Belg. J. Zool., 140 (1) : 59-64 January 2010

# The ultrastructural immunolocalization of loricrin in the hairy epidermis of the platypus (*Ornithorhynchus anatinus*, Monotremata) indicates it contributes to the formation of the cell corneous envelope

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ABSTRACT. The fine localization of loricrin, a major protein of the cell corneous envelope, is known for the epidermis of few species of placental mammals. The localization of this protein has been studied by immunocytochemistry in the hairy epidermis (orthokeratotic) of the platypus, a representative of monotremes, and compared with the localization of the protein in the epidermis of placental mammals. In the hairy epidermis small keratohyaline granules of 0.1-0.3μm are present in transitional cells of the stratum granulosum. An anti-loricrin antibody labels the pale component of keratohyaline granules, the corneous mass of transitional corneocytes, and mainly the corneous cell envelope of corneocytes in the stratum corneum. The last pattern resembles that of the hairy epidermis of placental mammals and differs from the diffuse distribution of loricrin previously described in the parakeratotic epidermis of the platypus. The study confirms that the final pattern of distribution of loricrin in the hairy epidermis of the platypus is as previously observed in corneocytes of placental mammals.

KEY WORDS: monotremes; hairy epidermis; loricrin; immunocytochemistry; immunogold

## INTRODUCTION

The evolution of the mammalian integument from that of its reptilian ancestors required numerous modifications in both the dermis and epidermis (CHUDINOV, 1968; FINDLAY, 1970; MADERSON, 1972). The epidermis of basic amniotes, either scaled or un-scaled, probably possessed only a form of alpha-keratinization (soft keratin). In comparison to the dry and resistant epidermis of extant reptiles, the general epidermis of mammals is soft, elastic and moisturized, properties that have been associated with the fine action of mammalian musculature, while the pelage has mostly taken over the role of mechanical protection together with thermal insulation (SPEARMAN, 1964; 1966; FINDLAY, 1970; MADERSON, 1972; SOKOLOV, 1982; ALIBARDI, 2006a). The softness of the mammalian epidermis may be related to the evolution of a granular layer that produced a soft stratum corneum (orthokeratotic) from the parakeratotic epidermis of reptilian progenitors (SPEARMAN, 1964). The latter was characterized by the presence of nuclei in cells of the stratum corneum (corneocytes) and lacked keratohyaline granules.

Keratohyaline-like granules characterize the granular layer of the epidermis of mammals, and have been categorised into F-granules, L-granules and composite granules (HOLBROOK, 1989; STEVEN et al., 1990; RESING & DALE, 1991; HARDMAN et al., 1998; ISHIDA-YAMAMOTO & IIZUKA, 1998).

Most morphological, physiological and molecular information is available for the epidermis of only a few mammalian species such as mouse, human, rat, and cow (MATOLTSY, 1986; HOLBROOK, 1989; FUCHS, 1990; MEHREL et al., 1990; RESING & DALE, 1991; DALE et al., 1994; ISHIDA-YAMAMOTO et al., 2000; KALININ et al., 2002). For the remaining mammals, only general histological information is available (SOKOLOV, 1982).

Those studies have shown that some proteins (filaggrin and trichohyalin) function as an interkeratin matrix for the formation of corneous material while other proteins (involucrin and loricrin) are mainly deposited along the plasmalemma to produce the resistant cell corneous envelope of mature corneocytes. Information on the expression and localization of keratins and associated proteins involved in the process of cornification is not available for most mammalian species, including marsupials and monotremes (ALIBARDI, 2004; ALIBARDI & TONI, 2005).

A previous study on the epidermis of the interdigital membrane of the Duckbill platypus (*Ornithorhynchus anatinus*) showed that the epidermis in this area is parakeratotic (nucleated) (ALIBARDI & MADERSON, 2003; ALIBARDI, 2006a). Loricrin was intracellularly diffuse in corneocytes instead of concentrated along the cell corneous membrane, as in corneocytes of the other mammals. A similar type of localization has been found in reptilian and avian corneocytes and may represent the primitive pattern of redistribution for this protein in the mature corneocytes of the epidermis.

In the present study, the previous observations on the localization of loricrin in the web epidermis have been compared with the localization of this protein in the epidermis of a hairy area of the platypus limb, the digit close to the interdigital membrane (web). This comparison allows us to establish whether or not the final pattern of distribution of the protein in monotreme epidermis is similar to that of other mammals.

## MATERIALS AND METHODS

The present study was conducted on samples of the skin from three adult individuals of platypus, *Ornithorhynchus anatinus*, (Shaw 1799), collected in the field around Sydney, Australia (see details in ALIBARDI & MADERSON, 2003; ALIBARDI, 2004).

Briefly, small pieces of digit skin containing some hairs (1x2mm) were collected from captured individuals of platypus that were re-marked and released in the field, and the tissues were immediately fixed for 8 hours in 2.5% glutaraldehyde in phosphate buffer 0.1M at pH 7.4, postfixed with 2% osmium tetroxide in water, dehydrated with ethanol, and embedded in Spurrs resin. Other samples were instead fixed in 3% paraformaldehyde in phosphate buffer for 4-5 hours, rinsed with phosphate buffer, dehydrated with ethanol, and embedded in the resin Lowicryl KM4 under ultraviolet light at 0-4ºC for three days.

Using an ultramicrotome, sections of 1-4μm were obtained and stained with 0.5% toluidine blue. Forty- to 90nm-thick sections were collected on copper grids, and stained with uranyl acetate and lead citrate according to routine methods for electron microscopic study of the skin. Other sections were collected on gelatin-coated slides from tissues embedded in Lowicryl KM4 for the following light microscopic immunocytochemical study.

Tissues were pre-incubated for 30 minutes in 5% normal goat serum with 2% BSA in 0.05M Tris/HCl buffer at pH 7.6, incubated overnight at 4°C in the buffer containing the primary antibody (rabbit anti-mouse loricrin 15-amino acid specific epitope, Babco, California, USA, diluted 1:300), which was omitted in the controls. After several rinses in the buffer, the sections were incubated for 1 hour at room temperature in the same medium, containing 1:50 anti-rabbit-IgG FITC-conjugated secondary antibodies. After rinsing, sections were mounted in Fluoromount (EM Sciences, USA) and observed under a Zeiss epifluorescence microscope equipped with a fluorescein filter.

For immuno-electronmicroscopy, 40-90nm-thick sections embedded in Lowicryl resin were collected on nickel grids, incubated for 10 minutes in 1% cold-water fish gelatin in Tris buffer as above, and immunostained with the anti-loricrin antibody (1:100 dilution). The primary antibody was omitted in controls. Anti-rabbit IgG conjugated to 10nm-large gold particles (Sigma, USA) was used as the secondary antibody at the concentration of 1:70 in buffer. The incubation of the tissue with the secondary antibody was carried out for one hour at room temperature. After a light staining with uranyl acetate (7 minutes) the sections were observed under a CM-100 Philips electron microscope.

## RESULTS

The multi-layered epidermis of the hairy digits of the platypus possessed a linear or undulating outline with some epidermal papillae, often containing numerous melanocytes (Fig. 1A). Generally a granular layer was not visible under the light microscope while a thin stratum corneum was present (Fig. 1B). The immunostaining for loricrin showed that only the transitional and corneous layers were positive while the other epidermal layers and the dermis were negative (Fig. 1C).

The ultrastructural analysis showed that many keratinocytes of the basal layer and of the spinosus layer contained numerous melanosomes which appeared even more numerous within the flattening cells of the upper stratum spinosus (Fig. 2A). The keratinocytes of the stratum spinosus contained mainly tonofilaments of low to medium electron-density, some of which converged to desmosomes. Numerous vesicular and some lamellar bodies were present in the cytoplasm of the upper keratinocytes of the upper spinosus layer, and were localized among the tonofilaments (data not shown, see ALIBARDI & MADERSON, 2003).

The keratinocytes of the upper layers of the stratum spinosus contained numerous dense tonofilaments and also sparse and dense keratohyaline granules, 0.1-0.2μm in diameter. These granules were more numerous, with variable but irregular size (0.1-0.3μm) in the transitional layer (pre-corneous) where they appeared merged with a pale corneous material (Fig. 2B). The latter corneous material did not contain tonofilaments but was mainly amorphous. Therefore these granules were identified as composite keratohyaline granules (darker F-granules surrounded by paler amorphous material). A 10-20nm thick cell corneous membrane was seen in corneocytes of the transitional layer (pre-corneous).

In the stratum corneum, cornified cells (corneocytes) showed an irregular or dentate surface that interlocked these cells to form a compact tissue. The corneocytes of the stratum corneum were surrounded with a 10-20nm thick and dense cell corneous membrane. The corneous material within corneocytes was not homogenous but contained irregular dense areas among paler material, probably derived from cell or nuclear degeneration.

The ultrastructural immunogold analysis showed that no specific labeling for loricrin was present in keratinocytes of the basal and spinosus layers. In the upper layers of the spinosus layer, and in the corneocytes of the transitional (pre-corneous) layer, the pale areas and the irregular granules present between the dense keratohyaline or between the condensing keratin bundles, were decorated with gold particles (Fig. 2C-D). Also in the composite granules, the labeling appeared mainly associated with the pale material present amongst the coarse network of dense material forming the F-granules (Fig. 2E).

In the transitional corneocytes the immunolabeling was diffuse (Fig. 3A) but in mature corneocytes of the stratum corneum, the labeling tended to localize along the plasma membranes or on the cell surface of mature corneocytes (Fig. 3B). Gold particles were also seen along parts of the desmosome remnants connecting the corneocytes of the stratum corneum, while the labeling for loricrin almost disappeared in the central corneous mass of mature corneocytes.

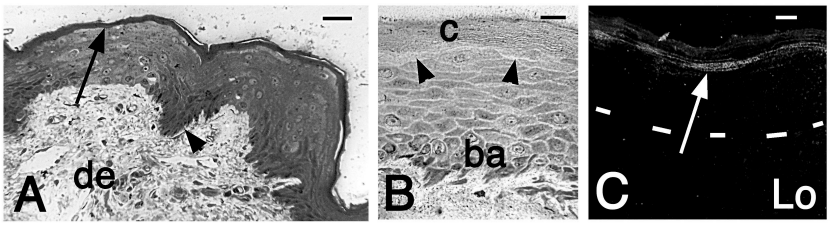


Fig. 1. – Light microscopy of the skin (hairy epidermis) of platypus (semithin sections). **A**, histological view of epidermis with thin stratum corneoum (arrow) and small papilla (arrowhead) into the dermis (de). Toluidine blue stain. Bar=10μm. **B**, detail of thick epidermis with irregular basal layer (ba), spinosus and transitional agranulated layer (arrowheads), and the corneous layer (c). Toluidine blue stain. Bar=10μm. **C**, Immunofluorescent staining for loricrin (Lo) of the corneous layer (arrow). Dashes underline the basal layer of the epidermis. Bar=10μm.

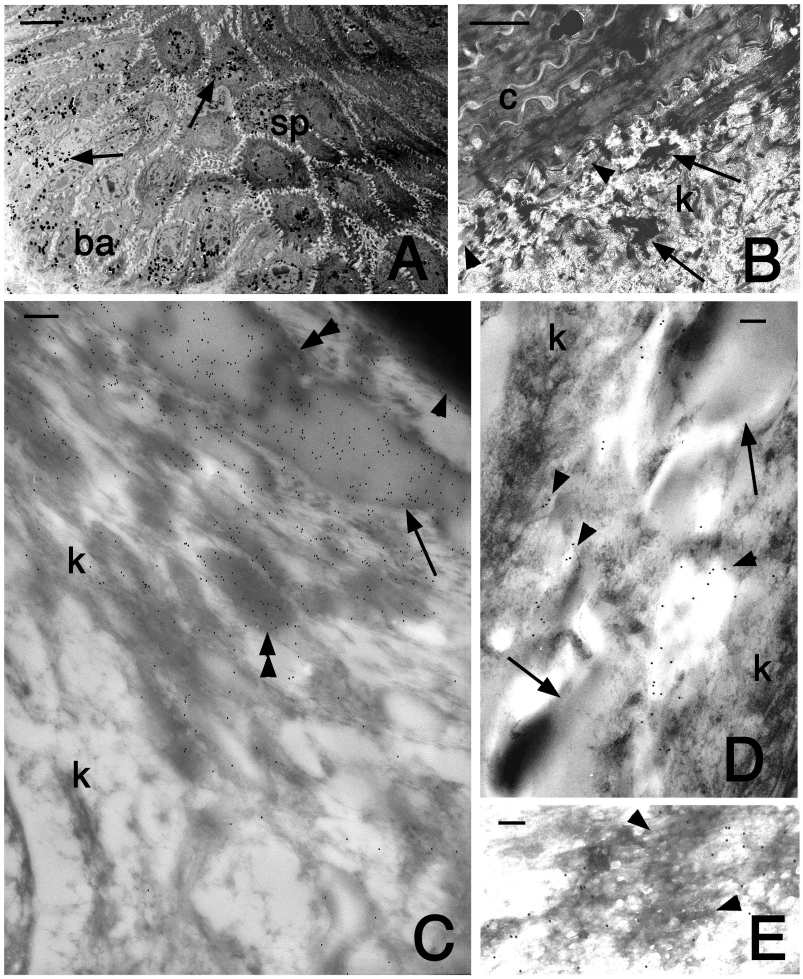


Fig. 2. – Ultrastructure (**A-B**) and Immunogold labeling (**C-E**) of the hairy epidermis of platypus. **A**, basal (ba) and spinosus (sp) layers of the thick epidermis. Arrows indicate the numerous melanosomes incorporated in these cells. Bar=5μm. **B**, detail of the transitional layer beneath the stratum corneum (c). Transitional cells contain pale granules (arrowheads) and composite keratohyaline granules (arrows) among bundles of keratin (k). Bar=1μm. **C**, diffuse loricrin immunolabeling in transitional cell (the arrowhead indicates the corneous layer). Most labeling is seen over pale material (arrows) associated with denser (double arrowheads) material forming the irregular keratohyaline granules (k). Bar=200nm. **D**, detail of cytoplasm of spinosus cell with diffuse labeling (arrowheads) among keratin bundles (k) and pale areas (arrows). Bar=100nm. **E**, detail of composite keratohyaline granule (arrowheads on coarse filaments forming the network) with labeling in the pale components. Bar=100nm.

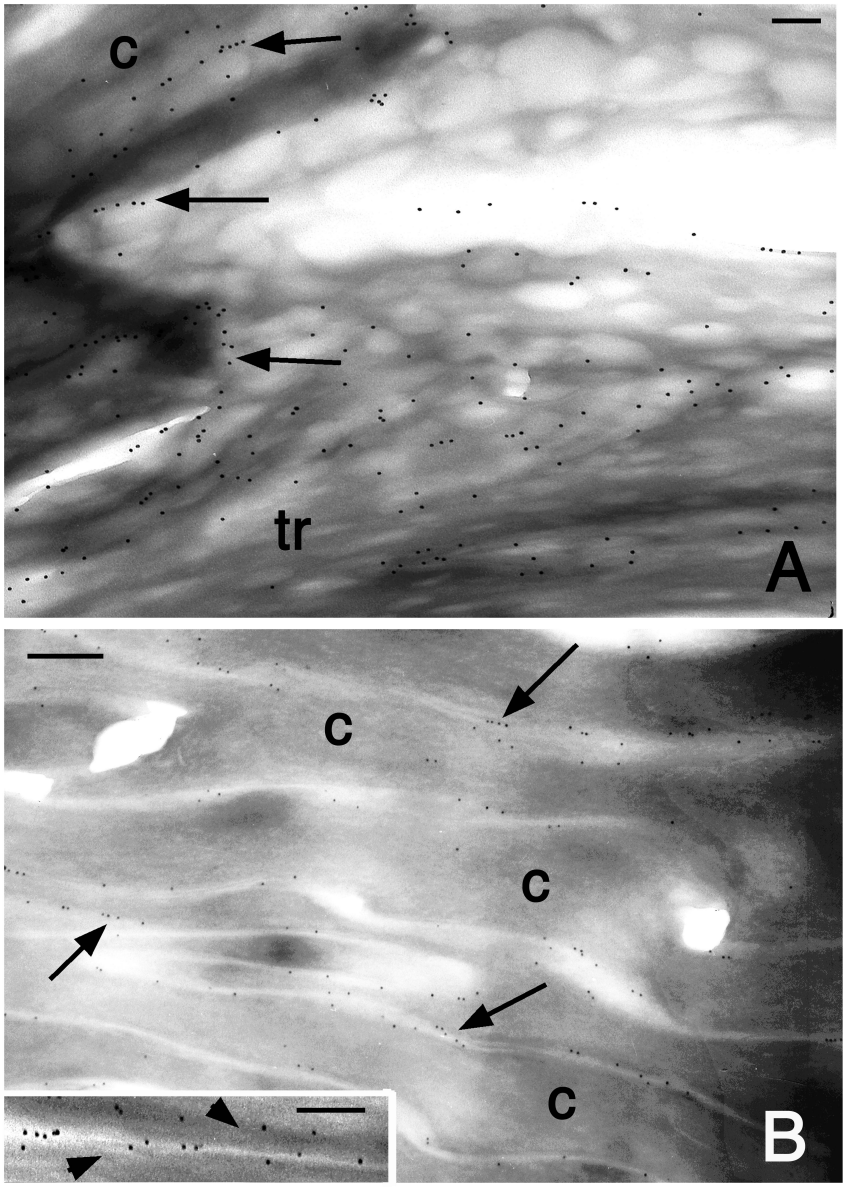


Fig. 3. – Immunogold labeling of external epidermal layers of the hairy epidermis of platypus. **A,** transitional layer (tr) with diffuse distribution of gold particles that tend to align along the plasma membrane of the corneocytes of the stratum corneum (arrows). **B**, loricrin-immunolabeling in corneocytes (c) of the stratum corneum where most gold particles are localized along the plasma membrane (arrows). Bar=100nm. The inset details the linear distribution of gold particles, mainly along the dense border (arrowheads) of corneocytes (Bar=100nm).

## DISCUSSION

The present study shows that keratohyaline granules in the hairy epidermis of the platypus are of small dimension (around 0.2mm), and therefore not easily detected under the light microscope. An extensive survey on the skin of many domesticated and wild mammals has shown some histological variations in the epidermis, in particular in relation to the presence, reduction or even disappearance of the stratum granulosum (SOKOLOV, 1982). In fact, the stratum granulosum is not clearly seen (in marsupials, pholidotans, dermopterans, chiropterans, cetaceans, sirenians, arctiodactylans, proboscideans), discontinuous (in insectivores, carnivores, xenartrans), or present with different degrees of size and location (in lagomorphs, primates, rodents, pinnipeda). It is possible, however, that, as in marsupials, so also in the epidermis of the placentals where the stratum granulosum is not visible under the light microcope, keratohyaline granules of submicroscopical dimensions may be present (smaller than 0.4μm), as in the case of bat epidermis (ALIBARDI, 2006b).

The scattered distribution of loricrin-immunolabeling previously detected in the parakeratotic interdigital (web) epidermis of the platypus resembles the immunolabeling of reptilian and avian corneocytes (ALIBARDI & MADERSON, 2003). In corneocytes of the hairy epidermis of the platypus, however, loricrin or loricrin-like immunolabeling is present over the electron-pale and amorphous material that is localized among keratin bundles and denser keratohyaline granules.

Parakeratosis in mammalian epidermis is often associated with pathological conditions (psoriasis, eczema, ichthyosis, and sometimes hyperkeratosis), and has been considered as a reversion to a more primitive form of cornification, possibly present in the first cotylosaurian reptiles from which therapsids and later mammals derived (SPEARMAN, 1964; 1966; MADERSON, 1972; ALIBARDI, 2003).

The process of terminal differentiation in both orto- and para-keratotic corneocytes eventually determines the formation of the cell corneous envelope, indicating that at least some proteins synthesized in these terminally-differentiated cells become cross-linked to the plasma membrane (KALININ et al., 2002). A loricrin-like immunoreactivity has been not only found in mammalian epidermis, but also in the epidermis of reptiles and birds, suggesting that a loricrin-like protein or a similar sulfur-rich protein could be an ancient structural protein necessary for the formation of the resistant cell corneous membrane of amniote epidermis (ALIBARDI, 2003; ALIBARDI & TONI, 2004). The immunological evidence on the presence of loricrin-like proteins in the epidermis of lower vertebrates, further suggests that its protective role was established very early during the evolution of the stratum corneum in tetrapods (ALIBARDI, 2006a).

It is, however, uncertain whether the diffuse distribution of loricrin is really primitive in mammals, since human and rat keratinocytes also show a diffuse labeling for loricrin in some body areas, and specific organelles (L-granules) are also not seen in some regions of the epidermis (STEVEN et al., 1990; HARDMAN et al., 1998; ISHIDA-YAMAMOTO et al., 1996; 2000). A random distribution of loricrin is also present in human corneocytes forming the stratum corneum in some pathological conditions, suggesting that the mechanism of distribution of loricrin is defective in these specific pathological conditions (ISHIDA-YAMAMOTO et al., 1996, 2000).

In conclusion, in the monotremes, here represented by the platypus, loricrin contributes to the formation of the cornified cell corneous membrane of the hairy epidermis of digits, as it does in the epidermis of the other mammals.

## ACKNOWLEDGMENTS

The study was partially funded (60%) by a University of Bologna grant and by self-support (Comparative Histolab). The skin tissues were collected in the field and generously supplied by Dr. Tom Grant (Cronulla, Sydney) under his permit to study the Australian platypus.

## REFERENCES

ALIBARDI L (2003). Adaptation to the land: the skin of reptiles in comparison to that of amphibians and endotherm amniotes. Journal of Experimental Zoology, 298B:12-41.

ALIBARDI L 2004. Fine structure and immunocytochemistry of monotreme hairs with emphasis on the inner root sheath and trichohyaline-based cornification during hair evolution. Journal of Morphology, 261:345-363.

ALIBARDI L (2006a). Structural and immunocytochemical characterization of keratinization in vertebrate epidermis and epidermal derivatives. International Review of Cytology, 253:177-259.

ALIBARDI L (2006b). Ultrastructure of bat (*Pipistrellus kuhlii*) epidermis with emphasis on terminal differentiation of corneocytes. Zoological Research, 27:86-93.

ALIBARDI L. & MADERSON PFA (2003). Distribution of keratin and associated proteins in the epidermis of monotreme, marsupial, and placental mammals. Journal of Morphology, 258:49-66.

ALIBARDI L & TONI M (2004). Immuno-cross reactivity of transglutaminase and cornification marker proteins in the epidermis of vertebrates suggests common processes of soft cornification across species. Journal of Experimental Zoology, 302B:526-549.

CHUDINOV PK (1968). Structure of the integuments of theromorphs. Doklady Akademii Nauk. USSR, 179:226-229.

DALE BA, RESING KA & PRESLAND RB (1994). Keratohyalin granules proteins. In: LEIGH I, LANE B & WATT F (eds), "The Keratinocyets Handbook", Cambridge University Press, pp 323-350.

FINDLAY GH (1970). The role of the skin in the origin of mammals. South African Journal of Science, 66:277-283.

FUCHS E (1990). Epidermal differentiation: the bare essentials. Journal of Cell Biology, 111:2807-2814.

HARDMAN MJ, SISI P, BANBURY DN & BYRNE DN (1998). Patterned acquisition of skin barrier function during development. Development, 125:1541-1552.

HOLBROOK KA (1989). Biologic structure and function: perspectives on morphologica approaches to the study of the granular layer keratinocyts. Journal of Investigative Dermatology, 92(4, supplement):84S-104S.

ISHIDA-YAMAMOTO A & IIZUKA H (1998). Structural organization of cornified cell envelope and alterations in inherited skin disorders. Experimental Dermatology, 7:1-10.

ISHIDA-YAMAMOTO A, EADY RA, WATT FM, ROOP DW, HOHL D & IIZUKA H (1996). Immunoelectron microscopic analysis of cornified cell envelope formation in normal and psoriatic epidermis. Journal of Histochemistry and Cytochemistry, 44:167-175.

ISHIDA-YAMAMOTO A, TAKAHASHI H & IIZUKA H (2000). Immunoelectron microscopy links molecules and morphology in the studies of keratinization. European Journal of Dermatology, 10:429-435.

KALININ AE, KAJAVA AV & STEINERT PM (2002). Epithelial barrier function: assembly and structural features of the cornified cell envelope. BioEssays, 24:789-800.

MADERSON PFA (1972). When, why, and how?: some speculations on the evolution of the vertebrate integument. American Zoologist, 12:159-171.

MATOLTSY AG (1986). The skin of mammals. Epidermis. In: MATOLTSY AG, BEREITHER-HAHN J & SYLVIA-RICHARDS K (eds), Biology of the Integument, vol B, vertebrates. Springer Verlag: Berlin and New York, pp 255-271.

MEHREL T, HOHL D, ROTHNAGEL JA, LONGLEY MA, BUNDMAN D, CHENG C, LICHTI U, BISHER ME, STEVEN AC, STEINERT PM, YUSPA SH & ROOP DR (1990). Identification of a major keratinocyte cell envelope protein, loricrin. Cell, 61:1103-1112.

RESING KA & DALE BA (1991). Proteins of keratohyalin. In: GOLDSMITH LA (ed), Physiology, biochemistry and molecular biology of the skin, vol 1, 2nd edition New York: Oxford University Press, pp 148-167.

SOKOLOV VE (1982). Mammalian skin. University of California Press, Berkley-Los Angeles-New York.

SPEARMAN RIC (1964). The evolution of mammalian keratinized structures. In: EBLING FJ (ed), The mammalian epidermis and its derivative. Symposium n. 12. London: Zoological Society, pp 67-81.

SPEARMAN RIC (1966). The keratinization of epidermal scales, feathers and hairs. Biological Review, 41:56-96.

STEVEN AC, BISCHE ME, ROOP DR & STEINERT PM (1990). Biosynthetic pathways of filaggrin and loricrin- two major proteins expressed by terminally differentiated epidermal keratinocytes. Journal of Structural Biology, 104:150-162.

*Received: January 30, 2009*
*Accepted: December 11, 2009*
*Branch editor: Schockaert Ernest*