed the strongest signal at the expected molecular weight of MMP 9 rather than MMP2 (KAK Hendry, unpublished observations). Infiltration of PMNs would be expected at sites of tissue trauma and inflammation, including ulcers, and elevation of MMP 9 seen in equine laminitis is reportedly due to the accumulation of PMN-derived MMP 9 in the dermis (Mungall et al. 1998; Mungall & Pollitt, 1999; Tarlton et al. 1997). Our microscopy evidence suggests that a similar situation exists in solear ulcers in the bovine claw. An infiltration of PMN and their secretion of MMP should aid the detachment of cells from the basement membrane, allowing them to participate in wound repair. MMP 9 is reported to be an indicator of chronic, infected and slow-healing post-operative wounds in human surgery (Tarlton et al. 1997). Therefore, alternatively, its elevation in the bovine claw might indicate the presence of chronic disease e.g. chronic sub clinical laminitis (Greenough, 1985).

Extracellular proteolysis catalysed by MMPs depends on the balance between active enzyme and the local concentrations of their inhibitors, TIMPs, which in normal physiological processes restrict MMP activity (Nagase & Woessner, 1999; Brew et al. 2000). For example, an imbalance of active MMPs and TIMPs has been associated with diseases involving breakdown and remodelling of the extracellular matrix, including rheumatoid arthritis and tumour invasion (Ishiguro et al. 1996; Stetler-Stevenson et al. 1993). The present study assayed TIMP 2, which inhibits the activity of all active MMPs and regulates the activation of pro-MMP 2 (Stetler-Stevenson et al. 1989; Stetler-Stevenson et al. 1993). ELISA detected both free TIMP 2 and TIMP 2 complexed to active forms of MMPs, and although there was considerable variation between animals, it was clear that TIMP 2 was depleted in claws that had a diseased area, the greatest reduction being found at ulcer sites. The balance between levels of protease and protease inhibitor had, therefore, shifted markedly in favour of increased MMP activity in the ulcerated tissue, and perhaps also in diseased tissue distal to ulcer sites.

An induction of MMP activity might well account for the loss of basement membrane integrity observed in ulcerated tissue. Circumstantial evidence suggests that this might lead to an impairment of keratin deposition in claw tissue. Specifically, keratinocytes in ulcerated bovine claw tissue were found to express basal undifferentiated keratinocyte type keratins in suprabasal locations (Hendry et al. 2001), and claw keratinocytes cultured in the absence of basement membrane express aberrant keratin types (Kitahara & Ogawa, 1994). A causal link between basement membrane integrity and keratinization has been established in other epithelia, where differentiated cell keratin types are not expressed until the establishment of integrin-mediated cell attachment to a basement membrane (Breitkreutz et al. 1997; Suter et al. 1997). Thus detachment of keratinocytes from the basement membrane would be expected to inhibit terminal differentiation of the keratinocytes and, indeed, may induce cell apoptosis (Meredith & Schwartz, 1997). To investigate a causal link between loss of basement membrane integrity and altered keratin deposition in bovine hoof epidermis, we examined the effects of antibodies to basement membrane signalling molecules. The effects, whilst equivocal with respect to the differing sensitivity of explant cultures to laminin and integrin function-blocking antibodies, suggested that, in some circumstances, impairment of basement membrane signalling can affect claw epidermal keratinization. The parameters chosen for assessment of antibody effect were protein synthesis and DNA synthesis, both of which we have previously observed to be altered *in vivo* in bovine claws, either as a result of lameness challenge (Hendry et al. 2001; MacCallum, 1999), or seasonally by factors as yet undefined (MacCallum, 1999). Targeting of laminin-integrin signalling was aimed at disrupting their function in assembly and adhesion of hemidesmosomes, which are numerous in the basement membrane of the claw dermal-epidermal junction (Hendry et al. 1995) and are likely, as in other keratinizing tissues, to play an important role in keratin filament assembly in the keratinocyte (Jones et al. 1991; Suter et al. 1997). Inhibition of protein synthesis by anti-laminin suggests that, in some circumstances, disruption of basement membrane signalling can alter protein, including keratin, synthesis in the bovine hoof. On the other hand, abrogation of laminin-mediated signalling had no effect on keratin localization in tissue explants (unpublished observations), unlike the re-distribution observed in ulcerated tissue *in vivo* (Hendry et al. 2001). This may reflect the redundancy inherent in signalling by basement membrane components (reviewed by Gille & Swerlick, 1996; Suter et al. 1997) or, alternatively, the realization of only partial inhibition of laminin signalling with this antibody concentration and duration of treatment. However, that disruption of basement signalling did influence keratinocyte protein synthesis to some extent suggests that MMP-mediated deterioration of the basement membrane may account, at least in part, for an impairment of keratin deposition that would promote lesion development.

In conclusion, we observed deterioration of the epidermal basement membrane in bovine claws associated with tissue ulceration. Deterioration of basement membrane is in turn

associated with altered keratinization of the epidermis, and is attributable to reciprocal changes in MMP and TIMP levels that favour extracellular proteolysis in ulcerated regions of the tissue. In addition, we observed changes in MMP and TIMP levels that may render apparently healthy tissue of diseased claws, and tissue of claws that had previously been damaged, more susceptible to extracellular proteolysis. This latent activity could promote loss of epidermal-dermal integrity during subsequent challenge by, for example, an imbalance in systemic calcium levels or local TIMP levels associated with physiological state such as pregnancy, or external challenge. This might provide a biochemical basis for the recurrence of ulcers in susceptible dairy cows during successive lactations.

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References

Agren MS 1994 Gelatinase activity during wound healing. *British Journal of Dermatology* **131** 634–640

Alexander CM & Werb Z 1989 Proteinases and extracellular remodeling. *Current Opinions in Cell Biology* **1** 974–982

Breitkreutz D, Stark HJ, Mirancea N, Tomakidi P, Steinbauer H & Fusenig NE 1997 Integrin and basement membrane normalization in mouse grafts of human keratinocytes. Implications for epidermal homeostasis. *Differentiation* **61** 195–209

Brew K, Dinakarpandian D & Nagase H 2000 Tissue inhibitors of metalloproteinases: Evolution, structure and function (I). *Biochimica et Biophysica Acta* **1477** 267–283

Gille J & Swerlick RA 1996 Integrins: Role in cell adhesion and communication. *Annals of the New York Academy of Sciences* **797** 93–106

Greenough PR 1985 The sub clinical laminitis syndrome. *The Bovine Practitioner* **20** 144–149

Hendry KAK, Knight CH, Galbraith H & Wilde CJ 2000 Basement membrane role in keratinization of healthy and diseased hooves *in* XIth International Symposium on Disorders of the Ruminant Digit & III International Conference on Bovine Lameness (Eds. CM Mortellaro, L De Vecchis & A Brizzi), pp. 128–130. University of Parma. Parma, Italy

Hendry KAK, Lancelott MJ, Knight CH, Kempson SA & Wilde CJ 1995 Protein synthesis in tissues cultured from the bovine hoof. *Cell and Tissue Research* **281** 93–99

Hendry KAK, MacCallum AJ, Knight CH & Wilde CJ 1999 Effect of endocrine and paracrine factors on protein synthesis and cell proliferation in bovine hoof tissue culture. *Journal of Dairy Research* **66** 23–33

Hendry KAK, MacCallum AJ, Knight CH & Wilde CJ 2001 Keratinocyte proliferentiation and differentiation in healthy and ulcerated bovine hoof epidermis. *Journal of Dairy Research* **68** 525–537

Ishiguro N, Ito T, Obata K, Fujimoto N & Iwata H 1996 Determination of stromelysin-1, 72 and 92 kDa type IV collagenase, tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-2 in synovial fluid and serum from patients with rheumatoid arthritis. *Journal of Rheumatology* **23** 1599–1604

Johnson PJ, Tyagi SC, Katwa LC, Ganjam VK, Moore LA, Kreeger JM & Messer NT 1998 Activation of extracellular matrix metalloproteinases in equine laminitis. *Veterinary Record* **142** 392–396

Jones JCR, Kurpakus MA, Cooper HM & Quaranta V 1991 A function for the integrin $\alpha_6\beta_4$ in the hemidesmosome. *Cell Regulation* **2** 427–438

Kempson SA & Logue DN 1993 Ultrastructural observations of hoof horn from dairy cows: Changes in the white line during the first lactation. *Veterinary Record* **321** 524–527

Kitahara T & Ogawa H 1994 Variation of differentiation in nail and bovine hoof cells. *Journal of Investigative Dermatology* **102** 725–729

Labarca C & Paigen K 1980 A simple, rapid and sensitive DNA assay procedure. *Analytical Biochemistry* **102** 344–352

Lee PPH, Hwang JJ, Mead L & Ip MM 2001 Functional role of matrix metalloproteinases (MMPs) in mammary epithelial cell development. *Jounal of Cellular Physiology* **188** 75–88

Li X, Zhao X & Ma S 1999 Secretion of 92 kDa gelatinase (mmp-9) by bovine neutrophils. *Veterinary Immunology and Immunopathology* **67** 247–258

MacCallum AJ 1999 A cell biological approach to studying lameness in the dairy cow. PhD thesis, University of Glasgow

Matrisan LM 1992 The matrix degrading metalloproteinases. *Bioessays* **14** 455–463

Meredith JEJ & Schwartz MA 1997 Integrins, adhesion and apoptosis. *Trends in Cell Biology* **7** 146–150

Mungall BA & Pollitt CC 1999 Zymographic analysis of equine laminitis. *Histochemistry and Cell Biology* **112** 467–472

Mungall BA, Pollitt CC & Collins R 1998 Localisation of gelatinase activity in epidermal hoof lamellae by in situ zymography. *Histochemistry and Cell Biology* **110** 535–540

Murphy G 1995 Matrix metalloproteinases and their inhibitors. *Acta Orthopaedica Scandinavica* (Supplement 266) **66** 55–60

Nagase H & Woessner JF 1999 Matrix metalloproteinases. *Journal of Biological Chemistry* **274** 21491–21494

Pollitt CC 1996 Basement membrane pathology: A feature of acute laminitis. *Equine Veterinary Journal* **28** 38–46

Pollitt CC & Daradka M 1998 Equine laminitis basement membrane pathology: Loss of type IV collagen, type VII collagen and laminin immunostaining. *Equine Veterinary Journal* Supplement **26** 139–144

Pollitt CC, Pass MA & Pollitt S 1998 Batimasttat (BB-94) inhibits matrix metalloproteases of equine laminitis. *Equine Veterinary Journal* Supplement **26** 119–124

Qin X, Chua PK, Ohira RH & Bryant-Greenwood GD 1997 An autocrine/paracrine role of human decidual relaxin. II. Stromelysin-1 (MMP-3) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). *Biology of Reproduction* **56** 812–820

Salo T, Makela M, Kylmaniemi M, Autio-Harmainen H & Larjava H 1994 Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Laboratory Investigation* **70** 176–182

Song L, Ryan PL, Porter DG & Coomber BL 2001 Effects of relaxin on matrix remodelling enzyme activity of cultures equine ovarian stromal cells. *Animal Reproduction Science* **66** 239–255

Stetler-Stevenson WG, Krutzsch HC & Liotta LA 1989 Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase family. *Journal of Biological Chemistry* **264** 17374–17378

Stetler-Stevenson WG, Liotta LA & Kleiner DEJ 1993 Extracellular matrix 6: Role of matrix metalloproteinases in tumor invasion and metastasis. *FASEB Journal* **7** 1434–1441

Suter MM, Crameri FM, Olivry T, Mueller E, von Tscharner C & Jensen PJ 1997 Keratinocyte biology and pathology. *Veterinary Dermatology* **8** 67–100

Tarlton JF, Vickery CJ, Leaper DJ & Bailey AJ 1997 Postsurgical wound progression monitored by temporal changes in the expression of matrix metalloproteinase-9. *British Journal of Dermatology* **137** 506–516