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Three-dimensional appearance of bovine epidermal keratinocytes in different stages of differentiation revealed by cell maceration and scanning electron microscopic investigation

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The epidermis of the modified skin of the bovine hoof is a highly mechanical loaded tissue. Consequently, all cell connections have to withstand high mechanical forces. As an adaptation to this stress, the epidermal keratinocytes show characteristic surface modifications. Furthermore, the tissue displays a complex three-dimensional architecture which is difficult to appreciate from histological sections. SEM-observation of macerated tissue samples is a fast, easy to use and reliable tool to receive three-dimensional information about the appearance and spatial relationship of cells within a tissue. Using cell maceration, the aim of this study was to separate individual as well as smaller groups of keratinocytes in order to reveal the formations of the cell surface, the appearance of individual cells and the spatial relationship of cells within the tissue. A NaOH maceration method described in literature was modified and applied to tissue samples from the wall and bulbar segment of the hooves of six cows. This method facilitated separation between the epidermal cells. Single cells as well as cell groups were available for SEM observation which revealed a three dimensional appearance characteristic for different stages of differentiation of the keratinocytes. The observed findings suggest that throughout the process of differentiation the surface modifications provide the basis for a stable cell to cell adhesion which is established by desmosomes and the intercellular cementing substance. Additionally, the broadened cellular surface area is related to the supply of the highly metabolic active living epidermal cells with nutrients and oxygen. Longer cell processes typically found in the central surface area of the keratinocytes may carry gap junctions and may be involved in cell communication. This, however, has to be clarified by further electron microscopic studies. The demonstrated appearance of individual cells and the complex architecture enable the hoof epidermis to fulfil its unique biomechanical functions.

key words: keratinocytes, epidermis, bovine, hoof, cell differentiation, desmosomes, intercellular cement, cell adhesion, cell maceration, scanning electron microscopy

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INTRODUCTION

The bovine hoof consists of skin covering the distal end of the limb in artiodactyls. It protects the enclosed elements of the skeleton and the soft tissue against mechanical and biological environmental influences and has important biomechanical functions during stand and locomotion [2,4,11,12,22].

As adaptation to these functions the skin of the hoof is extensively modified. The epidermis is a strongly cornified squamous epithelium with up to 60 layers of living epidermal cells (keratinocytes) and a stratum corneum with a thickness of approximately one centimetre. This way the tissue is adapted to the high mechanical forces it is loaded with particularly during locomotion [9,22]. On the cellular level all cell connections between living differentiating cells with a high metabolic activity as well as between the dead cornified cells have to withstand high mechanical forces [22]. Lying underneath the epidermis the dermis of the hoof is modified to a complex papillary body with finger like dermal papillae in most parts and dermal lamellae in one part exclusively which is the wall segment [1,2,11,13].

The histological structure and the ultrastructure of the dermis and epidermis of the bovine hoof are well documented [1–4,11,12,14,16]. However, there is little information available concerning the three--dimensional appearance of the individual epidermal cells and their spatial arrangement and interaction. From histological sections it is difficult to appreciate the three dimensional aspects of this tissue.

SEM-observation of macerated tissue samples has been described in literature for a variety of tissues and organs as a fast, easy to use and reliable tool to receive three-dimensional information about the appearance and spatial relationship of cells within a tissue [18,23,27]. However, until now there is no information available for hoof epidermis basing on SEM-examination of macerated tissue samples. Therefore, the objective of this study was to establish the appropriate maceration technique for hoof tissue and, using this method, to separate living as well as cornified keratinocytes in the intercellular space. This way the three-dimensional appearance, the spatial relationship and the surface modifications of bovine epidermal keratinocytes should be demonstrated in relation to different stages of cell differentiation.

MATERIAL AND METHODS

Tissue samples were obtained from the sound lateral and medial claws of the left fore and right hindlimb of six cows-from the local abattoir. All animals were cross-breed Holstein Friesian cows with an age ranging between 2 and 5 years. Using a band saw small tissue blocks were taken from the wall and bulbar segment of the hooves (Fig. 1). All samples consisted of dermis and epidermis and had a size of 0.6 x 0.6 x 0.6 cm. These samples were used for cell maceration and for light microscopy. Additionally, samples 0.3 x 0.3 x 0.3 cm in size were taken for transmission electron microscopic examination. All samples were fixed in 2.0 % glutaraldehyde in 0.1 m phosphate buffer (PB), pH 7.3, for 6 h at 4 °C and subsequently rinsed several times in PB. For light microscopy samples were routinely processed and embedded in Technovit 7100® (Kulzer, Werheim/Germany) as described by Mülling [11]. Two micrometer sections were prepared on a Polycut® microtome (Zeiss AG, Oberkochen/Germany) and stained with either haematoxylin eosin or with the periodic acid Schiff reaction [19]. For transmission electron microscopy samples were postfixed in a 1% aqueous solution of osmic acid (OsO_4) followed by dehydration in a graded series of ethanol and routine embedding in epoxy resin. Ultrathin section were prepared on a Ultracut® II microtome (Zeiss AG, Oberkochen/Germany) and stained with lead citrate and uranyl acetate [24] in an Ultrostainer® (Zeiss AG, Oberkochen/Germany). Transmission electron microscopic (TEM) examination was carried out with an EM 10 (Zeiss AG, Oberkochen/Germany). Cell maceration was performed using a modified NaOH maceration method originally described by Takahashi-Iwanaga and Fujita [23] and Ohtani [18] for liver. This method turned out to deliver the best results for hoof epidermis when the time used for maceration was adapted to the properties of hoof epidermis. For maceration the rinsed samples were incubated in 6 N NaOH for 60–120 min at 60 °C. Time particularly depended on which cell layer was supposed to be optimally macerated. This was followed by four times rinse in PB. Conductive-staining was carried out with the metal impregnation method according to Murakami [17]. Briefly, the samples were incubated with 1% tannic acid for 24 h at 4 °C; rinsed three times in PB followed by immersion in 2% osmic acid (OsO_4) for 2 h at 4 °C. The specimens were then rinsed three times in PB, dehydrated in a graded series of ethanol and critical point dried using liquid CO_2 [8]. The samples were glued onto aluminium plates with adhesive pads. The brittle tissue blocks were pricked with a thin needle and fractured into several fragments as shown in Figure 13 for a specimen from the wall segment. For SEM-observation the speci-

mens were coated with 10 nm gold and examined with a Nanolab 2000 (Bausch & Lomb) at an acceleration voltage of 10–15 kV.

RESULTS

The modified NaOH method facilitated separation between the epidermal cells clearly revealing their surface formations. Depending on the time incubated in the NaOH either cell groups in their characteristic association (e.g. Figs. 3, 4) or isolated smaller cell clusters (e.g. Figs. 6, 9) or even individual cells (e.g. Figs. 15, 16) were available for SEM-observation. In general SEM-investigation of macerated specimens significantly increased the information on three-dimensional appearance as compared to histological sections which display the two dimensional situation exclusively. The spatial arrangement of neighbouring cells as well as the relationship between the dermal papillary body and the living epidermal cells become clearly visible (Figs. 3, 4). Looking closer at groups of cells the characteristic shape and surface modifications related to different stages of differentiation were impressively revealed. In the wall and bulbar segment surface modifications common for all cells from the basal to the cornified layers were finger like processes covering the whole cell surface (Figs. 6, 9, 11). Most of these processes had a relatively uniform appearance with minor differences in their dimensions. However, in a distinct oval shaped central area the processes were significantly longer (Fig. 6). All cell processes showed intimate interdigitation between neighbouring cells.

Looking at the characteristic appearance of cells related to different stages of differentiation we start in the stratum basale of wall and bulbar segment, i.e. in the basal layer of keratinocytes covering the dermal laminae in the wall segment and the dermal papillae in the bulbar segment respectively. In the bulbar segment (Fig. 7) and even more significant in the wall segment (Fig. 18) the columnar basal cells showed long slim basal processes seming little feet or "footlets". These processes interdigitated with parallel running microridges on the surface of the dermal laminae in the wall segment (Figs. 14, 15) as well as on the dermal papillae in the bulbar segment (Fig. 3). These "footlets" deeply extended into the underlying dermis approaching the network of dermal blood vessels under the dermo-epidermal border. This way the distance between basal cell and supplying blood vessels is reduced (Fig. 17). The lateral aspects of the basal cells was relatively smooth (Fig. 17). On their apical surface the basal keratinocytes possess a relief of low "gyri-like" processes (Fig. 16) establishing the connection to the basal aspect of the suprabasal cells in the second layer of the stratum spinosum. These suprabasal keratinocytes are relatively small and of an irregular oval shape, their surface is completely covered by processes interdigitating with these of neighbouring cells. In the lower third of stratum spinosum (Fig. 8) the spiny cells display a polygonal shape, which changes to the typical and very uniform polyhedral appearance of spiny cells in the middle layers (Fig. 9). Compared to the transmission electron microscopic picture obtained from an ultrathin section it becomes obvious that SEM-observation of macerated specimen provides easily accessible information on both the shape of individual cells as well as their connection by numerous processes (Fig. 10–11). As shown by TEM-examination these processes interdigitate and provide surface area for numerous desmosomes establishing the cell to cell adhesion in stratum spinosum (Fig. 10). Moving to the upper third of stratum spinosum (Fig. 11) the picture recalls the situation in the middle layers with the exception of an increased irregularity of the shape of the cells. Finally, observing the cells undergoing the terminal stage of differentiation an abrupt change in their appearance becomes visible (Fig. 12). They become flat and somewhat elongated. It is difficult to differentiate individual cells because they are hardly separated by the maceration. Increasing the time of maceration beyond 2 h rather destroys the integrity of cells than facilitates a proper separation.

DISCUSSION

Compared to the already existing information on the structure of the hoof epidermis the results presented in this study increase the knowledge on the three--dimensional aspects of hoof epidermis. The understanding of the complex architecture and function of this tissue is improved. The SEM pictures provide direct information which is hard to achieve even from serial sections. However, considering the artefacts potentially produced by maceration techniques, for any quantitative examination the results have to be compared to those obtained from histological sections of corresponding samples. Particular attention has to be given to the volume shrinkage which can reach up to 25% [20] and may vary between the different layers of the epidermis according to the differences in water content from basal cells to the cornified layers. From our experience and the comparison of macerated cells to those in tissue sections,

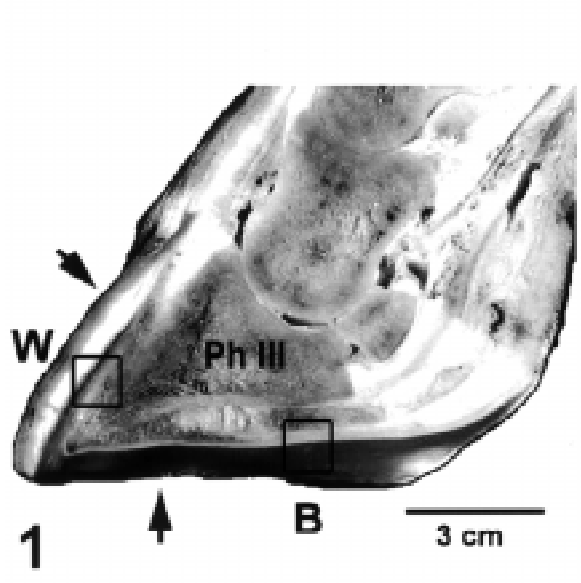

Figure 1. Sagittal section through the lateral claw of a hindlimb. The horny capsule (arrows) encloses the distal phalanx (Ph III) of the skeleton of the limb and the soft tissue elements. Sites where samples were collected are indicated by boxes. W — samples taken from the wall segment, B — samples taken from the bulbar segment.

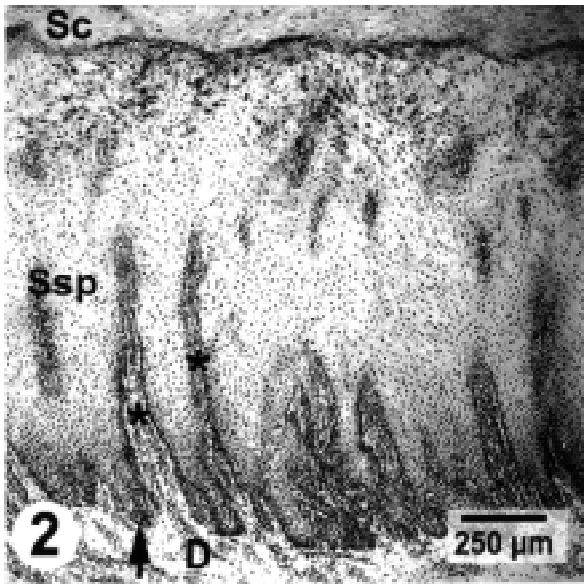

Figure 2. LM-micrograph of a transverse section of the bulbar epidermis covering long finger-like dermal papillae (asterisks). D — dermis. Within the epidermis the strata basale (arrow), spinosum (Ssp) and corneum (Sc) can be differentiated; haematoxylin eosin stain.

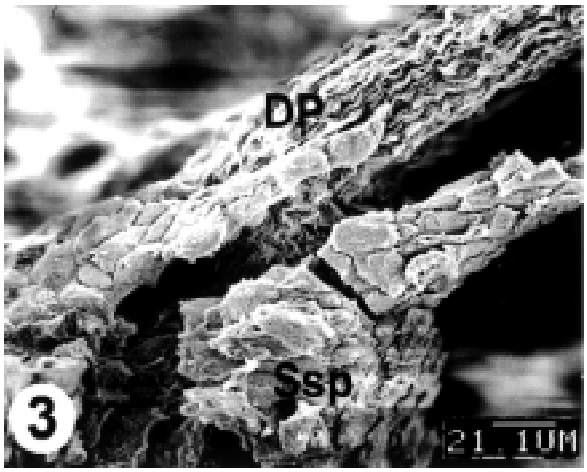

Figure 3. SEM-micrograph of a macerated specimen from the bulbar segment showing a dermal papillae (DP). The surrounding epidermal cells of the stratum spinosum (Ssp) have been separated and partly removed revealing the surface of the papilla which is covered by parallel running microridges.

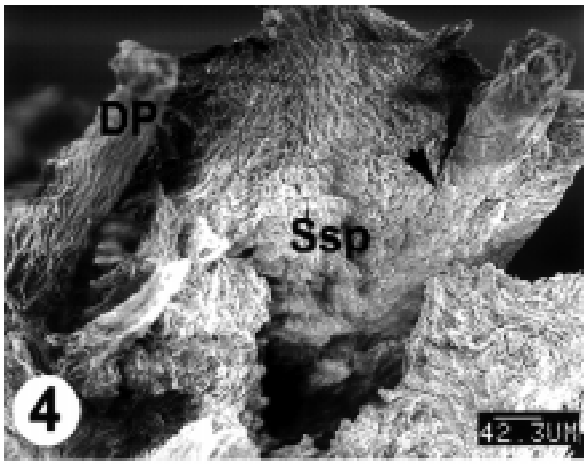

Figure 4. Low power SEM-micrograph showing the spatial arrangement of the dermal papillae (DP) and the surrounding epidermis of the stratum spinosum (Ssp) in the bulbar segment. Arrow indicates the dermo-epidermal interface where the separation by maceration occurs.

the shrinkage does not affect the characteristic shape and arrangement of cells. This is also shown in the direct comparison of the Figures 5 and 6. The percentage of shrinkage is approximately the same as in dehydrated and embedded tissue samples, i.e. for epidermal tissue 15–20% of the volume [11].

The observed findings suggest that the surface modifications increase the surface area of the individual cells to a large extent throughout the whole process of differentiation. The large surface area provides the structural basis for the cell to cell adhesion which is established by desmosomes and the intercellular cementing substance [14]. Furthermore a stable connection of all keratinocytes throughout the epidermis is the prerequisite for the biomechanical properties of hoof epidermis which enable the tis-

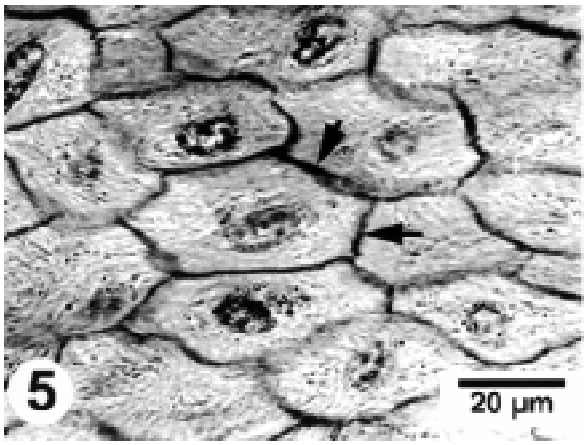

Figure 5. LM-micrograph of keratinocytes in the middle third of stratum spinosum. The spiny cells are connected by PAS-positive intercellular cementing substance (arrows).

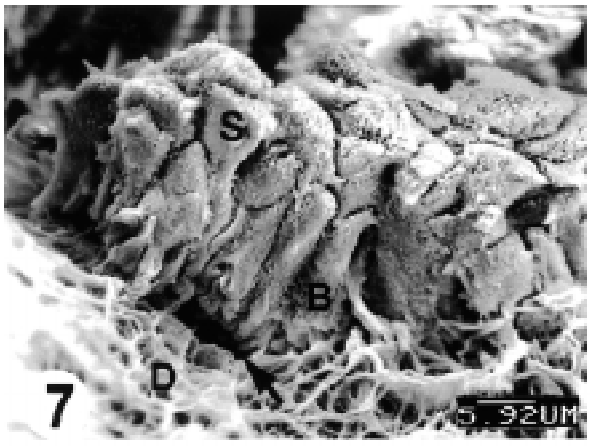

Figure 7. SEM-micrograph of an isolated group of columnar basal (B) and small polygonal suprabasal (S) keratinocytes attached to the surface of a dermal papilla (D). The basal processes have been partly pulled out of the underlying dermis (arrow).

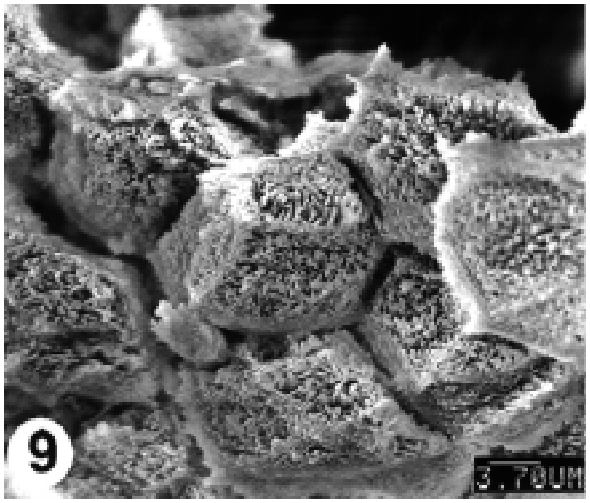

Figure 9. Typical appearance of cells in the middle third of stratum spinosum; their surface is covered by numerous cell processes interdigitating with those of neighbouring cells.

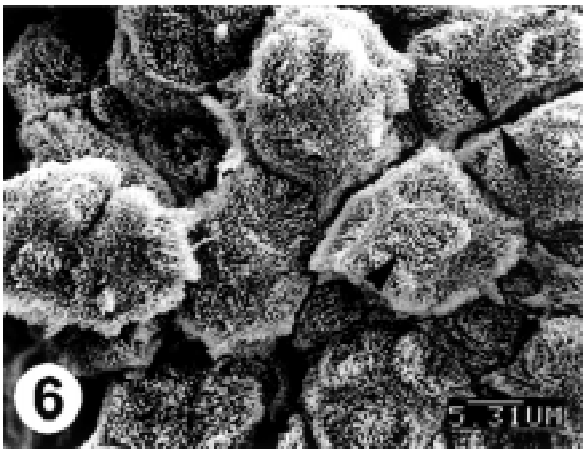

Figure 6. SEM-micrograph of spiny cells in the middle third of stratum spinosum corresponding to the light microscopic picture in Figure 5. Cells are separated long their boundaries, the artificially widened intercellular space is indicated by arrows. The cell surface is modified to densely arranged cell processes, which are significantly longer in the central area (arrowhead).

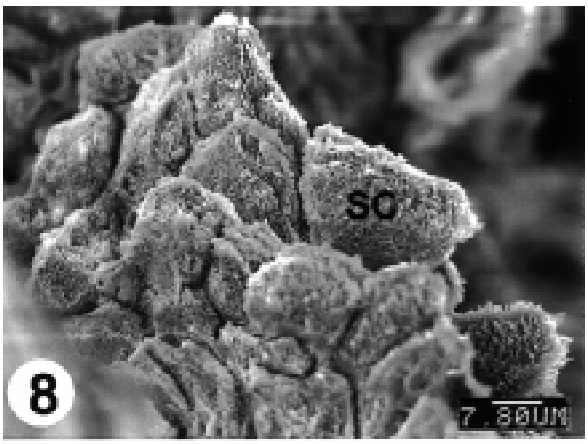

Figure 8. A group of spiny cells (SC) in the lower third of stratum spinosum of the bulbar segment.

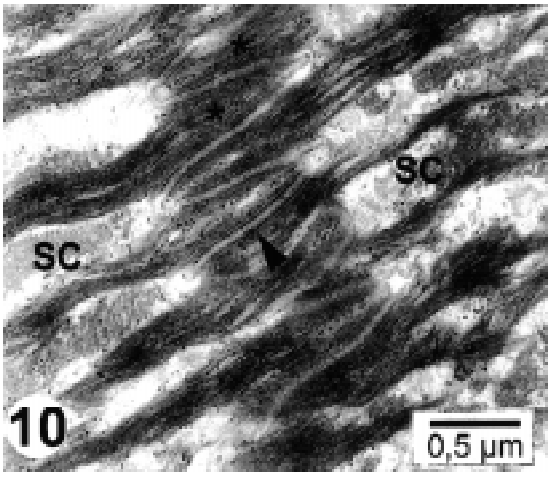

Figure 10. TEM-micrograph of two neighbouring keratinocytes (SC) in the middle stratum spinosum corresponding to Figure 9. The interdigitating cell processes (asterisks) are connected by desmosomes (arrowhead).

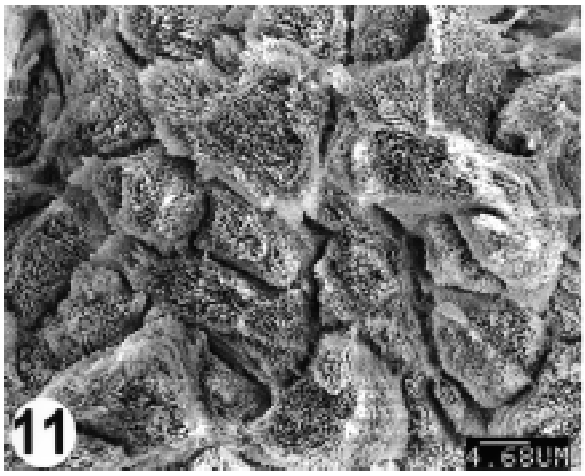

Figure 11. Cells in the upper third of stratum spinosum showing their typical spatial arrangement.

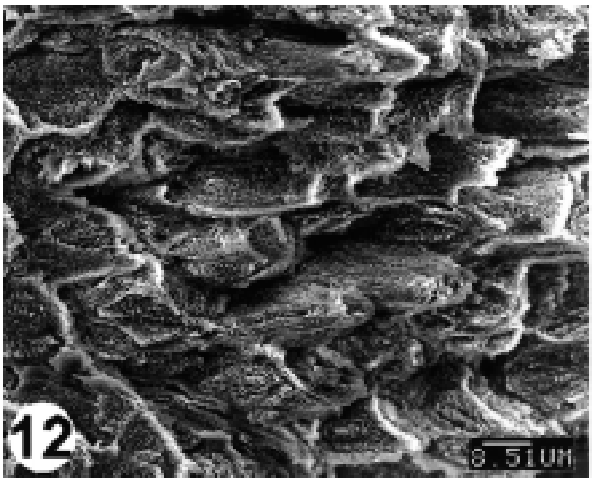

Figure 12. Keratinocytes in the uppermost layers of stratum spinosum undergoing terminal differentiation. The cells flatten and become elongated. Their surface is still covered by cell processes.

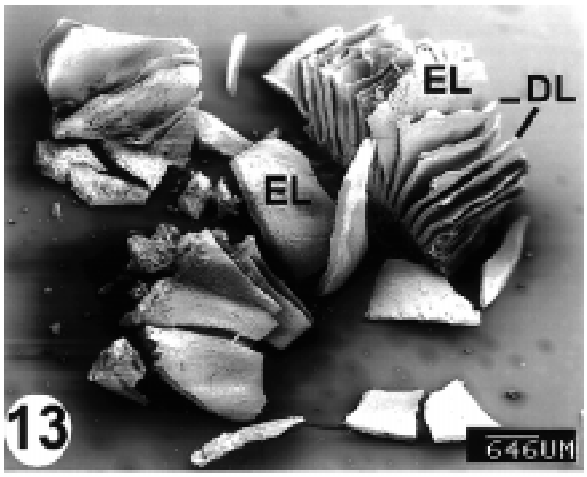

Figure 13. Specimen from the wall segment fractured in fragments. The epidermal laminae (EL) have been separated and removed from in between the dermal laminae (DL) revealing the surface on each site as well as the spatial relationship between dermis and epidermis.

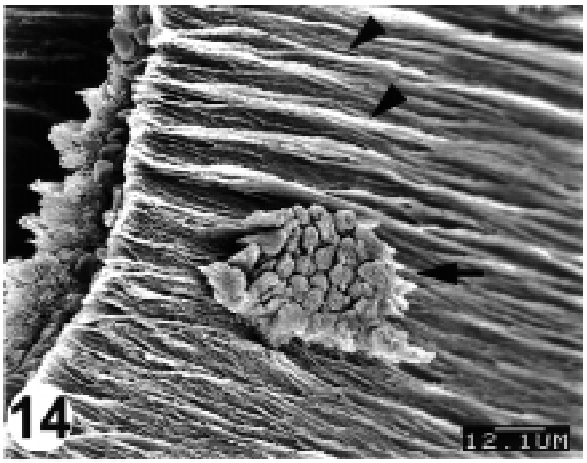

Figure 14. Oblique SEM view of a dermal laminae with parallel oriented microridges (arrowheads) on its surface. A group of basal keratinocytes (arrow) remained attached to the dermal surface.

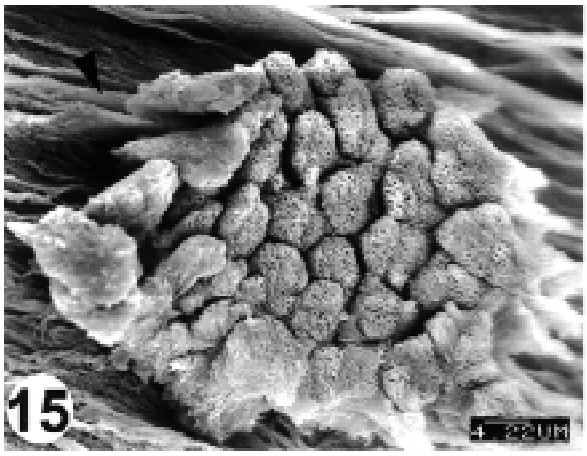

Figure 15. Close up of the group of basal cells shown in Figure 14 displaying the shape and spatial arrangement of the keratinocytes. Their basal processes extend into the dermal surface and are fixed between the microridges (arrowhead).

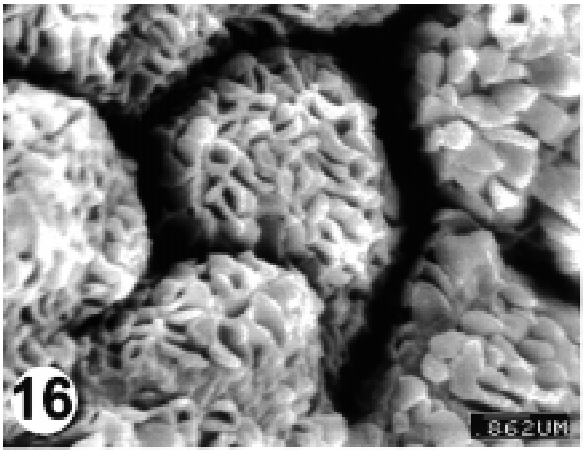

Figure 16. High power magnification of the apical surface of the basal keratinocytes showing the gyri-like surface modifications.

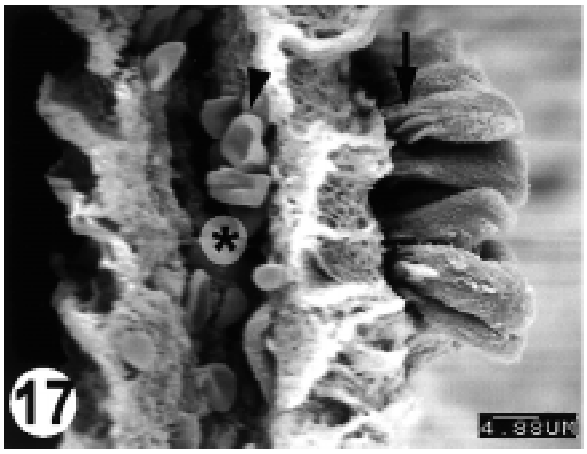

Figure 17. Lateral view of a group of basal cells fixed by their basal "footlets" (arrow) in the surface of a dermal lamina. Inside the cracked lamina a blood vessel (asterisk) filled with red blood cells (arrowhead) is visible.

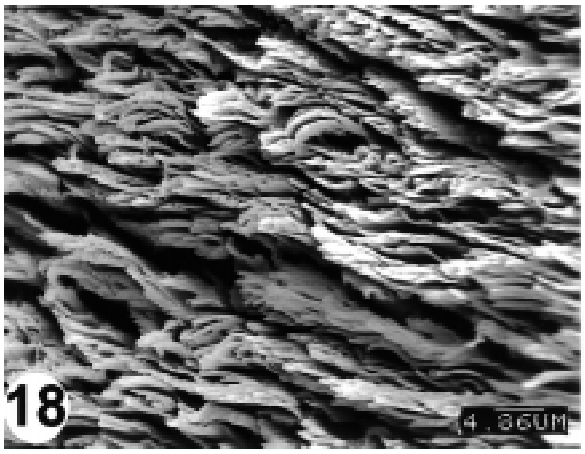

Figure 18. High power magnification of the basal surface of basal keratinocytes in the wall segment displaying the characteristic long processes ("footlets") of these cells which deeply extend into the underlying dermis.

sue to withstand the high mechanical stress exerted on it. The preferential sites of desmosomes are the finger like cell processes [11]. Increasing their number provides space for a higher number of desmosomes to settle on a broader contact area. Consequently, the mechanical stability is improved in particular between the living keratinocytes, where desmosomes are of critical importance for the connection of cells. However, the contact area between neighbouring cells is related to the second way cell to cell adhesion is established beginning in the upper stratum spinosum. Here the cells become biochemically glued by the intercellular cementing substance [14]. This intercellular material, also referred to as membrane coating material (MCM) [7,14], is extruded into the intercellular space at the level of the middle to upper third of stratum spinosum [2,11,14]. The MCM is rich in lipids and glycoproteins [7,14,21,25,26]. Besides the biochemical composition of the MCM the area of the surface of neighbouring cells available for connection by this MCM is of critical importance. Therefore, the high number of cell processes continuously covering the surface of keratinocytes provides the structural basis for mechanical stable cell to cell adhesion in living hoof epidermis. Besides the enlargement of the surface area the intimate spatial interaction of the cell processes neighbouring cells itself increases the stability of the hoof epidermis.

A further important role of the revealed surface modifications is elated to the supply of living epidermal cells with nutrients and oxygen. In the avascular epidermis the highly metabolic active cells essentially depend on a sufficient supply which is performed by diffusion [15]. Beside other factors, this mode of supply is dependent on the size of the cellular surface area. It can be significantly improved by a larger area available for the uptake of nutrients and oxygen.

Considering their functions it becomes obvious that the dimensions of cell processes reflect either the mechanical load or the local rate of supply. It is likely that both an increased or decreased mechanical stress and an increased or decreased nutritional demand will lead to adaptations i.e. structural alterations of the cell processes.

In addition to mechanical purposes and the supply, a number of cell processes may be involved in communication between individual cells within this complex tissue. In systematic ultrastructural examinations of the epidermis a high number of gap junctions in particular of annular gap junctions (AGJ) in the perinuclear cytoplasm has been demonstrated [3,6,11]. AGJ can be interpreted as gap junctions completely surrounding finger like cell processes extruding from one cell into the cytoplasm of other neighbouring cells [10,11]. Exchange of information between the remarkable number of cells is required for co-ordination of the process of cell differentiation in this complex multi-layered tissue [5]. Whether the longer processes in the central area of the cells carry gap junctions and are involved in cell communication has to be clarified by further electron microscopic studies.

Cell maceration in combination with SEM-observation has been proven to be a helpful tool to eluci-

date complicated three-dimensional aspects of the hoof epidermis. This study has added information on three-dimensional aspects which are essential for a better understanding of the complex architecture of this tissue. The demonstrated appearance of individual cells and the tissue architecture are directly linked to the biomechanical properties of this tissue enabling the hoof epidermis to fulfil its unique functions.

Further studies are needed directly comparing information revealed by SEM-investigation of macerated hoof tissue to results obtained from histological and transmission electron microscopic examination of tissue sections.

ACKNOWLEDGEMENTS

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