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Studies on the Cytokeratins of the Equine Hoof Wall, Chestnut and Skin, with Special Reference to Laminitis

Ove Wattle

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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av

Ove Wattle

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Abstract

Inhibited differentiation of the hoof keratinocytes has been proposed as a mechanism underlying the epidermal histopathology of equine laminitis. The aim of this thesis was to carry out morphological studies on the keratinocytes and immunohistochemical and biochemical studies on the cytokeratins of the equine hoof wall and chestnut in order to elucidate the differentiation of these cells in an acute attack of spontaneous laminitis.

The investigation comprised four studies. Study I was a histopathological analysis of the hoof wall, skin and chestnut in horses with spontaneous laminitis in the acute phase, i.e. within 48 hours of onset of lameness, and in horses with no symptoms of laminitis, with special emphasis on the epidermal basal cells. In studies II-IV, in the same groups of horses, an analysis was made of the cytokeratin composition of the corresponding tissues and of the cytokeratin distribution in these tissues with the aid of one- and two-dimensional electrophoresis and immunohistochemistry, respectively.

In horses with acute laminitis, inhibited differentiation of keratinocytes in the zone of cornification of the hoof wall and chestnut was observed morphologically. Further, there was an increased rate of proliferation in the stratum internum of the hoof wall as well as in the strata medium and externum and to some extent also in the chestnut. Moreover, in those horses with laminitis with inhibited differentiation of keratinocytes in the zone of cornification of the hoof wall and chestnut, the basal cell layer was affected at the same

time. The bio- and immunohistochemical studies in horses with laminitis showed that the types of cytokeratins present in the hoof wall and chestnut were identical to those in normal horses, but there was a change regarding the cell layers in which they were expressed.

Taken together, the increased rate of proliferation among epidermal cells, the change regarding the cell layers in which cytokeratins were expressed, the less differentiated appearance of the suprabasal cells and the probably primary changes in the zone of cornification that were observed early in acute spontaneous laminitis indicate that primary inhibition of differentiation of the hoof keratinocytes occurs in laminitis.

The observed change in the tissue distribution of cytokeratins was most likely caused by an increased proliferation among the epidermal cells. No "new" types of cytokeratins were produced in the tissues examined; hence it appears natural to consider the role of the cysteine-rich keratin-associated proteins in acute laminitis, and this issue is discussed in the present thesis.

Key words: Equine laminitis, lameness, hoof, cytokeratins, two-dimensional electrophoresis, immunohistochemistry, histopathology.

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Errata

- I regret the quality of the illustrations, which in print have not reached the standard of the originals. This has meant that some details are now not clearly discernible.

- The abstract in the thesis should be the same as the abstract on the loose page, but unfortunately there are some differences. It is the abstract on the loose page that should apply.

- p. 12. par. 3, line 5. The bracket after "rings" should be deleted.
  p. 14. par. 3, line 4 and 5, "Cardamine pratensis" should read "*Cardamine pratensis*" and "Berteroa incana" should read "*Berteroa incana*".
  p. 16. par. , line 15, "Streptococcus bovis" should read "*Streptococcus bovis*".
  p. 29. In reference "Katwa LC ...." The title is unfortunately missing. It should read "Expression of endothelin in equine laminitis".
  p. 29. Reference "Minnick PD, Brown CM" should read "Minnick PD, Brown CM, Braselton WE, Meerdink GL, Slanker MR"

- Paper 1. p. 1. Title: Line 4, "layer" should read "layers".
  p. 4. par. 1. Line 11, "• 48 hours" should read "< 48 hours".
  p. 5. Table 1. Horse 1, column 4. Line 2, " 6/4" should read " 26/4".
  p. 10. Figure 2. On lines 6 – 11, "•m" should read " µm".
  p. 25. Figure 6. On line 6, "•m" should read " µm".
- Paper 2. p. 68. column 2, line 3. "1; 1.5h" should read 1 - 1.5 h".
  p. 76. Figure B should be called Figure C. Figure C should be called Figure B.
  p. 77. Legend to Fig 6 a-n. Line 12, " Sagittal" should read " (g) Sagittal".
- Paper 3. p7. Legend to Figure 1 b. Line 1, "coronet" should read "coronary band".
  p. 18. Reference number 5 should read 4 and vice versa.
- Paper 4. p. 13. The legend to figure 6 a – e is unfortunately missing. It should read 6 (a-e): Cross-sections from the stratum internum. AE1/AE3 Mab.

(a) Control horse, Ip level. Central part of the laminar layer. Positive reaction with basal and suprabasal cells (bar = 63 µm).

(b) Control horse, Id level. Positive reaction with basal cells (bar = 20 µm).

(c) Horse 1, Id level. Relatively small morphological changes such as slightly stretched SEL with round appearance of basal cell nuclei; compare (b). Positive reaction with basal cells (bar = 20 µm).

(d) Horse 2, Id level. Innermost part of the displaced laminar layer. Positive reaction with basal and suprabasal cells (bar = 63 µm).

(e) Horse 1, Ip level. Outermost part of the laminar layer. Hyperplasia and cellular oedema are observed among the epidermal cells (curved open white arrow). Positive reaction in suprabasal cells except for the 2-3 cell layers adjacent to the cornified PEL. These latter 2-3 cell layers correspond to the normal number of suprabasal cells in this position at the Ip level (bar = 63 µm).

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Abstract

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The investigation comprised four studies. Study I was a histopathological analysis of the hoof wall, skin and chestnut in horses with spontaneous laminitis in the acute phase, i.e. within 48 hours of onset of lameness, and in horses with no symptoms of laminitis, with special emphasis on the epidermal basal cells. In studies II-IV, in the same groups of horses, an analysis was made of the cytokeratin composition of the corresponding tissues and of the cytokeratin distribution in these tissues with the aid of one- and two-dimensional electrophoresis and immunohistochemistry, respectively.

The bio- and immunohistochemical studies in horses with laminitis showed that the types of cytokeratins present in the hoof wall and chestnut were identical to those in normal horses, but there was a change regarding the cell layers in which they were expressed. In horses with acute laminitis, inhibited differentiation of keratinocytes in the zone of cornification of the hoof wall and chestnut was observed morphologically. Further, there was an increased rate of proliferation in the stratum internum of the hoof wall as well as in the strata medium and externum and to some extent also in the chestnut. Moreover, in those horses with laminitis with inhibited differentiation of keratinocytes in the zone of cornification of the hoof wall and chestnut, the basal cell layer was affected at the same time.

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Key words: Equine laminitis, lameness, hoof, cytokeratins, two-dimensional electrophoresis, immunohistochemistry, histopathology.

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Till Sebastian och Jonathan
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till minnet av min Far Evert

Contents

Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I Wattle O. and Funkquist B. 2000. Histopathology of equine hoof wall, skin and chestnut in acute spontaneous laminitis, with special reference to the living epidermal cell layer. Manuscript.

II. Wattle O. 1998. Cytokeratins of the equine hoof wall, chestnut, and skin: bio- and immunohisto-chemistry. Equine Vet. J. Suppl, 26: 66-80.

III. Wattle O. 2000. Cytokeratins of the matrices of the chestnut and periople in acute laminitis. Accepted for publication in Am. J. Vet. Res.

IV Wattle O. 2000. Cytokeratins of the stratum medium and stratum internum of the equine hoof wall in acute laminitis. Accepted for publication in Acta vet. Scand. 41: xx-xx.

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Abbreviations

The following abbreviations are used in the text:

PEL	primary epidermal laminae
SEL	secondary epidermal laminae
PDL	primary dermal laminae
SDL	secondary dermal laminae
Mab	monoclonal antibody
zoc	zone of cornification
M_r	molecular weight

The following terms are used in the text for the anatomy of the hoof wall

Stratum externum = periople
Stratum medium
Stratum internum = stratum lamellatum ≈ PEL + SEL
Laminar layer ≈ PEL + SEL + PDL + SDL

Introduction

Basic concepts of laminitis

The term laminitis refers to the inflammation that can be seen in the laminar region of the hoof after the condition has become manifest. Equine laminitis is a common cause of lameness, and affected horses run a considerable risk of chronic lameness. In severe cases, the terminal result of laminitis may be a permanent displacement of the third phalanx, the dermal laminae and the deepest layer of the epidermis (Fig. 1).

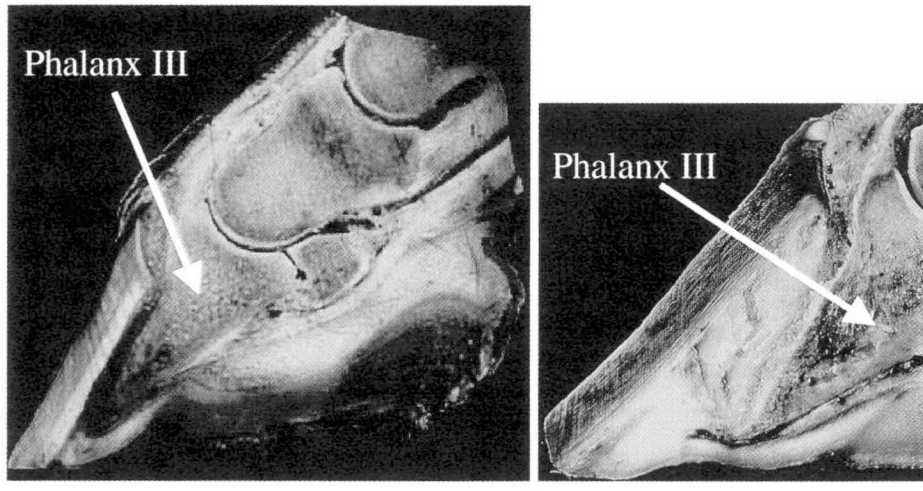

Figure 1. A sagittal section of the equine hoof. The photograph on the left shows a normal hoof. The right-hand photograph shows the front part of a hoof affected with laminitis. The third phalanx is vertical and rotationally displaced.

The proposed cause of this displacement is that the epidermal laminae are weakened to such an extent that they are no longer able to support the body weight (Obel 1948), leading to an elongation of the laminar layer. The clinical picture of laminitis is a horse showing characteristic signs of lameness with warm feet and increased pulsation of the digital arteries. In addition, fever is often present in the acute phase (Möller 1880; Obel 1948).
To facilitate comparisons between horses, Obel (1948) established a grading system for classification of the clinical signs of lameness:

1. In a standing position, the horse lifts its feet incessantly, often at intervals of but a few seconds ("paddling"). At a walking pace it does not show any lameness but the trotting gait is short and stilty.
2. The horse moves quite willingly at a walking pace but the gait is characteristic for laminitis. A forefoot may be lifted without difficulty.

3. The horse moves most reluctantly and vigorously resists attempts to lift a forefoot.
4. At this stage, the horse does not move without being forced to.

The disease can be divided into three phases- developmental, acute and chronic. The first and the third phases can be defined, but the definition of the acute phase is more obscure.
The "developmental phase" is the period between the initial causative insult and the first appearance of acute lameness identifiable as laminitis. This phase is for natural reasons observable only in studies of experimentally induced laminitis. Depending on the method used to induce the disease, the developmental phase has been reported to vary between approximately 12 hours (Galey et al. 1991) and 60 hours (Trout et al. 1990)

The term "chronic laminitis" is used for horses that after the end of the acute phase show clinical or radiographic evidence of displacement of the third phalanx. The clinical evidence includes depression of the dorsal coronary band, dropping of the sole, widening of the white line at the solar surface, and "founder rings"). Although radiographic methods for evaluating the degree of dislocation of the third phalanx have been described (Linford 1987; Cripps and Eustace 1999), it is difficult, to identify mild dislocations even on radiographs (Baxter 1986; Cripps and Eustace 1999).

The "acute phase" has been considered to last until there is evidence of displacement of the third phalanx or until a certain number of hours have elapsed since the first signs of lameness, for instance 48 hours (Stashak 1987) or 72 hours (Hood 1999a). The 72-hour duration of the acute phase has been based on clinical experience, indicating that if a patient survives this long without suffering mechanical or structural failure of the foot, full recovery is most likely to occur (Hood 1999a). However, since elongation of the laminar layer already occurs during the developmental phase of experimentally induced laminitis (Linford 1987), it is difficult to determine the duration of the acute phase, and thereby set a time-limit for this phase. Even though it is not obvious on radiographs of the hoof taken in the acute phase, it is reasonable to believe that elongation of the laminar layer cannot occur without concurrent rotation or "sinking" of the third phalanx. Moreover, the degree of severity of the morphological changes in the laminar layer varies between hooves within the same horse (Pollitt 1996), a fact that might make it even more difficult to decide whether the horse is suffering from acute or chronic laminitis.

Even though a majority of horses affected with laminitis are able to return to athletic soundness, 26-40 horses/year out of 10,000 insured horses (correlated to the total population of horses in Sweden this means about 520-800 horses/year) are subjected to euthanasia because of chronic laminitis (figures based on statistics from the Swedish insurance company AGRIA for the years 1980-1989, personal communication).

The terminology of the disease from a historical view

Laminitis has been referred to in the literature for a number of centuries. The ancient Greeks described a disease with clinical symptoms that resemble those of laminitis, which they named χριθιασις *(krithiasis)*, after the Greek word χριθ'η (krithé = barley), which was one suspected aetiological factor in cases of surfeit (Aristotle; Ruellius 1530). The disease has been named after barley in other languages also, for instance, *hordeatio* (Latin) (Ruellius 1530) *l´orzuolo* (Italian) (Möller 1880) and *fourbure* (French), which according to Huzard fils (1820, cited by Möller 1880) is the result of a corruption of the Latin word Hordeum to Forbeum. The Italian chief veterinarian Jordanus Ruffus (-1256), who explained the disease from a physical point of view in claiming that the circulating body fluids sank down to the hooves, called the disease *infusio* (cited by Molin 1818). In the older Germanic literature the disease was mainly named after its characteristic symptoms. For instance, the German farrier and veterinarian Martin Böhmes (1559-1636) used the name *Rehe* for the disease (Behm 1648), whereas in the English literature the name *founder* (Markham 1662) or *morfounde* (Wagner and Heymering 1999) was applied. Since the older Swedish literature in which the disease was mentioned often consisted of translations of German works, the word Rehe was used to describe this disease (Behm 1648; Robertson 1772). However, in the late 18th century the name "Rehe" was replaced by the word *fång* in the Swedish literature (von Sind 1774; Swederus 1794), sometimes together with the word *förfångad* (Berling 1776). The Swedish word *fång,* the word *forfangenhed*(t) used in Denmark and Norway, the Dutch word *hoefbevangenheid* and the German term *Verfangen* all indicate the stiff movements of a horse with laminitis (Åkerblom 1977). The first known monograph concerning "Rehkrankheit der Pferde" was written by a German colonel named von Sind in 1768, and the first detailed description of sections from horses with laminitis to be found in the literature is said to have been made by Jakob Clark (1777, cited by Gutenäcker 1901). In the 18th century the term *laminitis* was also first applied in reference to the disease (Wagner and Heymering 1999).

The names mentioned above are only a limited number of all names given to the disease, based on suspected aetiological factors, clinical signs and histopathological features that have been described in the literature. The word laminitis has come to be used in several countries and to be referred to the entire syndrome and not just to the inflammation of the laminar layer. Moreover, nowadays the terms chronic laminitis and founder are used synonymously.

Predisposing factors, a historical review

Ever since the ancient Greeks associated the disease with indigestion, laminitis of alimentary origin has been discussed in the literature (Aristotle; Ruellius 1530). However, "new" suspected aetiological factors have gradually been proposed over the years. Vegetius (450, cited by Gutenäcker 1901) mentioned mechanical factors and Ruffus (cited by Molin 1818) concluded that laminitis could also be

caused by over-intake of water and develop as a consequence of other diseases. In Hippiatria (1555) the disease was divided into *Futter-, Wind-* and *Wasserrehe.* Fanser (1576, cited by Möller 1880) added *Blutrehe* to this list. If a horse that had been ridden or driven hard was suffered to stand in the cold or if a cold wind struck the horse during hard work, it could become affected with *Windrehe.* If a sweaty horse were ridden or driven in cold water, especially if the water reached the chest or abdomen, or if it were given cold water to drink, it could become affected with *Wasserrehe* (Swederus 1794). According to Spinola (1858, cited by Möller 1880), water from certain wells implied a higher risk for *Wasserrehe.* The disease refered to as *windrehe* was later considered a disease of its own, i.e. *"tying up"* (rhabdomyolysis)(Möller 1880; Tidholm 1888). Von Sind (1768) added *Stallrehe* to the list of different variants of laminitis, but he regarded the different types of Rehe as being one disease (Möller 1880). In 1677, Solleysel (cited by Garsault 1797) expressed his opinion that laminitis was a rheumatic disease. This idea seemed to have lost supporters in the early 19th century when several authors considered that the disease was mainly focused in the hoof (Möller 1880). Several decades later laminitis was described as a disease in which different aetiological factors, through different pathways, caused a pododermatitis (Möller 1880; Eberlein 1908). However, the term rheumatic laminitis was used off and on in the literature in connection with the systemic symptoms (Åkerblom 1977). According to Eberlein (1908), three principal forms of laminitis can be seen: Laminitis caused by concussion or mechanical overloading of the laminar layer; laminitis caused by a toxic or chemical agent (i.e. through feedstuff); or metastatic laminitis (i.e. secondary to another affliction, such as pneumonia, abortion or post-partum complications). In the 20th century "new" predisposing factors were proposed, such as endocrine disorders and drug treatments (Eyre et al. 1979; Stashak 1987). However, the major suspected causative factors are still found within the above- mentioned categories. A modern literature overview of suspected aetiological factors of spontaneous laminitis is given below:

- Mechanical overload of the foot: Excessive unilateral weight-bearing (Peloso et al. 1996) and road founder (Hood 1999b).
- Alimentary factors: for instance overfeeding with cereals (especially fresh cereals), lush growing grass or legumes; or consumption of products containing toxic substances, such as mould (Hood 1999b), fresh black walnut shavings (True et al 1978) or the flower Cardamine pratensis in a fresh condition (Stottmeister 1901). The flower Berteroa incana has also been considered to cause laminitis (Geor et al. 1992).
- Disease processes involving another body system: for instance proximal enteritis (Cohen et al 1994), colitis, small intestinal strangulation/obstruction, puerperal metritis, myositis and pulmonary diseases (Hood 1999b), with laminitis as a possible complication.

In experiments conducted to study the pathogenesis of alimentary laminitis and the therapeutic effect of medications and treatments, the disease has been induced by feeding water mixed with fresh rye (Durcio 1840, cited by Möller 1880), fresh rye and colibacilli (Åkerblom 1934; Obel 1948), a "compound" of 85%

carbohydrate and 15% fibre (Coffman et al. 1970), a combination of 85% corn starch and 15% wood cellulose flour (Garner et al. 1975), a combination of 85% ground maize, 13% soybean meal and 2% minerals and vitamins (Rowe et al. 1994), and ground wheat (Pollitt and Davies 1998). These methods are nowadays classified as carbohydrate overload models. Laminitis has also been induced experimentally by feeding fresh Cardamine pratensis (Stottmeister 1901) and water extract of fresh black walnut shavings (Minnick et al. 1987; Galey et al. 1991). The exact nature of the laminitis-triggering factors and the mechanism of development of the disease spontaneously or through the experimental methods mentioned above, have not been established but a number of different hypotheses concerning the pathogenesis have been put forward. A summary of some of the theories is given below.

Hypotheses on the development of the early laminar changes

A widely spread concept of the pathogenesis of alimentary laminitis is that the primary problem lies in dysfunction of the digital vasculature accompanied by shunting of blood away from the laminar capillaries via arteriovenous anastomoses and a digital ischaemia that in turn causes degeneration of the laminae. This hypothesis is based on studies with angiographic (Coffman et al 1970) and scintigraphic techniques (Hood et al 1978). The following have all been suggested as possible causes of reduced blood perfusion in the laminar region: a digital vasospasm (Hood et al. 1993; Hood 1999b) possibly caused by vasoactive peptides such as 5-hydroxytryptamine (Bailey and Elliott 1998) and endothelin (Katwa et al 1999); a digital venoconstriction causing an increase in capillary pressure (Allen et al 1990; Hunt 1991; Eaton et al. 1995); and microthrombosis (Weiss 1997). The role of endotoxaemia caused by gastro-intestinal disorders (Garner 1980) or different infections (Baxter 1994) has been discussed in connection with the development of laminar ischaemia. However, endotoxinaemia cannot be consistently detected during the developmental phase of experimentally induced laminitis (Weiss 1997), and endotoxin does not induce laminitis when injected intravenously (Hood 1984). A previously proposed theory of vascular damage is that of histamine-produced capillary damage (Åkerblom 1934); but like endotoxin histamine has not induced laminitis when injected intravenously (Åkerblom and Sjöberg 1938). It is worthy of mention in this context that the results of direct measurement of digital blood flow (Robinson et al. 1976) in acute laminitis, and of non-invasive scintigraphic studies (Trout et al 1990) of digital blood flow and of measurements of hoof wall temperature (Pollitt and Davies 1998) during the developmental phase, did not support lamellar ischaemia as a primary cause of the disease.

Pollitt (1996) introduced a new theory for the pathogenesis of laminitis. In the early stage of experimentally induced laminitis he observed detachment between the epidermal basal cells and their basement membrane and considered that activation of laminar enzymes, which lyse the attachments between the distal phalanx and the inner hoof wall, might explain the morphological changes seen in the laminar layer in acute laminitis. Pollitt and co-workers have found an increase

in the production of metalloproteinases, which are capable of destroying the cell to cell, and cell to basement membrane attachment (Pollitt 1999). The laminar basal and parabasal cells lose their normal shape, become elongated and appear to slide over one another, and as a consequence the secondary epidermal laminae (SEL) become elongated. The laminar basement membrane loses its attachment to the basal cells initially at the part of the SEL that is closest to the primary epidermal laminae (PEL). The detachment of the basement membrane appears to progress from the epidermal side and the sheets of loose lamellar basement membrane remain attached to the connective tissue (Pollitt 1999). During displacement of the basement membrane and the connective tissue between the SEL, the laminar capillaries are affected, which may explain the increased resistance to blood flow mentioned above (Allen et al 1990; Hunt 1991; Eaton et al. 1995) and this might also explain why blood was bypassing the capillary bed through dilated arteriovenous anastomoses in the study of Hood et al. (1978). Streptococcus bovis, which is one of the microorganisms responsible for the rapid fermentation of carbohydrate to lactic acid in the equine hindgut, has been suggested as a potential metalloproteinase activator in acute laminitis (Pollitt 1999).

Findeisen (1915) suggested a primary disturbance of the process of cornification in the laminar layer as a causative factor of laminitis, but it was Obel (1948) who provided the first scientific support for this theory. Obel (1948) and later Ekfalck (1991) proposed an inhibition of epidermal differentiation as a primary event in laminitis. This hypotheses was mainly based on the finding by Obel (1948) in his histopathological studies on acute spontaneous and experimentally induced laminitis that the primary morphological change, which occurs early in an acute attack of laminitis, appears in the epidermis and consists of disappearance of the onychogenic acidophilic fibrillar structures. These structures, according to Obel, normally occur in the suprabasal cells of the secondary epidermal laminae and also in the zone of keratinisation of the matrix of the stratum medium of the hoof wall. Obel believed that the disappearance of the acidophilic fibrils in the laminar layer leads to a reduction in the tenacity of the SEL, which in turn leads to overstretching of these laminae. He also detected a change in the appearance of the zone of cornification (zoc) of the periople and chestnut. There were no pathological alterations in the laminar corium in the early stage of laminitis and no changes whatsoever in the corium of the chestnut. Obel and co-workers also reported a reduced incorporation of cystine in this inner part of the zoc of the stratum medium in acute laminitis (Larsson et al. 1956; Ekman et al. 1958), corresponding to the localisation of the normally occurring acidophilic fibrillar structures.

Cytokeratins in keratinocyte differentiation

The acidophilic fibrils normally seen in the suprabasal cells of the SEL and in the matrix of the stratum medium (Obel 1948) are most likely composed of cytokeratins that have polymerised into intermediate filaments and an interfilament matrix of keratin-associated proteins. The epidermal laminae as well

as the rest of the hoof wall and the chestnut mainly consist of keratinocytes. The proliferating keratinocytes are chiefly located in the basal cell layer, and the differentiating cells are arranged in several suprabasal cell layers, where they undergo an orderly sequence of maturation described by the term keratinisation. During this process, several specific cell components (which are important for the mechanical properties of the tissue) are synthesised and modified, and contribute to the eventual formation of the stratum corneum. The intracellular skeleton of the keratinocytes is one such component. The main part of the cytoskeleton of the living keratinocytes is composed of keratin intermediate filaments. A web of these filaments stretches out across the cell, extending from the nuclear envelope in the centre of the cell to the desmosomes and hemidesmosomes at the cell periphery (for a review concerning skin, see Fuchs 1997). Two distinct types of cytokeratins have been recognised: 1) the hard cytokeratins that are present almost exclusively in hair- and nail-forming cells and 2) the soft cytokeratins of the skin and other epithelial tissues. Hard and soft cytokeratins are closely related (Hanukoglu and Fuchs 1982), but are divergent enough to be divided into two separate groups (Yu *et al.* 1991). Using two-dimensional gel electrophoresis and monoclonal antibodies, both types of cytokeratins (in human and other species) can be divided into two broad groups, one acidic and one basic, according to their isoelectric point (pI). As far as soft cytokeratins are concerned, the cytokeratins of a given cell type are expressed in pairs, and each pair contains at least one member of the acidic and one corresponding member of the basic group (Sun *et al.* 1985; Eichner *et al.* 1986; Steinert and Freedberg 1991). If there is a disturbance in the expression of cytokeratins in a cell layer, for example when other cytokeratins are synthesised, the mechanical properties of the tissue seem to be changed (Fuchs 1995).

As mentioned above, Obel (1948) and Ekfalck (1991) proposed an inhibition of epidermal differentiation as a primary event in laminitis. Since in man the expression of cytokeratins varies with the grade of keratinocyte differentiation, it was considered of interest to test the keratinocyte differentiation by studying the expression of cytokeratins in normal horses and in horses with acute laminitis.

Aims of the investigation

The overall aim of the present investigation was to carry out morphological, immunohistochemical and biochemical studies on the keratinocytes of the equine hoof wall and chestnut in order to elucidate the differentiation of these cells in an acute attack of laminitis. The specific objectives of the present studies were:

* to examine the morphological changes in the living cell layers of the hoof wall, chestnut and skin, in acute spontaneous laminitis.

* to determine the biochemical properties of the cytokeratins of the hoof wall and chestnut by one- and two-dimensional electrophoresis, and the tissue distribution, of the cytokeratins by immunohistochemical staining, in normal horses and in horses with acute spontaneous laminitis.

Methods and results

The investigation comprised four studies. Study I was a histopathological analysis of the hoof wall, skin and chestnut in horses with spontaneous laminitis in the acute phase and in horses with no symptoms of laminitis, with special emphasis placed on the epidermal basal cells. In studies II-IV, in the same groups of horses, an analysis was made of the cytokeratin composition of the corresponding tissues and of the cytokeratin distribution in these tissues with the aid of one- and two-dimensional electrophoresis and immunohistochemistry respectively.
Acute laminitis is defined in this work as beginning with the first clinical signs of lameness according to the grading system of Obel (1948) and continuing until 48 hours have elapsed. Since elongation of the laminar layer can be seen already during the developmental phase of experimentally induced laminitis (Linford 1987), it seemed more appropriate to use a time limit for the definition of the acute phase rather than the criterion that it lasts until there is evidence of displacement of the third phalanx. The 48-hour interval was chosen since this is the shortest time limit given for the acute phase in the literature.

Histopathological studies of the hoof wall, the chestnut and the skin (Study I)

For the histopathological investigation tissue samples were collected from skin and chestnuts of 10 horses with acute laminitis and from the hooves of 5 of these horses within 48 hours after the first clinical signs of lameness. Comparable biopsies from 9 horses without signs of ongoing laminitis and with no previous history of this disease were used as controls. The samples were sectioned for histological examination. The sections were stained with haematoxylin-eosin (H&E) and with periodic-acid Schiff (PAS) and with periodic acid silver methanamine (PASM) for evaluation of the epidermal cells and their basement membrane. For studies of cell proliferation an immunohistochemical examination was performed in certain tissues, using a monoclonal antibody against Ki-67 nuclear antigen as a marker. In addition the number of cells in mitosis was counted in H&E and PASM stained sections.

The hooves in the horses with laminitis, both macro- and microscopically, showed morphological changes of different degrees of severity, such as oedema and stretching and tearing of the laminar layer. Many of the basal cells of the SEL showed the same changes as observed by Obel (1948) early in experimentally induced laminitis, i.e. a more cuboidal shape with more rounded cell nuclei and a more coarsely meshed chromatin network compared with controls. The same observations were made in many of the innermost basal cells in the chestnut, periople and stratum medium of horses with laminitis. Necrotic areas were seen only in the epidermal part of the laminar layer. Comparison of the basal cells in the two groups of horses gave the overall impression of increased proliferation in the examined tissue in the periople and the strata medium and internum of horses with laminitis, and in some cases also in the chestnut and skin. Regarding the

stratum internum, the greatest increase in the number of mitoses was observed in its distal parts. The result of the immunohistochemical examination of the keratinocyte proliferation corresponded well with the picture of increased proliferation that was obtained on counting mitotic cells. The immunohistochemical examination, however, gave better information on where in the tissue the proliferative cells occurred. It was judged that the increased proliferation in the lamellar layer could only partly be secondary, i.e. caused by regeneration. The basement membrane of the examined epithelial cells showed no differences between the horses with and without laminitis with respect to the skin, chestnut, and strata externum and medium. The basement membrane of the stratum internum displayed changes, such as detachment from the epidermal basal cells, that could be related to the degree of tearing between the epidermal and dermal tissues of the laminar layer. In the corium, changes were almost exclusively seen in the laminar layer. The remaining histopathological changes were in general in accordance with Obel's findings in 1948, including disappearance of the acidophilic fibrillar substance of the inner part of the zoc in the matrix of the wall and a decreased number of keratohyalin granules in the stratum granulosum of the matrices of the periople and chestnut.

Expression of cytokeratins in the normal hoof wall, chestnut and skin (Study II)

This investigation of cytokeratins in normal horses comprised 8 of the horses that were used as controls in study I and also a newborn foal. Cytokeratins was extracted from the chestnut and strata medium and internum of the hoof wall and then separated by one- and two-dimensional gel electrophoresis. Silver staining and seven monoclonal antibodies, one polyclonal antibody and immunoblot analysis were all used to characterise the extracted cytokeratins. Regarding the normal occurrence of different cytokeratins, differences were found between tissue with "soft" keratinisation (the chestnut) (Table 1) and that with "hard" keratinisation (i.e. the strata medium and internum) (Table 2). In addition, a difference was found between the stratum medium and the stratum internum in that the stratum medium also contained a basic, 68 kDa cytokeratin (Table 2). The tissue distribution of the cytokeratins was studied by immunohistochemical staining of sections from the skin, the chestnut, the periople, the matrix of the stratum medium of the hoof wall, and the stratum internum of the hoof wall. Within the groups of tissues with "soft" (besides the chestnut, also the skin and periople) and "hard" keratinisation, there were differences between cell layers in respect to occurrence of those cytokeratins included in the tissue. This could be explained by the degree of differentiation of the included cells and the degree of proliferation of the different tissues. For example the immunohistochemical staining of the basal cell layer of the tissues differed from that of more differentiated adjacent cell layers, and the proximal part of the laminar layer differed in its immunohistochemical staining from the normally less proliferative distal parts.

Table 1. Silver-stained proteins classified as cytokeratins in extracts from chestnuts.

	Basic M_r (kDa)			Acidic M_r (kDa)			
Chestnut	**71.0**	**69.0**	**65.0**	65.0	61.0	56.5	52.0

Table 2. Silver-stained proteins classified as cytokeratins in extracts from stratum medium and stratum internum.

	Basic M_r (kDa)				Acidic M_r (kDa)					
Str. medium	**68**	**65**	**62.2**	**59.5**	59.5	56.5	55.3	52	49.6	47.2
Str. internum		**65**	**62.2**	**59.5**	59.5	56.5	55.3	52	49.6	47.2

Expression of cytokeratins in the chestnut and the periople (stratum externum) in acute laminitis (Study III)

In this study cytokeratins were analysed in tissue samples from chestnuts of eight horses with acute laminitis and from stratum externum of nine hooves taken from three of these horses. The tissue samples were collected within 48 hours after the first signs of lameness. Controls for this study consisted of the biochemical and immunohistochemical results in study II, with the exception of biochemical data on the periople, since this was not investigated in that study. Periople-related control data were obtained by biochemical analysis of the matrix of the periople taken from one of the front hooves of four horses with no history or evidence of disease in the hoof area. The horses with laminitis were found to have an identical biochemical cytokeratin content in the matrix of the chestnut and of the periople to that of the healthy horses. The matrix of the periople in both the control and the laminitic horses differed from the chestnut matrix in that the periople also contained a basic, 64 kDa cytokeratin (Table 3). On the other hand the immunohistochemical analysis showed that those laminitic horses that exhibited the most distinct morphological changes in the cornification zones of the chestnut and stratum externum, i.e. a large reduction of the number of keratohyalin granules in the papillary tips, also showed a difference in the expression of cytokeratins at the basal cell level in corresponding regions. The difference consisted in a decrease in the reaction with the A 5/6 monoclonal antibody (Mab).

Table 3. Silver-stained proteins classified as cytokeratins in extracts from the chestnut and the periople in laminitic and control horses.

	Basic M_r (kDa)				Acidic M_r (kDa)			
Chestnut	**71.0**	**69.0**	**65.0**		65.0	61.0	56.5	52.0
Periople	**71.0**	**69.0**	**65.0**	**64.0**	65.0	61.0	56.5	52.0

Expression of cytokeratins in the strata medium and internum in acute laminitis (Study IV)

In this study the cytokeratins were analysed in the strata medium and internum of the hoof wall from the same nine hooves as were used in the investigation of the stratum externum in study III. The results from the horses in study II were used as controls. Irrespective of the degree of tearing of the dermal and epidermal laminae, no difference was found between laminitic and control horses regarding the biochemical cytokeratin content in the strata medium and internum of the hoof wall. On the other hand the basal cells in the matrix of the stratum medium in the laminitic horses showed decreased reactivity with one of the five monoclonal antibodies (AE1/AE3 Mab) that was used in the immunohistochemical analysis. In the stratum internum of the hoof wall, the basal and suprabasal cells displayed a reaction pattern against the CK 8.12, AE1/AE3 and CK 14 Mabs that differed from that in the control horses in that they stained similarly in the proximal and distal parts of the stratum internum. Moreover, at the distal part of the laminar layer in the horses with laminitis, the CK 8.12 Mab differed from the AE1/AE3 and CK 14 Mabs in that a shift in staining between cell layers was seen also in SEL showing only relatively slight morphological changes.

Complementary immunohistochemical study of the stratum internum

In addition to study IV, an immunohistochemical analysis was performed in one more horse with acute laminitis, which was included as horse 5 in study I and had shown clinical signs of lameness for 10-13 hours. With the same immunohistochemical technique as in studies II to IV, the CK 8.12 Mab, which gave the most marked change between horses with laminitis and control horses in study IV, was used on one front and one hind hoof of this horse. The result of the immunohistochemical staining in the front hoof of this horse was in accordance with the result in study IV, i.e. an immunohistochemical reaction mainly with the suprabasal cells of the SEL. In the hind hoof, which showed only very slight morphological changes, the immunohistochemical results differed somewhat from those in the other laminitic hooves examined. Namely, in some areas of the laminar layer the cells of the SEL in this hoof displayed a reaction pattern comparable to that in the control horses (Fig. 2a), and in other areas, in relation to the observed degree of cellular proliferation, a gradual increase in reaction with suprabasal cells (Fig. 2b and c).

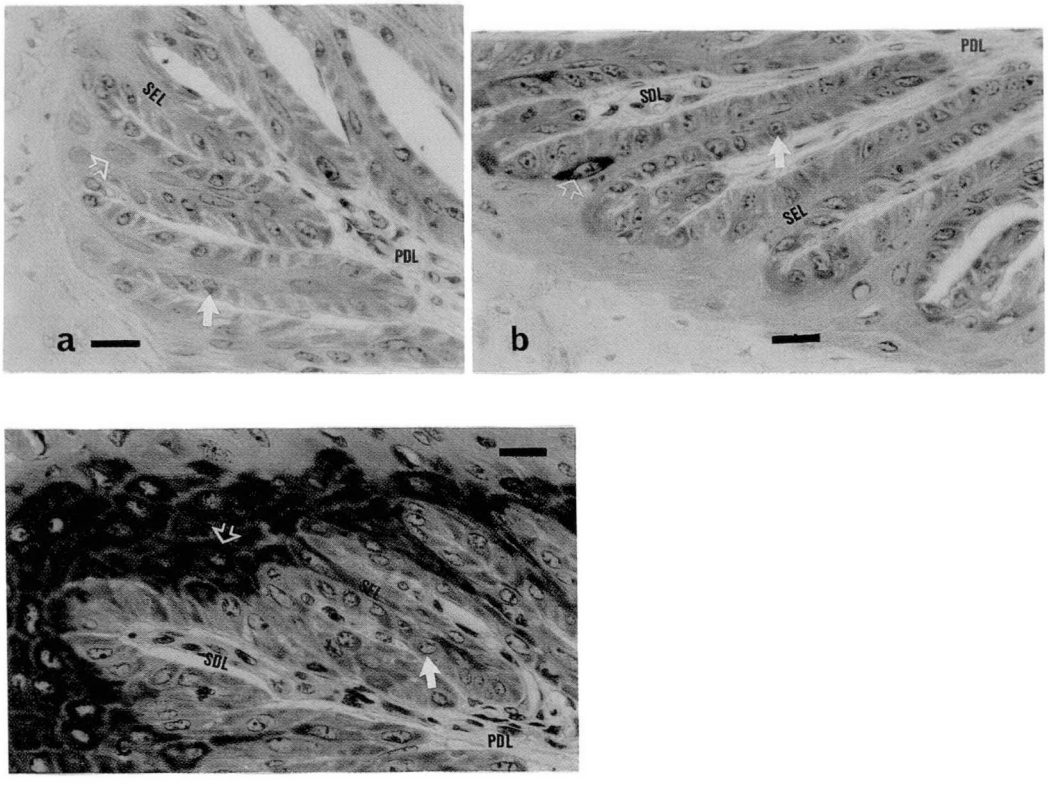

Figure 2 a – c. Right hind hoof from the additionally examined horse with acute laminitis; cross-sections from the outermost part of the laminar layer. The right-hand sides of the figures are directed towards the third phalanx and the left-hand sides towards the dorsal hoof wall. Immunohistochemical staining using the avidin-biotin-peroxidase complex (ABC) procedure with the CK 8.12 Mab and Mayer`s haematoxylin as counterstain (bar 20 µm). A positive reaction gives a brown product. Common symbols: PDL, primary dermal lamina; SDL, secondary dermal lamina; SEL, secondary epidermal lamina; white arrow, basal cells; open white arrow, suprabasal cells.
a) Mid level of the dorsal hoof wall. The CK 8.12 Mab reacts mainly with the basal cells of the SEL.
b) Proximal level of the dorsal hoof wall. The CK 8.12 Mab reacts mainly with the basal cells of the SEL, but also reacts distinctly with a suprabasal cell (open white arrow).
c) Distal level of the dorsal hoof wall. The CK 8.12 Mab reacts markedly with suprabasal cells and to a lesser extent with basal cells.

Discussion

The overall results of the four studies clearly indicate that the epidermal basal cells in the hooves and chestnuts show distinct changes during the acute phase of laminitis. Together with the verification of the morphological changes in the zone of cornification described by Obel (1948), and the finding that the changes in the basement membrane and the dermal tissues were especially restricted to those parts of the laminar layer of the laminitic horses where tearing of the tissue was seen, the observations in the above studies favour rather than contradict the theory that the epidermal changes in an attack of laminitis are primary.

In the present studies it was found that biochemically the types of cytokeratins present in the laminitic horses did not differ from those in the control horses. Nor were any "new" types of cytokeratins expressed by the proliferating keratinocytes in connection with the regeneration in the laminar layer that occurred at an early stage of acute laminitis. This finding was interesting, since in connection with regeneration in the skin in man, new types of cytokeratins are produced (Martin 1997). The reason why no new types of cytokeratins are formed in laminitic horses is considered to be that the distal cells of the stratum internum, which are now activated, retain the potential for proliferation, with expression of the same type of cytokeratins as normally are exhibited only by the most proximal cells. An alteration concerning the cell layer in which the different cytokeratins were expressed (i.e. the tissue distribution) was demonstrated immunohistochemically, however, in the horses with laminitis. The morphological changes in the laminar basal cells, that is, a cuboidal shape, with more rounded cell nuclei and a more coarsely meshed chromatin network, the increased number of mitotic figures and the increased reaction among the suprabasal cells and decreased reaction among the basal cells at the distal level with three of the anti-cytokeratin monoclonal antibodies used in study IV might be explained by a cellular hyperproliferation. This hyperproliferation could at least partly be a stage in an initiated tissue regeneration caused by the severe tearing in the laminar layer. However, the increased proliferation in the strata externum and medium can hardly be explained as caused by regeneration, since these tissues do not display as severe tissue damage as the stratum internum. Furthermore, in spite of the fact that the disease does not cause any mechanical lesions in the chestnut, similar morphological changes among the epidermal basal cells and an altered staining reaction with one of the Mabs were seen in the chestnuts of those of the laminitic horses that showed a large decrease in the number of keratohyalin granules. Moreover, in some of the chestnuts from horses with laminitis there was also an increased number of mitotic figures among the basal cells.

The complementary immunohistochemical staining with the CK 8.12 Mab in the laminar layer in the right front hoof of horse number 5 (used in study I) was in accordance with the results of the staining with this Mab in study IV. The results of this staining answered a question that was put forward in study IV, namely, whether this change in staining reaction with CK 8.12 occurs before the SEL becomes elongated. The laminar layer of the right hind hoof of this horse, in

which there were only very slight epidermal changes and no changes whatsoever in the corium, included SEL with normal as well as SEL with abnormal staining patterns. It was therefore shown in this case that the shift in staining with the CK 8.12 Mab appeared not before, but very early in connection with proliferation in the SEL (Fig. 2). Moreover, an increased rate of proliferation was evident despite the fact that there were only slight morphological changes in this hoof.

The above discussion may be summarized as follows: Together, the increased rate of proliferation among epidermal cells, the less differentiated appearance of the suprabasal cells and the most likely primary changes in the zoc that are observed early in acute spontaneous laminitis indicate that primary inhibition of differentiation of the hoof keratinocytes occurs in laminitis.

As the types of cytokeratins present are the same in laminitic horses as in healthy horses, the interest becomes focused on the keratin-associated proteins. Of these, the basic cysteine-rich keratin-associated proteins are especially interesting. Besides cytokeratins, these proteins are contained in the acidophilic structures that were found by Obel (1948) to disappear in the early stage of the disease. It is supposed that the cysteine-rich keratin-associated proteins play an important role, at least in hair, through stabilisation of the protein structures by disulphide bonds (Powell and Rogers 1994). A disturbed interaction between the intermediate filament and the keratin-associated proteins might explain the elongation of the SEL that Linford (1987) demonstrated in the developmental phase and the elongation in the early clinical phase of acute laminitis (Obel 1948; Linford 1987). By contributing to the reduction of the disulphide bonds, activation of the thioredoxin/thioredoxin reductase system - possibly in combination with a decrease in the pH of the tissue - could be a common causative factor in the different types of laminitis. It is also conceivable that activation of the thioredoxin/ thioredoxin reductase system, by reason of the cell growth promoting activity of thioredoxin, might explain the observed increase in proliferation, which cannot be fully explained as a regeneration phenomenon. The presence of thioredoxin reductase in, for example, chloroplasts in growing grass, in newly threshed grain, in the placenta (at least in man) and in bacteria (e.g. *Escherichia coli*) (Mustacich and Powis 2000) could explain the occurrence of the different types of spontaneous and experimental laminitis. It is also possible that laminitis caused by an aqueous extract of black walnut shavings could be explained as a thioredoxin effect, since an "laminitis causing" effect of this extract requires freshly made shavings (True et al. 1978; Minnick et al. 1987). Further, it has been found on culture of cells from the endometrium in man that when ovarian steroid hormones are added, the human thioredoxin protein is up-regulated (Maruyama et al. 1999).

If this theory of the reducing effect of the thioredoxin/thioredoxin reductase system on the disulphide bindings as a cause of laminitis holds true, new pathways for medical treatment of horses with laminitis may be opened.

A treatment that can already be tried is to keep the horse recumbent to prevent stretching of the weakened laminar layer during the first 2-3 days of the acute

attack of laminitis. A method for keeping ponies recumbent, without causing further harm to the animal, has been described by Wattle et al. (1995).

Conclusions

The following conclusions may be drawn from the results of this morphological study on the keratinocytes of the hoof wall and chestnut with special emphasis placed on the epidermal basal cells, and the biochemical and immunohistochemical investigation of the cytokeratins of these tissues:

- In horses with acute spontaneous laminitis, the rate of proliferation is increased, not only in the traumatised stratum internum of the hoof wall but also in the strata medium and externum and to some extent also in the chestnut.

- The differentiation of keratinocytes in the zone of cornification of the hoof wall and chestnut is inhibited in horses with acute spontaneous laminitis. In those horses with laminitis that show distinctly inhibited differentiation in the zone of cornification, the basal cell layer of the hoof wall and chestnut have a more cuboidal shape, with more rounded cell nuclei and a more coarsely meshed chromatin network, compared with controls.

- Both in normal horses and horses with acute laminitis, there is a difference in the types of cytokeratins in soft and hard keratinised tissues, but there is also a difference in this respect between the chestnut and the stratum externum of the hoof wall (soft keratinised tissues) and between the stratum medium and stratum internum of the hoof (hard keratinised tissues). No "new" types of cytokeratins are expressed by the living epidermal cells of the hoof wall in acute spontaneous laminitis.

- Both in normal horses and in horses with laminitis, there is a difference between cell layers in respect to the distribution of the cytokeratins present in the tissue. This difference is most likely due to a difference in the degree of differentiation of the included cells.

Taken together these results indicate that an inhibition of differentiation, marked by a disturbance of cornification and a simultaneous increase in cell proliferation, with a change in the tissue distribution of cytokeratins, occurs in the acute phase of spontaneous laminitis.

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