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Immunocytochemistry and Keratinization in the Epidermis of Crocodilians

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Lorenzo Alibardi (2003) Immunocytochemistry and keratinization in the epidermis of crocodilians. *Zoological Studies* **42**(2): 346-356. The distributions of alpha and beta keratins, filaggrin and loricrine are described in crocodilian epidermis by immunocytochemistry and electron microscopy. Many layers of beta keratin cells are produced in the outer surface of the scutes, but in the hinge region between scales they are reduced to thin cells of alpha keratin. Alpha keratins are expressed mainly in the basal and a few suprabasal layers, but disappear in the upper spinosus, prekeratinized and keratinized layers. The production of beta keratin, as studied using an antibody against a chicken scale beta keratin, decreases in hinge regions where alpha keratin, including weak keratin-AE2, filaggrin-like, and loricrin-like immunoreactivities are seen. These immunoreactivities are probably associated with a common, unknown antigen. Beta keratin is deposited on bundles of tonofilaments and desmosomes in cells of the upper part of the stratum spinosus which are turned into electron-pale beta keratin packets with the typical 3-4-nm keratin pattern. Some desmosome remnants are present among beta cells of the stratum corneum. Lipid material is also deposited in early beta-keratinizing cells, especially in the hinge regions where beta keratin is replaced by alpha keratin and thin alpha keratinocytes are produced like in the apteric epidermis of birds. Melanosomes derived from epidermal melanocytes are incorporated in maturing keratinocytes from the lowermost level of the stratum spinosus into the stratum corneum, and this produces the skin pigmentation. Although beta keratinization is similar in both neck (softer scales) and tail verticils (harder scales) a heavier condensation of pale beta packets is present in the larger and spinulated beta cells of the tail epidermis. http://www.sinica.edu.tw/zool/zoolstud/42.2/346.pdf

Key words: Crocodilian epidermis, Keratins, Associated proteins, Immunocytochemistry, Ultrastructure.

During adaptation to terrestrial environments, the vertebrate epidermis has improved its protective system in its capacity to limit water loss, to stop microbical invasion, to develop a chemical protection or communication, and to increase mechanical resistence to wear and external agents (physical substrate, predators, temperature) (Lillywhite and Maderson 1982, Maderson 1985, Matoltsy 1987).

The epidermis of basal amniotes was probably capable of limiting water loss more efficiently than that of ancestral amphibians, and was also more efficient in mechanical resistence, probably with the loss of the breathing capacity of the epidermis of ancestral and modern amphibians. The chemical nature of keratinization therein is unknown, but probably was a variably advanced form of alpha keratinization, as beta keratin probably evolved later in sauropsid reptiles (Maderson and Alibardi 2000).

The modality of alpha keratinization can produce forms of extremely hard keratin, and has evolved in the therapsid lineage from which mammals evolved. Hard alpha keratin is present in mammalian hairs, quill, horns, nails, claws, and scales (Baden and Maderson 1970, Marshall et al. 1991). Mammalian hard keratins are made of alpha keratin intermediate filaments embedded in an amorphous matrix composed of high-sulphur and high-tyrosine proteins.

By the time reptilian radiation took place in the Mesozoic, several lineages (turtles and diapsids) had probably already developed a tough epidermis where beta keratin, a new form of hard keratin, was produced in some types of epidermal cells, later inherited by birds (Schweitzer et al.

1999, Sawyer et al. 2000). This new gene product, beta keratin, presents specific genomic, chemico-physical, ultrastructural, morphological, and staining properties (Matulionis 1970, Frazer et al. 1972, Wyld and Brush 1979 1983, Gregg and Rogers 1986, Sawyer et al. 2000).

Extanct primitive reptiles (crocodilians, chelonians, and the tuatara) can offer some testimonial of primitive forms of hard keratinization (beta keratinization) in the amniote epidermis. On this ground, the present study reports some morphological and immunocytochemical observations on the process of hard keratinization in scales of the neck and tail of the Australian saltwater crocodile and midbody areas of post-hatchling alligators.

The process of keratinization in crocodilians is poorly known, despite their scientific importance (they are archosaurs like birds) and economic value (for the leather industry) (Spearman 1966, Spearman and Riley 1969, Alexander 1970, Baden and Maderson 1970, Landman 1986). Recent ultrastructural studies have shown variations of skin morphology during embryogenesis in the American alligator (Alibardi and Thompson 2000 2001 2002). However no data on the distribution of alpha and beta keratins and of possible interkeratin matrix proteins and cell corneous envelope proteins are known in crocodilians. The present study deals with keratin distribution, and with the possible participation of filaggrin (a mammalian inter-keratin matrix molecule) and loricrin (a mammalian molecule involved in the formation of the cell corneous envelope) in the formation of the corneous layer in crocodilians. This is compared with the process of keratinization in other reptiles, birds, and mammals.

MATERIALS AND METHODS

For this study 2 young adults of the Australian saltwater crocodile, *Crocodyle porosus* Schneider, 1801, and 3 juvenile (1-2-d-old) individuals of the American alligator, *Alligator mississippiensis* Daudin, 1802, were used. The male crocodile was 1.37 m and the female 1.29 m in total length. The post-hatchling of alligators were 21-24 cm long. The skin of crocodiles was collected from the neck (softer scales) and tail (harder scales) after biopsies, while that of alligators was derived from the belly, lateral midbody wall, back, and proximal tail, after killing the animals by decapitation.

Some tissues were fixed for 6-10 h in Carnoy's fluid or in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4, dehydrated, and embedded in Lowcryl KM4 or Bioacryl resin under UV polymerization at 0-4°C (Scala et al. 1992). Others were fixed in 2.5% glutaraldehyde in buffer, post-osmicated, stained in toto with 1% uranyl acetate for 60-90 min, and embedded in Spurrs' resin. The latter tissues were used for ultrastructural analysis, after collecting thin sections (40-70 nm) on copper grids, and routine staining of the grids with uranyl acetate and lead citrate.

Thick sections (1-5 μ) were collected with an ultramicrotome on gelatin-coated slides for immunocytochemistry. On some uncoated slides, thick sections were stained with 0.5% toluidine blue for general histology. Thin sections (40-90 nm) were collected on nickel grids for the following immunogold procedure (see details in Alibardi 2000a b).

Immunocytochemistry on thick sections was done using large-spectrum antibodies against alpha keratins (AE1, AE2, AE3; Sun et al. 1983; purchased from Progen, Heidelberg, Germany), beta keratins (a generous gift of Dr. R.H. Sawyer, Univ. of South Carolina, Columbia, SC, USA; see Sawyer et al. 2000), filaggrin (antibody #466, a generous gift of Dr. B.A. Dale, Univ. of Washington, Seattle, USA), and loricrine (a generous gift of Dr. E. Fuchs, Univ. of Chicago, IL, Chicago, USA; see Mehrel et al. 1990). The anti-alpha keratin antibodies were used at a dilution of 1: 50-1: 100, the anti-beta at 1: 100, the anti-filaggrin at 1: 500; the anti-loricrin at 1: 500).

In order to carry out immunofluorescence labelling, sections were pre-incubated for 20 min in buffer containing 5% normal goat serum and 2% BSA in order to block unspecific groups. Then sections were incubated overnight at 0-4°C in the primary antibody in buffer with 2% BSA (this was omitted in the controls), rinsed, and incubated for 1 h with the secondary antibody (1: 50-100, goat anti-rabbit or anti-mouse according to the primary antibody, conjugated to fluorescein thiocyanate). Sections were observed under a Zeiss epifluorescence microscope (Jena, Germany) equipped with a filter for the visualization of fluorescein.

In order to label sections with immunogold, the sections on grids were incubated overnight in the primary antibody in Tris buffer at pH 7.6 containing 1% cold-water fish gelatin. In controls, the primary antibody was omitted. The sections were then washed carefully in buffer, and incubated with goat anti-rabbit or anti-mouse secondary antibodies (1: 40-50) for 1 h. After extensive rinsing, the grids were stained for 5 min in 2% uranile acetate

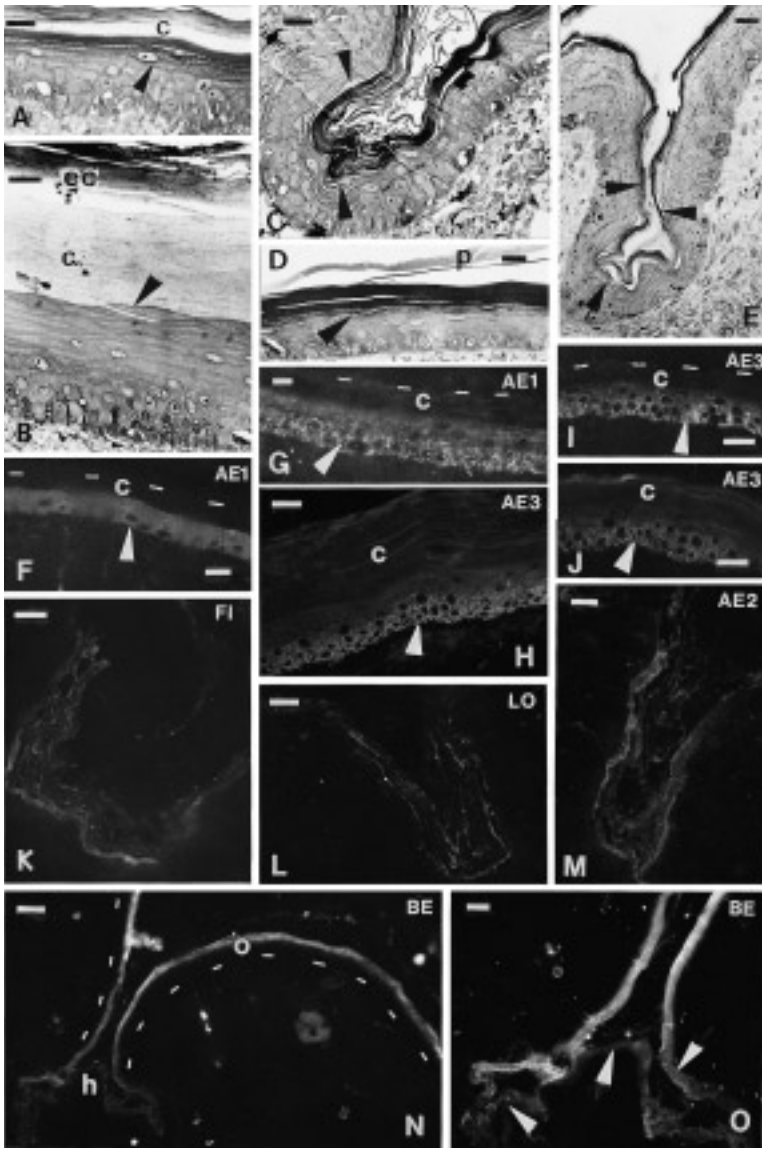

Fig. 1. Light microscopic images of toluidine blue-stained (A-E) and immunofluorescent (F-O) epidermis. A, Longitudinal section of a crocodile neck scale showing prekeratinized cells (arrowhead) under the corneous layer (c). Bar, 10 μ. B, Longitudinal section of a tail scale of the crocodile showing the thick pale corneous layer (c) and darker external corneous (ec) layer. The arrowhead indicates a polygonal, incompletely mature, beta cell. Bar, 10 μ. C, Cross section of the crocodile hinge region showing the flattening of pale prekeratinized cells (arrowheads) and the dark keratinized layer. Bar, 10 μ. D, Longitudinal section of a lateral scale of a hatched alligator still showing a detaching periderm (p) with a dark beta layer underneath (arrowhead). Bar, 10 μ. E, Longitudinal section of a belly scale of the alligator showing the ends of the beta keratin corneous layer (arrowheads) which is replaced by a paler corneous layer in the hinge region (arrow). Bar, 10 μ. F, AE1 immunofluorescent basal (arrowhead) and suprabasal layers of alligator ventral scale epidermis. The dots indicate the external limit of the immunonegative corneous layer (c). Bar, 10 μ. G, AE1 immunofluorescent basal (arrow) and suprabasal layer of crocodile neck scale epidermis. The dots indicate the external limit of the immunonegative corneous layer (c). Bar 10 μ. H, AE3 immunofluorescent basal (arrowhead) and suprabasal layers of a crocodilian tail scale. The thick corneous layer is negative. Bar, 20 μ. I, AE3 immunofluorescent basal (arrowhead) and suprabasal layers of lateral alligator scale. The corneous layer, whose external limit is indicated by dots, is negative. Bar, 10 μ. J, AE3 immunopositive basal (arrowhead) and suprabasal layers, and negative corneous layer (c) of a crocodilian neck scale. Bar, 10 μ. K, Weak filaggrin-like immunoreactivity (FI) of the corneous layer in the hinge region of an alligator scale. Bar, 20 μ. L, Weak loricrin-like immunoreactivity (LO) of the corneous layer of the hinge region of a crocodile scale. Bar, 20 μ. M, AE2 immunofluorescent corneous layer of an alligator lateral scale. Bar, 20 μ. N, Beta-1 (BE) immunofluorescent corneous layer in the outer scale surface (o) of an alligator lateral scale with fluorescence that disappears in the hinge region (h). Dots underlie the basal layer which is negative as well as the non-corneous layers. Bar, 20 μ. O, Detail of the hinge region showing the disappearence of beta-1 immunofluorescence (arrowheads). Bar, 10 μ.

at room temperature, and observed under a Philips EM-100 electron microscope (Eindhoven, The Netherlands).

RESULTS

Light microscopy and immunofluorescence

In the crocodile, the epidermis of the neck comprises 2-3 polygonal suprabasal cell layers before cells flatten under the pale corneous layer. Pre-keratinized cells show a granulosus cytoplasm due to beta-keratin accumulation (Fig. 1A). The thickness varied between 40 and 70 μ, with the corneous layer occupying about 1/2 or more of the entire thickness. The external part of the corneous layer was often darker than the thicker inner part. The tail epidermis, especially that of scale verticils, was much thicker (commonly over 100-150 μ) than that of the neck (Fig. 1B). Above the germinative layer, often containing cylindrical cells, 2-3 layers of suprabasal polygonal cells were found, followed externally by 6-9 layers of fusoid and toluidinophilic beta cells. The latter become abruptly unstained and compacted into the pale corneous layer, but conserved their cell boundaries. In contrast to the outer scale surface, the hinge regions were composed of a thinner epithelium (30-40 μ) for which pre-keratinized and keratinized layers occupy about 30%-50% of the entire thickness (Fig. 1C). Pre-keratinizing cells became very elongated along the curved outline of the hinge region; they flattened and remained pale (did not become granulated like those of the outer surface), until they piled up into the dark corneous layer. In the hinge region, keratinocytes were often loose so that the stratum corneum often appeared frayed.

Alligator epidermis is similar to that of the crocodile, although the thickness of the outer scale surface of hatchling epidermis was thinner (30-40 μ in ventral and lateral scales, and over 40 μ in dorso-caudal scales) with respect to that of older crocodile specimens (Fig. 1D). The hinge region of alligator scales also showed thinning of the corneous layer when the entire epidermal thickness was 20-30 μ (Fig. 1E). Analysis under a stereomicroscope revealed that the dark banding pattern of the skin was due to concentration of epidermal melanocytes in the epidermis of these areas, while dermal melanophores were sparsely distributed in these areas like in non-pigmented areas.

The AE1 immunolabelling of the epidermis in both species was mainly seen in the basal and first 2-3 suprabasal cells (those still polygonal) (Fig. 1F-G), and resembled that after AE3 staining (Fig. 1H-J). The corneous layer remained completely unstained with AE1, and low to unstained with AE3. The AE2 antibody did not stain the living layers of the epidermis, but weakly stained the corneous layer of the hinge regions, a pattern similar to that seen after filaggrin and loricrin immunolabelling (Fig. 1K-M). Finally beta-1 immunolabelling was only seen in the external corneous layer, and lower into the hinge region, where the fluorescence gradually disappeared (Fig. 1N-O).

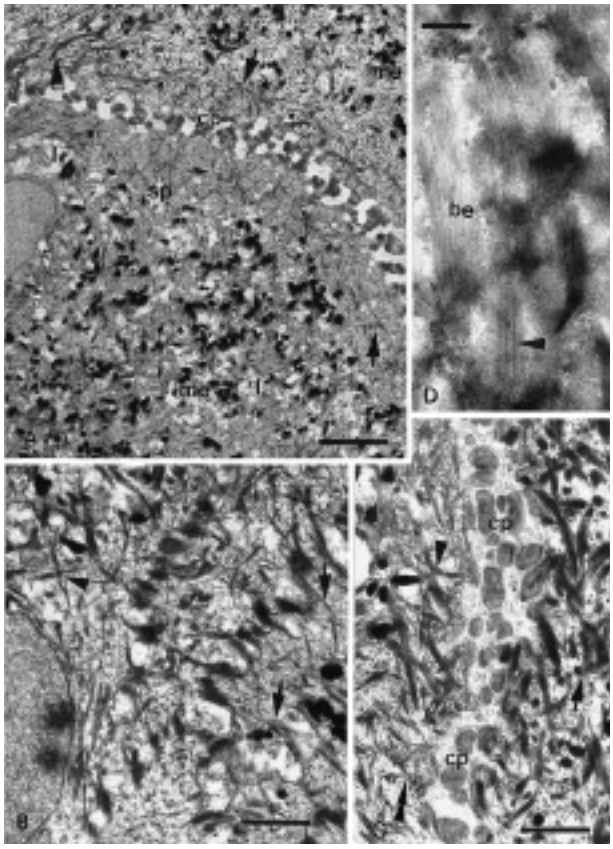

Fig. 2. Ultrastructural analysis of keratin accumulation in cells of the suprabasal layers of crocodile epidermis. A, Spinosus cell filled with melanosomes (me), tonofilaments (arrow), and lipid vacuoles (l). The upper cell is also rich in tonofilament bundles (arrow) but a few thicker filaments (arrowhead) can also be seen. cp, Cytoplasmic processes joining the spinous cells. Bar, 2 μ. B, Details of bundles of alpha-keratin filaments (arrows) and of rare thicker homogeneous beta-filaments (arrowhead) in an upper spinosus cell. Bar, 1 μ. C, Homogeneous beta keratin filaments (arrowhead) mix to tonofilaments (double arrowhead) in a cell of the upper spinosus layer. Numerous cytoplasmic processes (cp) contact a pre-keratinizing cell rich in darker beta-keratin bundles (arrow). Bar, 2 μ. D, Details of merging beta-keratin filaments (be) which are surrounding desmosomes (arrowhead). Bar, 0.25 μ.

Ultrastructure

The basal and the first 3-4 suprabasal cells layers showed many bundles of tonofilaments, composed by 7-10-nm dark keratin filaments (Fig. 2A-B). Some of these tonofilaments converged into desmosomes of the cytoplasmic processes joining spinous cells. Many melanosomes could be seen, especially where the skin was dark, while they were very scarce in paler skin.

In cells of the upper spinosus layers (4-7 layers above the germinative) most keratin bundles became larger; dark keratin filaments disappear and were replaced by a homogenous, electron-pale or electrondenser keratinaceous material in which filaments were no longer visible. At high magnification this keratin showed 3-4-nm electron-lucent filaments with sparse darker matrix (Fig. 2C-D). Beta-keratin bundles 50-300 nm in diameter were irregularly distributed in the cytoplasm of beta cells and in that of the uppermost flat cells, while most organelles had disappeared and few ribosomes remained (Fig. 2D). Also the cytoplasmic components of desmosomes, the dense attachment plaque became embedded within the beta keratin filaments.

Pale vacuoles, or even denser lipid droplets, remained sparse among irregular or roundish beta keratin bundles of spinosus and pre-keratinized cells (Figs. 2A, 3A). These lipid-containing organelles also remain inside the mature keratinocytes of the stratum corneum, and were often associated with degraded melanosomes (Fig. 3A-B). Beta keratinocytes of the hinge region and inner scale surface were thinner and had a smoother surface compared to those of the central part of the scale which had a spinous outline (Fig. 3A-B). In particular, keratinocytes of the outer scale surface of tail and dorsal scales had a very spinulated outline and showed desmosomal remnants. The beta layer resulted in a pluricellular stratum made of interlocking spinulated beta cells (Fig. 3C). The electron-dense material mixed with the electron-lucent beta keratin also remained adherent to the cell membrane to form an 8-15-nm dense band.

Cells of the hinge region contained few beta keratin filaments but were richer in pale vacuoles, lipid droplets, and small bundles of alpha keratin (Fig. 3D). While the lipid material remained in the core of the maturing keratinocytes, the keratin material lost its fibrillar aspect, became homogeneously dense, and aggregated along the cell periphery, as in avian (sebo) keratinocytes (Menon et als. 1986 1996). The 0.05-0.2 μ thick mature keratinocytes were more electrondense than were beta keratinocytes, and their surfaces were undulated or irregular but not spinulated as in beta cells (Fig. 3E). Lamellar material was rarely seen in the extracellular space, and cell corneous envelopes were scarcely developed or were completely

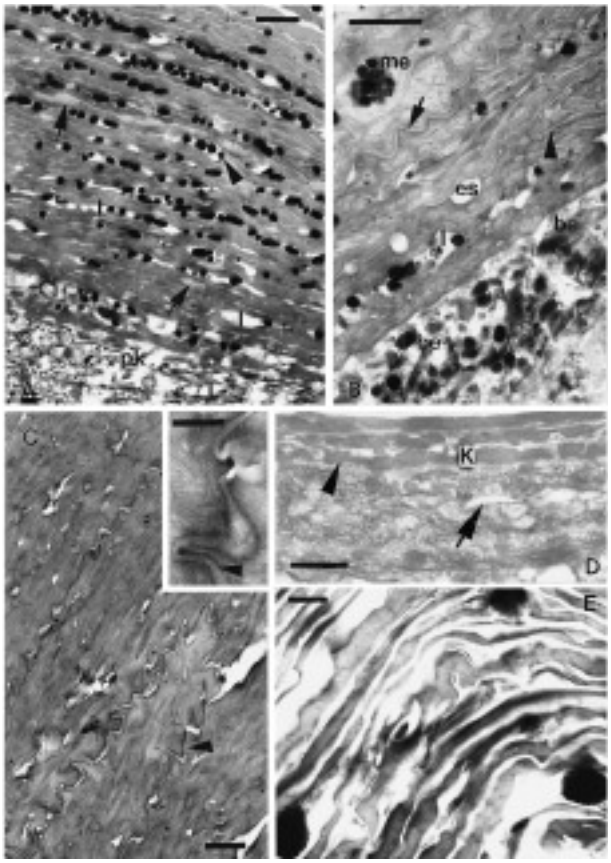

Fig. 3. Ultrastructure of pre-keratinized and keratinized layers. A, Pigmented (arrowhead on melanosomes) corneous layer of the lateral border of the outer surface of a neck scale of the crocodile. Arrows indicate intercellular lipid-mucous material. l, Indicates lipid within keratinized and pre-keratinized (pk) cells. Bar, 1 μ. B, Central part of the outer scale surface of a crocodilian neck scale showing spinulated (arrow) beta cells (es, extracellular space) containing a few lipids (l) and melanosomes (me). Some desmosomal remnants can be seen (arrowhead). In pre-keratinized cells beta-keratin filaments are mixed with electrondense material (be). Bar, 1 μ. C, Details of the corneous layer of the outer surface of a crocodilian tail scale. The arrowhead indicates the spinulated surface of single beta cells. Bar, 0.5 μ. The arrowhead in the inset details the desmosome remnants present along the spinulae; Bar, 250 nm. D, Alligator hinge region showing the packing of keratin (arrowhead) in alpha keratinocytes (K). The arrow points to lipid vesicles. Bar, 0.5 μ. E, Thin alpha keratinocytes of the stratum corneum of the hinge region of an alligator ventral scale. Bar, 0.5 μ.

absent along the plasma membrane.

Ultrastructural immunocytochemistry

Localization of AE3 labelling for keratin, as seen with the light microscope, was uneven over the tonofilament bundles of basal and 1st suprabasal cells (Fig. 4A-B). Instead, the keratin bundles of upper suprabasal cells tended to loose their immunolabelling (Fig. 4 C), and those of pre-keratinizing cells and corneocytes were little or not decorated at all with gold particles (Fig. 4 D).

By contrast, with beta-1 antiserum no labelling was seen in the lowermost keratin bundles but increased labelling was evident in those cells from the upper spinosus layers, 4-5 cells

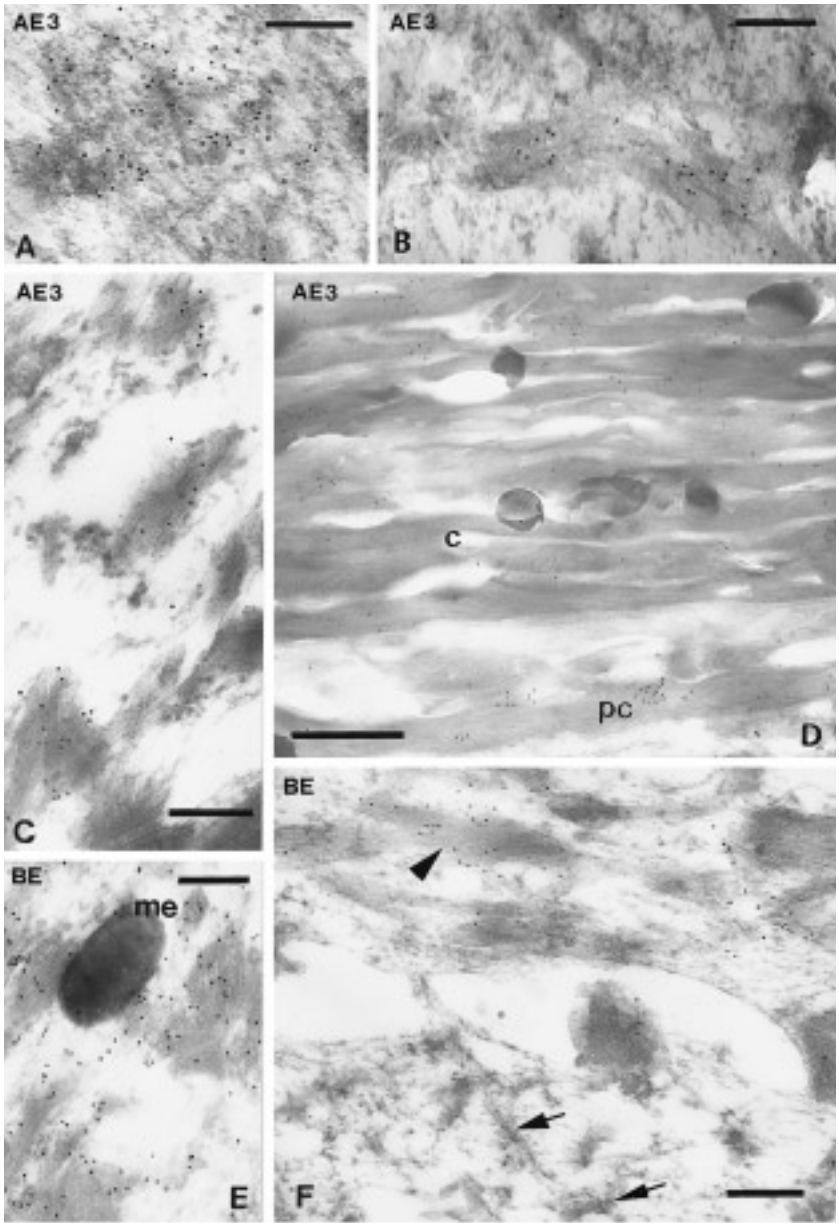

Fig. 4. Ultrastructural immunocytochemical distribution of alpha and beta keratins in alligator epidermis. A, AE3-labelled filament bundle in a basal cell. Bar, 250 nm. B, AE3-labelled bundles in a suprabasal cell. Bar, 200 nm. C, Sparce AE3-labelled keratin bundles in a cell of the upper spinosus layer. Bar, 200 nm. D, Low AE3-labelling of the pre-corneus (pc) and corneous (c) layers. Bar, 0.5 μ. E, Intense labelling of keratin filaments in cell of the upper spinosus layer using the beta-1 antibody (BE). me, Melanosome. Bar, 200 nm. F, Beta-1 immunonegative keratin filaments (arrows) in a low spinosus cell which contacts an upper cell containing immunopositive bundles (arrowhead). Bar, 250 nm.

above the germinative layer (Fig. 4E-F). This was indicated by the appearence of immunofluorescence for the beta-1 antibody in fusoid cells of the 2-3 prekeratinizing layers of crocodile tail scales (Fig. 5A). Beta-1 immunofluorescence, like that seen in alligator scales, disappeared in the hinge regions of crocodile scales (Fig. 5B). The intense immunofluorescence of the corneous layer of the outer scale surface (Figs. 1N-O, 5C) was confirmed by the strong immunolabelling with gold particles of pre-keratinized and keratinized materials. Also, desmosomal cytoplasmic attachment filaments were immunolabelled for beta keratin (Figs. 2D, 5D). The immunolabelling was also intense in keratin bundles of pre-keratinizing cells but decreaseed in those of the lowermost spinosus cells (Figs. 4F, 5D-E).

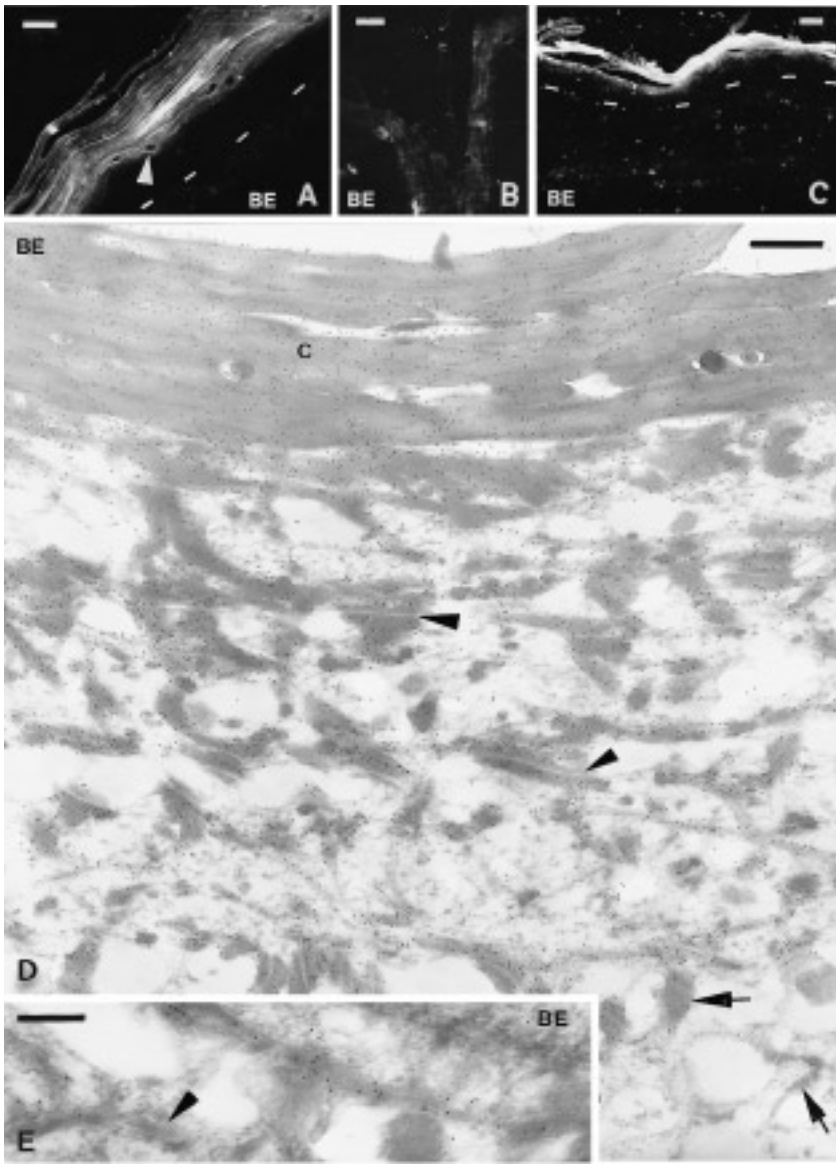

Fig. 5. Distribution of beta-1 labelling (BE) in upper keratinizing layers. A, Immunofluorescence only in the corneous and precorneous (arrowhead) layers of a crocodilian tail scale. Dots underlie the basal layer. Bar, 20 μ. B, Disappearance of immunofluorescence in the hinge region of a crocodile neck scale. Bar, 20 μ. C, Intensely labelled corneous layer of an alligator lateral scale. Dots underlie the basal layer. Bar, 10 μ. D, Intensely labelled keratin filaments in pre-keratinized and corneous (c) layers of an alligator ventral scale. Also the cytoplasmic component of the desmosomes is labelled (arrowheads). The labelling decreases until it disappears in bundles of the lowermost spinosus cells (arrows). Bar, 0.5 μ. E, Details of unlabelled (arrowhead) keratin bundles in lower spinosus cell as compared to the labelled filament of the next upper cell. Bar, 250 nm.

DISCUSSION

Keratinization in crocodilians

The present study on adult crocodilian skin completes previous analyses on embryonic skin (Alibardi and Thompson 2000 2001 2002). The thickness of the epidermis increases with age due to thickening of the corneous layer. Also, larger beta cells (e.g. those of the caudal and dorsal scales) have a more spinulated surface than those of smaller scales (e.g. those of the neck and lateral midbody wall), and they interlock more tightly with each other so that the resulting tissue may be more resistent and tougher.

The distribution of alpha keratins described herein roughly resembles that seen in mammalian epidermis (Sun et al. 1983). The AE1 and AE3 immunostaining patterns also resemble those described in other reptiles, such as lizards and turtles (Alibardi 2000a 2002c). The AE1 and AE3 patterns of fluorescence partially overlap suggesting that 50-kDa and 56.5-kDa keratins (AE1, acidic) and 58-kDa and 67-kDa keratins (AE3, basic) are not so precisely localized as in mammals where AE1 remains only in the basal layer, an artifactual staining pattern as both acidic and basic keratins have been biochemically detected in all epidermal layers (Sun et al. 1983). The AE3 antibody also stains the upper suprabasal layers, suggesting that basic keratins (AE3 positive) are associated with beta keratin. In turtles, only a 61 kDa keratin (probably basic) has been detected as the keratin isoform of higher molecular weight (Fuchs and Marchuck 1983), and it is associated with beta keratin in the uppermost layers (Alibardi 2002c). Biochemical studies on keratins in crocodilians are required to understand the mechanism of association between alpha and beta keratins during the progressive phases of keratinization.

The disappearance of AE2/AE3 immunolabelling from the stratum corneum, which is instead labelled in mammalian epidermis, confirms that a different form of keratinization takes place in the epidermis of crocodilians, with replacement of alpha with beta keratin as indicated by the beta antibody. In fact, AE2 and AE3 labelling is still present in the hinge regions of crocodilians, where beta keratin tends to disappear. Ultrastructurally, this keratin replacement is manifested by the transition from tonofilament bundles with more or less visible alpha keratin filaments into a homogenous mass of beta keratin (Alexander 1970, Baden and Maderson 1970, Matulionis 1970, Frazer et al. 1972, Baden et al. 1974, Landmann 1979 1986, Alibardi and Thompson 2001 2002). This replacement of keratins typically takes place in scutate scales of the chick, which are considered more similar to reptilian scales (Baden and Maderson 1970, Wyld and Bush 1979 1983, Sawyer et al. 1986 2000, Shames et al. 1988 1989). It is also confirmed in adult epidermis that, as in embryonic epidermis, beta keratin synthesis tends to disappear in hinge regions where the softer alpha keratin prevails. In these areas, a form of elastic keratin (alpha) is useful for stretching under mechanical/tensional demands. In these same areas, the presence of some AE2-positive alpha keratin, and of filaggrin-like immunoreactivity is probably related to some cross-reactivity of common antigenic epitopes between these proteins (Dale and Sun 1983). Another marker of alpha keratinization, loricrin, is weakly expressed in these same regions where beta keratin tends to disappear. Loricrine immunoreactivity may also indicate the presence of very small amounts of molecules with some cross-immunoreactivity to this antibody. Similar results have also been seen with soft alpha keratinized layers of other reptiles, such as lizards and turtles, and birds (Alibardi 2000b 2002a b c).

Other studies using tritiated histidine (a marker of histidin-rich proteins associated with alpha keratinization) have shown that reptilian beta keratinocytes are those that take up the least amount of this amino acid in comparison to alpha keratinocytes (Alibardi 2000b 2001 2002a). It remains to be biochemically demonstrated whether or not proteins similar to those of mammals are really present in these regions of crocodilian epidermis.

Neither lamellar bodies nor keratohyalin are present in keratinizing cells of outer surfaces, inner surfaces, or hinge regions of crocodilian scales. Lipids remain intracellular, among keratin filaments, especially in the hinge region. The lack of extracellular lipid lamellae, which are considered the most effective hydrophobic barrier in amniotes (Landmann 1980, Menon et al. 1986 1996), may be responsible for the relatively poor water barrier capacity of crocodilian skin in comparison to that of other reptiles (Bentley and Schmidt-Nielsel 1966, Davis et al. 1980, Lillywhite and Maderson 1982, Grigg et al. 1986, Dunson and Mazzotti 1988). It is not known whether the barrier can vary according to changes in ecological conditions, from wet to dry. In some birds, under xeric adaptation or water deprivation, the water barrier can increase within 12-24 h because of the extrusion of intercellular

lipids from lamellar granules (which normally remain intracellular), a phenomenon termed "facultative waterproofing" (Menon et al. 1996). Nothing similar is known when crocodilians move away from rivers or the sea for migration or when rivers dry up during the dry season.

Comparative aspects of beta keratinization

The cross reactivity after beta-1 antibody application further confirms the relationships between avian and reptilian beta keratins (Carver and Sawyer 1987, Sawyer et al. 2000).

Based on observations presented in this and other studies on reptilian epidermis, it can be postulated that beta cells of early progenitors of lepidosaurians, as in extant but ancient reptiles (Sphenodontia, Chelonia, and Crocodilia), possessed a completely spinulated cell surface. The latter cells, that we may indicate as beta echinokeratinocytes, probably had both alpha and beta keratin synthetic capabilities. This condition is today present in the intermediate epidermal region of the epidermis of *S. punctatus* (Alibardi 1999, Alibardi and Maderson, pers. observ.). The latter represents cells which progessively loose the alpha keratin characteristics to completely acquire beta keratin characteristics. In beta echinokeratinocytes of turtles and crocodilians (Alibardi and Thompson 1999 2001, Alibardi 2002c), and in beta cells of bird scales (Shames et al. 1988 1989, Sawyer et al. 2000), the production of beta keratin instead prevails over that of alpha keratin.

Finally, during the evolution of the integument in lepidosaurian reptiles, the progressive separation between beta- and alpha-keratin synthetic capabilities initially produced a pluricellular intermediate region like in *Sphenodon*. This region was progressively reduced to a single cell layer (the oberhautchen), and a shedding complex was formed. In fact, the 1st cell layer that accumulates beta keratin, the oberhautchen, is still in contact with the last alpha layer (the clear). The oberhautchen still shows a spinulated surface, as in beta echinokeratinocytes of turtles and crocodilians. This spinulated surface disappears in subseguent beta cells due to their fusion into a syncitial layer, termed the beta layer (Maderson 1985, Maderson et al. 1998). In lepidosaurian reptiles, there is probably a refined mechanism that switches on beta keratin synthesis while that of alpha keratin is maintained at a lower rate or is switched off.

Pigmentation

The present study confirms the observations on the epidermis of early embryos of the alligator (Alibardi and Thompson 2001 2002) which indicated that epidermal melanocytes determine the dark-banded pattern of the skin. While melanosomes can spread or condense within the cytoplasm of dermal melanophores after light exposition in amphibians and some reptiles, those injected into beta cells of lepidosaurians (Szabo et al. 1973), turtles, and crocodilians (Cooper and Greenberg 1992, present observ.) remain in place and are lost when superficial beta cells are desquamated due to wear. In this way, the pigmentation pattern is mainly a property of epidermal melanocytes, and the pattern is preserved as long as these cells in the germinative layer maintain their position and area of pigmentation.

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