REVIEW ARTICLE

Laminitis in the dairy cow: a cell biological approach

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INTRODUCTION

Lameness is a major welfare concern in dairy cattle. Estimates of the annual incidence of lameness range from 4 to 30%, and even in well managed herds as many as 15% of animals can be affected (Esselmont, 1990). In addition to the cost in animal suffering, lameness is accompanied by loss of production on a scale comparable, in temperate countries, with that caused by mastitis. Lost production, veterinary charges and milk discard costs coupled with reduced fertility or premature culling in turn make lameness a major economic factor in dairy farming. In the UK alone, the estimated cost in lost production is £44–£90 million per annum, equivalent to £10–20 per cow (Booth, 1989; Esselmont, 1990).

Lameness is the ultimate manifestation of a variety of conditions which may have distinctly different origins. However, the majority of these conditions are associated to a lesser or greater degree with lesions within the horny tissue of the hoof (Logue *et al*. 1993) and, of these, one of the most common afflictions (especially in lactating dairy cows) is laminitis (Booth, 1989; Greenough, 1991; Vermunt & Greenough, 1994). Laminitis (*pododermatitis aseptica diffusa*) is essentially a consequence of poor quality horn. It is assumed to be the result of impaired synthesis or increased breakdown of keratins, the structural proteins of the hoof, with resultant deterioration of the macromolecular organization that confers the tissue's mechanical strength. The cellular and molecular processes of horn synthesis, its physiological regulation and the biochemical lesion(s) that precipitate laminitis are the subject of this review.

HOOF ANATOMY AND CELL BIOLOGY

Anatomy

Each horny claw of the hoof is composed of a wall and a sole, the junction between them being called the white line (Kempson & Logue, 1993*a*). The horn

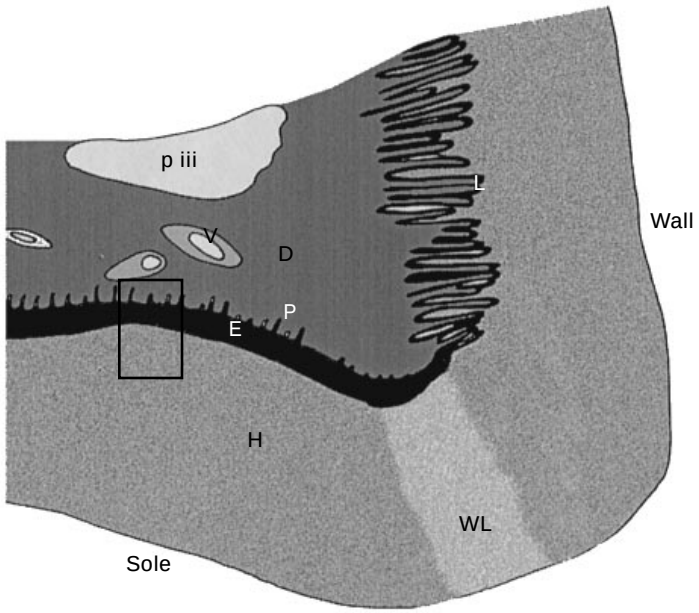

Fig. 1. Diagrammatic representation of cross section of bovine hoof. D, dermis; E, epidermis; H, horn; WL, white line; L, laminae; P, papillae; v, blood vessel; p iii, pedal bone iii. The boxed area is shown in Fig. 2 as a micrograph.

capsule is joined to the underlying connective tissue through epidermal and dermal folds of tissue. In the wall, these folds are regularly arranged and are termed laminae; in the sole they are irregular and called papillae (Fig. 1). Both structures are highly vascularized. They act as shock absorbers, dissipating the impact of the hoof's contact with the ground.

Horny tissue is generated by keratinization of cells generated in the basal (i.e. germinal) layer of the epidermal tissue. The germinal layer is located on the laminar or papillary surface of the dermis (Calhoun & Stimson, 1981), and is the sole site of cell division. All distal layers of the epidermis are derived from these cells by a process of differentiation (Fig. 2). During this process the columnar basal cells, which have apically situated oval nuclei, reorientate to lie perpendicular to the basal cell layer and become enlarged, flattened and polygonal (squamous) in shape. Keratinization involves the progressive replacement of most of the cell contents by keratin proteins, their macromolecular organization into tonofilaments, and subsequent incorporation into the cell cytoskeleton by intermediate filament-binding proteins (Budras *et al*. 1989; Grosenbaugh & Hood, 1993; Kempson & Logue, 1993*a*), such that the tonofilaments are aligned parallel to the long axis of the squame. Each squame is tightly bound to its neighbours by desmosomal intracellular junctions (Budras *et al*. 1989; Leach, 1993) and secretes a lipid-rich extracellular matrix in which mature keratinocytes become embedded (Elias, 1983; Grosenbaugh & Hood, 1993; Mülling *et al*. 1994). Horn is composed of tubular and intertubular arrangements of cells. The tubular horn is derived from the cells originating on the lamellar or papillar surface and intertubular horn is derived from those at the tips of the lamellae (Budras *et al*. 1989). The mechanical strength of the hoof is primarily dependent upon the integrity of the cell–cell interactions of the keratinocytes and the organization of keratin filaments within these cells (Fraser & McCrae, 1980).

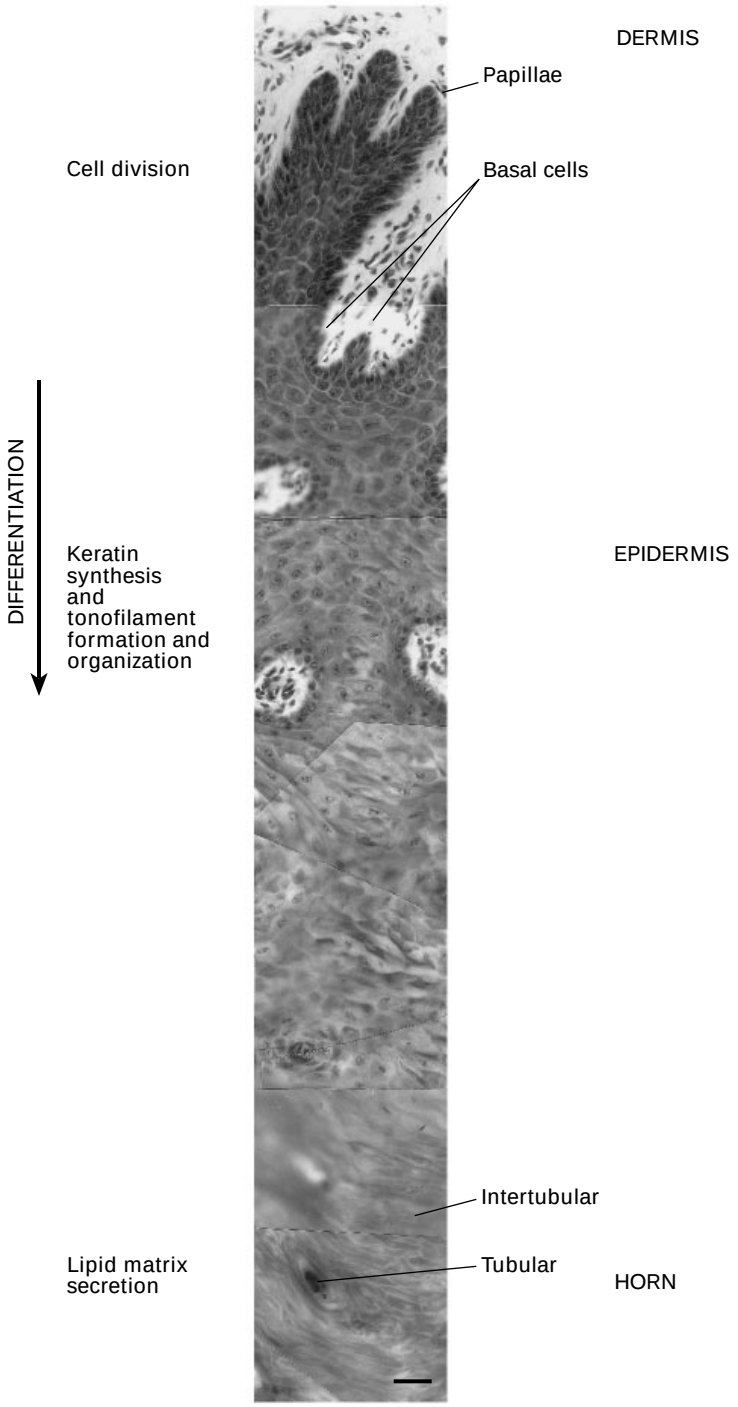

Fig. 2. Micrographic montage of the sole of the bovine hoof. Anatomical regions of the epithelium are labelled and the stages of keratinocyte differentiation indicated. Methylene blue stain; bar, 30 μm.

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Keratin structure

Keratin proteins differ widely in their physicochemical properties, including molecular mass (M_r 40000–70000) and isoelectric point, the latter serving to identify acidic (pI, 4·5–5·5) and basic (pI, 5·5–7·5) subtypes of the proteins. A principal characteristic of the keratins is their ability to form stable macromolecular structures by the heterodimerization of two molecules (one of each subtype) to form a coiled coil, which then complexes with another to form a four-polypeptide complex stabilized by disulphide bonds and hydrophobic interactions. Thus, acidic and basic subtypes are in equimolar proportions (Fuchs, 1983). Keratins are present in all epithelial cells. However, not all members may be represented in each cell type, and epithelia or regions thereof are characterized by specific expression of particular keratin subtypes (Lane *et al*. 1985; Smack *et al*. 1994). Similarly, in some pathological conditions diseased epithelium is distinguished by aberrant keratin expression. For example, in cases of human basal cell hyperplasia, basal cell keratins are expressed in suprabasal layers (Wetzels *et al*. 1991).

Hoof keratins

Immunohistochemical analysis of equine hoof tissue has shown different patterns of keratin subtype expression in the basal epidermis and the suprabasal keratinizing tissue (Pellman *et al*. 1993): cytokeratin 10 (CK10) is expressed preferentially in those epidermal keratinocytes generating intertubular horn and not in basal (i.e. germinal) cells nor in tubular horn, whereas CK4 is found principally in the basal and immediately suprabasal strata. CK14 is also localized in the germinal cell layer in healthy equine tissue but, notably, was detected suprabasally in tissue from horses with chronic laminitis (Grosenbaugh & Hood, 1992, 1993). Abnormal suprabasal CK14 expression is also observed in pathological conditions of epithelial cell hyperplasia (Wetzels *et al*. 1991).

CK14 and other common keratin subtypes, including CK1, CK6, CK7, CK10 and CK16, are also found in bovine hoof tissue (Kvedar *et al*. 1986; Kitahara & Ogawa, 1994). CK1 and CK10 are, as in equine hooves, suprabasally expressed (K. A. K. Hendry, unpublished results). However, the precise location of most of the bovine keratin subtypes is uncertain, either because observations were made in comparative studies of bovine horn and human nail bed differentiation, with human anatomical terms applied to both (Baden & Kubilus, 1984; Kitahara & Ogawa, 1994), or because the results are preliminary. We have detected CK14 in the epidermal cells of the sole but, unlike in the horse, expression is not solely restricted to the basal cells of the laminae (K. A. K. Hendry, unpublished studies; see Fig. 3). Bovine tissue also contains a group of low molecular mass acidic keratins named a1–4, which are localized in differentiating epidermal cells, and a hitherto uncharacterized basic keratin designated b2 (Kvedar *et al*. 1986). Novel low molecular mass keratin subtypes are also reported elsewhere, but again their precise location and structural role are uncertain because of the use of human rather than bovine anatomical descriptors (Baden & Kubilus, 1984; Kitahara & Ogawa, 1994).

Laminitis

Laminitis is the generic term for conditions in which the sensitive laminae of the hoof are damaged. In its most common subclinical form, locomotion may appear unaffected. Nevertheless, there are distinct changes in the claw horn, which typically

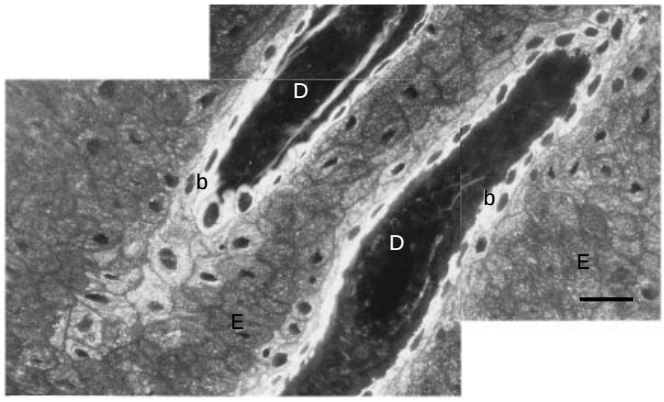

Fig. 3. Indirect immunofluorescent detection of cytokeratin 14 in basal and suprabasal epidermal cells in the bovine hoof. D, dermis; E, epidermis; b, basal cells. Bar, 30 μm.

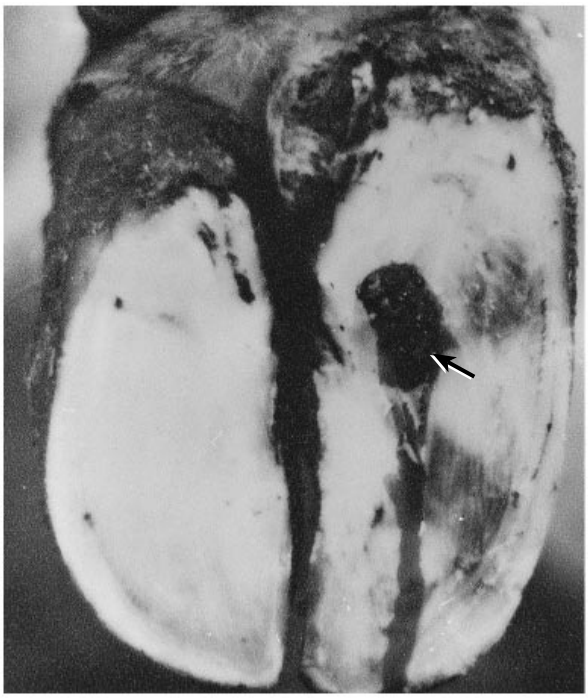

Fig. 4. Sole of a bovine hoof showing a solar lesion (arrowed).

becomes softer, yellowed and waxy. Softening of the horn makes the hoof prone to wear and damage, causing ulceration (Fig. 4), heel erosion and haemorrhaging in the weight-bearing surfaces, particularly the white line and toe. Subclinical laminitis can progress to the acute and chronic forms in which the claw becomes misshapen owing to abnormal growth, and the sole is typically flat and broadened, often with distinctive grooves and ridges (Greenough, 1987, 1991). Histological examination shows marked changes in the microvasculature of the dermal laminae resulting in oedema (Vermunt & Greenough, 1994) and distension and subsequent necrosis of adjacent epidermal laminae (Obel, 1948). At an ultrastructural level, the basal cells

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of the germinal layer appear abnormal, with large centrally located nuclei and vacuolated cytoplasm (Obel, 1948; Marks & Budras, 1987; Pollitt, 1996).

Gross biochemical examination indicates that poor horn quality in laminitic cows is due to a disruption of keratin metabolism in the epidermis (Obel, 1948; Marks & Budras, 1987) and a reduced proportion of cystine and methionine (MacLean, 1971). Electron microscopic examination of white line slivers from precalving heifers with poor quality horn showed that the normal organization of keratin fibres is disrupted and irregular (Kempson & Logue, 1993*a*, *b*). Impaired hoof keratinization appears, therefore, to be a key factor in the development of laminitis. Remarkably, however, there are no detailed descriptions published of the aetiology of the disease, the regulators of keratin deposition or the factors that cause impaired keratinization. In the following sections we consider the information available, and identify areas deserving detailed investigation.

KERATINIZATION

Experimental approaches

The dearth of information on hoof keratinization reflects both the difficulty of conducting detailed experiments *in vivo* and the absence of a culture system suitable for studying the processes of keratin synthesis and deposition. Hoof cell culture techniques adapted from other systems may maintain cell viability, but compromise the cells' differentiated function, including keratinization. Mixed cell preparations from hooves of healthy cattle, when cultured in suspension, incorporated radiolabelled methionine into cellular protein (Ekfalck *et al.* 1985*a*, *b*, 1988*a*), and this process was inhibited by serum and serum fractions from laminitic animals (Ekfalck *et al.* 1985*b*), suggesting that the process was responsive *in vitro* to pathophysiological influences. However, suspension cultures are short-lived, and by their nature lack the cell–cell contacts and interaction with basement membrane important for maintenance of differentiated function (see below). This deficiency has been addressed by culturing cells on feeder layers (Kubilus *et al.* 1979; Baden & Kubilus, 1983) or on collagen matrices (Kitahara & Ogawa, 1994), but on these substrata keratin subtype expression is different from that *in vivo*. A better approach is to culture hoof tissue as explants (Ekfalck *et al.* 1990; Hendry *et al.* 1995), thereby maintaining the cells' contact with each other and the extracellular matrix and also, potentially, retaining the autocrine, paracrine and juxtacrine regulation of cell function existing *in vivo*. We have prepared tissue explants by microdissection so as to contain the epidermal tissue responsible for keratin deposition and the adjacent regions of vascularized corium and horny hoof (Hendry *et al.* 1995). When cultured in a defined, serum-free medium, explants behaved *in vitro* as *in vivo*, both in terms of the identity and localization of synthesized protein and the site of cell proliferation. The rate of protein synthesis, measured by [^{35}S]methionine incorporation, was maintained for several days in culture (Table 1). Radiolabelled proteins were characterized by SDS-PAGE and fluorography and shown to be representative of the proteins found in hoof tissue. Three prominent radiolabelled bands were identified as keratins and actin by Western blotting. Immunohistochemistry showed that keratin was localized principally in the epidermal layers, and microautoradiography indicated that this was the major site of protein synthesis (Hendry *et al.* 1995; Fig. 5). In contrast, DNA synthesis, measured by incorporation of [^{3}H]thymidine, was localized to the germinal layer of the tissue, the site of cell proliferation *in vivo* (K. A. K. Hendry, unpublished results). By culturing explants on a semiporous membrane and

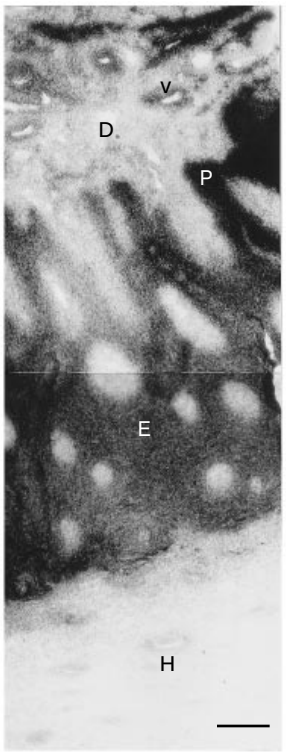

Fig. 5. Protein synthesis in cultured bovine hoof explants studied by incorporation of [^{35}S]methionine. ^{35}S-labelled protein was visualized by microautoradiography, and is localized predominantly in the epidermis. D, dermis; E, epidermis; H, horn; P, papillae; v, blood vessel. Bar, 130 μm. (From Hendry *et al.* 1995, © Springer-Verlag and reproduced by permission.)

including serum and hormones in the culture medium, the lifespan of explant cultures can be extended to several weeks (Ekfalck *et al.* 1990). Under these conditions, there is extensive cell proliferation, with outgrowth of keratinocytes at the dermal–basal cell junction. An alternative method for studying hoof tissue biochemistry *ex vivo* is to graft explants on to athymic mice (Kvedar *et al.* 1986). These explants retained the morphology of normal hoof tissue, produced cornified tissue and remained distinct from surrounding murine tissue. However, the types of keratins within the hoof tissue graft, while distinctly of bovine origin, differed from that of normal hoof tissue *in situ*. Therefore, at present the most useful technique for study of hoof cell biology appears in fact to be the most straightforward, i.e. explant culture, requiring only a source of tissue freshly obtained post mortem, and facilities for microdissection to obtain the epidermal and surrounding tissues.

Regulation by hormones and growth factors

Study of other keratinizing tissues has identified a number of potential regulators of hoof epidermal keratinization, including hormones (hydrocortisone, prolactin, thyroid hormones), growth factors (epidermal growth factor (EGF), transforming growth factors), micronutrients (vitamin D_3, retinoic acid) and cell microenvironment (for reviews, see Green, 1980; Ekfalck *et al.* 1991). There is as yet no definitive evidence to implicate any of these factors in the physiological regulation of hoof keratinization, with the possible exception of EGF. Receptors for this growth factor have been demonstrated in both bovine (Ekfalck *et al.* 1988*b*) and equine hoof tissue

Table 1. *Protein synthesis in cultured bovine hoof explants.* [^{35}S] *methionine incorporation*† *measured as trichloroacetic acid-precipitable radioactivity in tissue explant homogenates*

(Values are expressed as radioactivity incorporated, dpm/μg DNA, means ± SEM for $n = 9$)

Time, h		
0	24	48
154 ± 63	245 ± 53	335 ± 94

† Incorporation was measured by pulse labelling at the times indicated (Hendry *et al.* 1995).

(Grosenbaugh *et al.* 1991), but in different locations. We have observed EGF binding throughout the epidermis in bovine hoof tissue, and found that this growth factor stimulates protein synthesis in hoof tissue explants (K. A. K. Hendry, unpublished results). In equine tissue, receptors are located mainly in the basal region, and EGF is reported to suppress keratin expression in healthy tissue (Grosenbaugh *et al.* 1991). It should be noted, however, that an altered (i.e. more extensive) epidermal distribution of EGF receptors in laminitic equine hooves is associated with a stimulatory effect of EGF in tissue explants from some such animals (Grosenbaugh *et al.* 1991). Thus, the contrasting EGF responsiveness of bovine and equine tissue probably reflects the tissue localization of growth factor receptors in the two species.

Preliminary experiments show that hoof tissue explants respond not only to EGF but also to exogenous hormones, including those whose characters are likely to change during pregnancy and lactation. Prolactin, a key lactogenic hormone in ruminants (see Cowie *et al.* 1980), decreased protein synthesis of bovine hoof explants (K. A. K. Hendry, unpublished results). Hydrocortisone, a potent stimulator of growth and inhibitor of differentiation in cultured keratinocytes (Rheinwald & Green, 1975), also inhibited protein synthesis in explant culture (K. A. K. Hendry, unpublished results). Glucocorticoid concentrations are reported to be elevated during lactation (Wagner *et al.* 1972; Wagner & Oxenreider, 1972) and are higher in high yielding cattle (Johnson & Vanjonack, 1976). Moreover, the chronic pain and stress of lameness are associated with elevated corticosteroid levels in sheep (Ley *et al.* 1994), although not in cattle (Ley *et al.* 1996), and steroid treatment is reported to exacerbate equine laminitis (Milne, 1985). Insulin at physiological concentrations stimulated both protein and DNA synthesis *in vitro*, so that the decrease in systemic insulin during lactation (see Cowie *et al.* 1980) and the inverse relationship between circulating insulin and animal productivity (Hart *et al.* 1978) would be predicted to compromise hoof keratinization. On the other hand, circulating hormone concentration is not necessarily an indicator of tissue response. Although we have demonstrated the presence of insulin receptors in hoof tissue sections by binding of fluorescently labelled hormone (K. A. K. Hendry, unpublished results), the tissue may nevertheless share the insulin resistance shown during lactation by other tissues, including adipose tissue and skeletal muscle (see Vernon, 1988). Overfeeding during the dry period gives rise to hyperinsulinaemia and hyperglycaemia (two classic signs of insulin resistance) in early lactation (Holtenius *et al.* 1996). High energy feeding also appears to predispose cows to laminitis (for review, see Vermunt & Greenough, 1994). If, however, the preliminary findings from hoof explant cultures are confirmed, the generally adverse effect of key lactogenic hormones on germinal cell proliferation and keratin synthesis appears to provide an endocrine basis for the increased susceptibility of lactating animals to lameness.

Demonstration of endocrine regulation will, of course, ultimately depend on experiments *in vivo* to determine the physiological interaction of putative regulatory hormones. However, hoof tissue explant culture arguably offers a means of screening individual hormones, investigating their likely interaction in the hormonal milieu of the hoof tissue and, not least, the interplay between endocrine regulation and locally active factors. For example, steroid hormones elevated during pregnancy may influence local production of EGF, as in other bovine tissues (Plaut, 1993). Similarly, a high systemic prolactin concentration in lactation could inhibit EGF-dependent cell differentiation, as it does in mammary tissue (Fenton & Sheffield, 1993).

Tissue microenvironment

The basement membrane forms a continuous partition between the dermis and epidermis of the hoof. In normal hoof tissue, it is bound by a network of crosslinking fibrils to the underlying connective tissue of the dermis (Pollitt, 1994), and performs a structural function, anchoring and promoting cytoskeletal organization of the epidermal cells. This structural organization is consistently seen to deteriorate in both equine (Pollitt, 1996) and porcine laminitis (Kempson & Johnston, 1990). In laminitic equine hooves, there is a progressive separation of the basement membrane from the epidermal laminae, beginning at the tips of the secondary epidermal laminae and culminating in complete dissociation of the primary epidermal and dermal laminae, so as to leave isolated fragments previously associated with the tips of epidermal laminae (Pollitt, 1996). The extent of basement membrane disruption is related to the severity of laminitis. Moreover, basement membrane damage and failed attachment of basal epidermal cells are among the first detectable pathological events in these animals; they occur in the absence of oedema and appear to precipitate collapse of the lamellar architecture of the hoof. Early deterioration of the epidermal basement membrane is also observed in precalving heifers, allowing infiltration of amorphous extracellular material between adjacent squames (Kempson & Logue, 1993*b*; Logue *et al*. 1993). The cattle in this study were clinically sound, but had poor quality horn exhibiting disorganized keratin fibres and irregular arrangement of the squamous epidermal cells, and these animals were subsequently most susceptible to lameness during lactation.

On this evidence, basement membrane disruption could be a causative factor in development of laminitis, rather than the result of a primary lesion elsewhere. Study of other tissues supports such a role, in that basement membrane performs not only a structural role but a signalling function, and is indeed essential for proper differentiation of many epithelial tissues. Accordingly, without the presence of a basement membrane in hoof cell or tissue cultures, keratinocyte proliferation is inhibited (Ekfalck *et al*. 1990) and an abnormal pattern of keratin subtypes is produced (Kitahara & Ogawa, 1994). Conversely, the structural and biochemical fidelity of hoof tissue explants may reside, at least in part, in their retention of basement membrane elements essential for epidermal anchorage and differentiation (Ekfalck *et al*. 1990; Hendry *et al*. 1995).

AETIOLOGY OF LAMINITIS

It is apparent from the foregoing discussion that changes in hoof morphology and epidermal cell ultrastructure occur in advance of any detectable clinical signs of laminitis. Precalving heifers with poor quality horn are more likely to contract laminitis during lactation (Kempson & Logue, 1993*b*; Logue *et al*. 1993). Thus, the

high incidence of bovine lameness in early lactation may stem from earlier, predisposing biochemical events, but be precipitated by calving or changes in husbandry or animal physiology associated with parturition and lactogenesis (Kempson & Logue, 1993*b*). The fact that many lame cattle can substantially recover later in lactation and then relapse in the following lactation also suggests that the endocrine and metabolic demands of pregnancy and early lactation adversely affect horn growth (Logue *et al*. 1993). Similarly, in the ewe, keratinization in the form of wool fibre diameter, tensile strength and sulphur content are all depressed during pregnancy and lactation (Masters *et al*. 1993). Beyond this, however, the aetiology of laminitis remains poorly defined. At the tissue level, while ultrastructural study of bovine and equine hooves provides persuasive evidence that loss of basement membrane integrity is an important contributory factor, this may arguably be secondary to capillary bed damage or a general impairment of blood circulation (Nilsson, 1963; Edwards, 1981; Greenough *et al*. 1990; Vermunt, 1996). Indeed, there is considerable evidence relating laminitis to dietary deficiency (e.g. retinoic acid, vitamin D_3), nutrient supply (possibly mediated by histamine, serotonin or nitric oxide) or local accumulation of toxic metabolites such as lactic acid or endotoxins (see, for example, Nilsson, 1963; Maclean, 1965, 1966; Edwards, 1981; Hinckley *et al*. 1996).

Looking forward

Elucidation of the cellular basis of laminitis and its aetiology will depend in part on detailed examination *in vitro*. As already discussed, recent development of a tissue culture system that reflects the tissue's performance *in vivo* allows examination of the regulatory roles of hormones, growth factors and basement membrane in hoof development. Supplementation or depletion of macronutrients or micronutrients in hoof tissue culture media also provides a convenient method for screening other systemic factors for a direct effect on epidermal keratinization. In the longer term, this culture system should also allow examination of the factors that regulate keratin gene expression (for review, see Blumenberg *et al*. 1993), and the downstream processing of keratin into mature keratin fibres, events mediated by small, cysteine-rich intermediate filament-associated proteins (Smack *et al*. 1994). These proteins have been identified in the equine hoof, and are particularly abundant in the more keratinized hoof wall tissue (Grosenbaugh & Hood, 1992). Significantly, this was also the major site of [^{35}S]cysteine incorporation both *in vivo* (Larsson *et al*. 1956) and in hoof cultures (Grosenbaugh & Hood, 1992), raising the possibility that deficiency of this amino acid could, by limiting synthesis of intermediate filament-associated proteins, disrupt squame formation despite a normal rate of keratin synthesis.

Clearly, however, detailed longitudinal studies *in vivo* will also be necessary in order to identify the hierarchy of causative factors. This is now practicable: the advent of a procedure for hoof tissue biopsy (Singh *et al*. 1993) makes possible non-terminal tissue collection, and allows serial sampling of individual animals in longitudinal studies. We have recently combined this biopsy technique with tissue explant culture to examine the influence of pregnancy and lactation on hoof tissue biochemistry. Preliminary results indicate that hoof protein synthesis and cell proliferation vary through the lactation cycle. Further analysis will, it is hoped, relate this developmental pattern to the endocrine status of the animals. These initial results illustrate the potential value of such an approach, particularly if combined with manipulative treatment *in vivo* and detailed examination of developmental

regulation *in vitro*. With this dual approach, there should in due course be the possibility of developing effective rather than empirical treatment of laminitis, so as to improve the welfare of dairy cattle.

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