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Keratinization in crocodilian scales and avian epidermis : evolutionary implications for the origin of avian apteric epidermis

Lorenzo Alibardi

Dipartimento di Biologia evoluzionistica sperimentale, University of Bologna, via Selmi 3, 40126, Bologna, Italy
Corresponding author : Lorenzo Alibardi, e-mail: Alibardi@biblio.cib.unibo.it

ABSTRACT. Terminal differentiation of keratinocytes of avian apteric epidermis occurs with the accumulation of little keratin and much lipids so that a soft and elastic corneous layer is produced. The distribution of keratins and some proteins associated with cornification has been studied in crocodilian scales, in ratite and zebrafinch apteric epidermis by means of light and ultrastructural immunocytochemistry. Soft (alpha)keratinization in apteric epidermis of birds resembled the process occurring in hinge regions of crocodilian scales where the stratum corneum was thin and beta(hard)-keratin disappeared. Acidic and basic alpha-keratins were seen in living pre-corneous layers. Instead, keratins typical for cornification, loricrine, transglutaminase, and sometimes filaggrin-like immunoreactivities, were present in the transitional and lowermost corneous layer of crocodilian hinge and apteric avian epidermis. Trichohyalin, involucrin, and iso-peptide bond immunoreactivities were absent. Loricrine-like and transglutaminase labelling were generally absent in the corneous layer, but were weakly present among keratin bundles and lipids in the transitional layer of apteric epidermis. Transglutaminase immunolabelling was present in condensing nuclear chromatin of transitional corneocytes of apteric epidermis, suggesting that these cells undergo terminal differentiation or even apoptosis. Sulfydryl groups in keratins, or specific sulfur-rich proteins of loricrin-type, were scarce in apteric epidermis. This suggests that the cornified cell envelope of avian keratinocytes is more simplified than that of mammalian keratinocytes. In the latter, numerous proteins concentrate along the corneous cell envelope to enhance mechanical and chemical resistance of the stratum corneum. This mechanism probably is more simplified in apteric epidermis of birds, and the mechanical protection of the epidermis is taken over by the plumage, while apteric epidermis has mainly a role as barrier against water loss. It is speculated that avian interfollicular and apteric epidermis has evolved from interscale hinge regions of proto-avian archosaurian ancestors.

KEY WORDS : crocodilians, ratite birds, epidermis, keratinization, immunocytochemistry.

INTRODUCTION

From the hard, scaled epidermis of archosaurian, and perhaps of theropod reptiles, scales and feathers in birds have probably evolved (SPEARMAN, 1966; MADERSON, 1972; BRUSH & WYLD, 1980; BRUSH, 1993; CHUONG et al., 2000; PRUM, 2002). It is thought that the evolution of the avian integument from scaled archosaurian reptile ancestors (MADERSON, 1972; MADERSON & ALIBARDI, 2000) has originated a soft epidermis (apterilae) among feathers (pterylae), while scales have remained in the skin of the hindlimb (ALIBARDI, 2003a, b, 2004a, b).

Previous morphological and molecular studies (SPEARMAN, 1966; MADERSON, 1972; GREGG & ROGERS, 1986; SAWYER et al., 1986; SAWYER et al., 2000; MADERSON & ALIBARDI, 2000), together with recent embryological, ultrastructural and immunocytochemical evidence (ALIBARDI & THOMPSON, 2000, 2001; ALIBARDI, 2003a, 2004b), suggest that the expansion of the hinge regions among scales of the proto-avian archosaurians might have originated the apteric epidermis. This pliable and elastic epidermis has transformed the rigid and scaled reptilian armour, into a flexible and articulate skin. While plumage and scales are epidermal appendages specialized for flying and mechanical protection, the lipid-rich and delicate apteric skin hosts the permeability barrier against water loss (MENON et al., 1986; MENON et al., 1996; MENON & MENON, 2000). The apteric and interfollicular epidermis also gives the mechanical plasticity necessary for feather orientation during flight (HOMBERGER & DE SILVA, 2000), participates in the homeothermic regulation by heat dissipation, secretes specific lipid-rich materials, and hosts special glands (LUCAS & STETTENHEIM, 1972; SPEARMAN & HARDY, 1985).

Presently, much information is available on the ultrastructure, biochemistry and molecular biology of feather and scale epidermis (MATULIONIS, 1970; BRUSH & WYLD, 1980; GREGG & ROGERS, 1986; SAWYER et al., 1986; 2000; CHUONG, 1993; CHUONG et al., 2000; ALIBARDI, 2002a). As opposed, except from the ultrastructure and lipid composition (LAVKER, 1975; ELIAS et al., 1987; SPEARMAN & HARDY, 1985; MENON et al., 1986, 1996; PELTONEN et al., 1998, 2000; MENON & MENON, 2000), little is known on the expression of specific proteins during the terminal differentiation of keratinocytes in apteric epidermis.

In mammalian epidermis, the synthesis of different proteins (involucrin, loricrin, filaggrin etc.) from the upper spinosus or in the granular layer, determines the formation of the transitional and corneous layer (FUCHS,

1990; RESING & DALE, 1991; RAWLINGS et al., 1994; KALININ et al., 2002; ALIBARDI & MADERSON, 2003). The production of similar proteins during maturation of keratinocytes in apteric epidermis of birds is not well known although new information is emerging (ALIBARDI, 2004a, b, ALIBARDI & TONI, unpublished observations). To address some of these questions, an ultrastructural and immunocytochemical study has been done on the distribution of keratins, loricrin, filaggrin, transglutaminase and isopeptide-bond in apteric epidermis of ratite birds, in comparison with the epidermis of crocodilian scales. Such a comparison aims to check whether the characteristics of keratinocytes of crocodilian hinge regions resemble those of keratinocytes of apteric epidermis in birds, termed sebokeratinocytes (MENON & MENON, 2000). This further supports the hypothesis that apteric epidermis is derived from hinge regions of proto-avian archosaurs (ALIBARDI, 2003a).

MATERIAL AND METHODS

Two young Australian saltwater crocodile, *Crocodyle porosus*, and 2 late embryos of the American alligator, *Alligator mississippiensis*, were used in the present study. The skin was collected from biopsies of the neck, belly, lateral mid body (softer scales), and from back and tail scales (harder scales), as previously detailed (ALIBARDI & THOMPSON, 2000; ALIBARDI, 2003a). Tissues (2-4 mm in length) were fixed for 5-8 hours in Carnoy fluid (9 parts 80% ethanol + 1 part acetic acid), dehydrated, and embedded in Lowcryl K4M resin under UV for 2-3 days at 0-4°C. Other tissues were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 post-fixed in osmium, dehydrated, and embedded in Durcupan resin.

Two zebrafinch embryos, from 12 days post-deposition eggs, were fixed in 2.5% glutaraldehyde as above. Other four zebrafinches were injected in the abdomen with of a Ringer solution containing tritiated histidine (L 2,5 3H-histidine, specific activity 40-60 Ci/mole, Amersham, UK) at a dosage of 2-4 μCi/g body weight. Samples of skin from the ventral and pectoral regions were collected 4 hours after the injection, for the histoautoradiographic study on skin sections.

From four adult emus (*Dromaius novae-hollandiae*) little pieces (3-5 mm) of skin were collected from a featherless area about 12 cm above the wing toward to the neck, and immediately fixed. Some tissues were fixed in 10% formaldehyde for 5-6 hours, stored in 0.1 M phosphate buffer for a few days, post-fixed in 2.5% glutaraldehyde for 2 hours, then in 2% osmium for 2 hours, dehydrated and embedded in Spurr resin. Other tissues were fixed in Carnoy's fluid, dehydrated, and embedded in Lowcryl K4M resin under UV-light. Fresh skin samples were obtained from the head and neck of four ostriches (*Struthio camelus*). Some tissues were fixed in 2.5% glutaraldehyde as above, post-fixed in osmium, dehydrated and embedded in Durcupan resin. Other pieces were fixed in 3% paraformaldehyde in phosphate buffer or in Carnoy's for 4-6 hours, and then embedded in Lowcryl K4M resin as above.

Semithin sections of the embedded tissues (1-4 μm thick) were obtained using a LKB-Nova ultramicrotome, and attached to gelatin-coated slides for the following immunocytochemical stain. From some areas of the skin, thin sections (40-90 nm thick) were collected on copper or nickel grids, stained with uranyl acetate and lead citrate, and observed under a CM-100 Philips electron microscope.

For autoradiography, tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for about six hours, post-fixed in 1% osmium tetroxide for one hour, dehydrated and embedded in the resin Durcupan. Sections of 2-4 μm thickness were coated with Ilford K5 Nuclear Emulsion, exposed in a darkroom for 1-2 months, developed in Kodak D19 and fixed in Ilfor fixer. Sections, either unstained or lightly stained with 1% toluidine blue, were studied under the light microscope for detection of the silver grains derived from the autoradiographic process. Other thin sections (40-90 nm thick) were collected onto collodion-coated slides and coated with Ilford L4 Nuclear Emulsion for ultrastructural autoradiography. After 3-4 months exposure, sections were developed as above, collected on nickel grids, stained and observed under the electron microscope.

For light microscopic immunocytochemistry, anti-keratin mammalian antibodies (AE1, AE2, AE3) were purchased from Progen (Heidelberg, Germany). They recognize most acidic and basic alpha-keratins (SUN et al., 1983; O'GUIN et al., 1987). The anti-filaggrin antibody was produced and generously supplied by Dr. BA Dale (Department of Oral Biology, University of Washington, Seattle, USA). It is a polyclonal antibody (#466) produced in rabbit and directed against rat filaggrin in both sections and blottings. The anti-loricrin antibody, produced in rabbit, was generously supplied by Dr. E. Fuchs (Howard Hughes Medical Institute, University of Chicago, USA), and recognizes a 15 amino acidic sequence toward the C-terminal of mouse loricrin (MEHREL et al., 1990). The antitrichohyalin antibody was kindly supplied by Dr. G. Rogers (University of Adelaide, Australia), and recognizes trichohyalin from sheep, rat and other mammals (ROTHNAL & ROGERS, 1996; A ALIBARDI, personal observations). The anti-involucrin antibody is a mouse monoclonal antibody directed against human involucrin (I-9018, Sigma, USA). The antibody ab421 against transglutaminase 1 of guinea pig liver (rabbit polyclonal), and the ab422 against N-?-(?-glutamyl)-lysine isopeptide bond (isopetide-bond, mouse monoclonal) were purchased from Abcam Limited, Cambridge, UK. Finally, the beta-1 is a rabbit polyclonal antibody directed against a chicken scale beta-keratin, and was generously supplied by Dr. R. Sawyer (University of South Carolina, Columbia, USA; SAWYER et al., 2000).

Tissues were preincubated for 30 minutes in 5% normal goat serum in 2% BSA in 0.05 M Tris/HCl buffer at pH 7.6, incubated overnight at 4° C in the buffer containing the primary antibody (dilutions, 1 : 50-100 for alpha-keratin antibodies, 1 : 500 for filaggrin, 1 : 200-300 for loricrin, 1 : 150 for transglutaminase, 1 : 150 for iso-peptide, 1 : 500 for trichohyalin, 1 :200 for beta-1 keratin, and 1 : 100 for involucrin). After several rinses in buffer, the sections were incubated in the same medium for one hour at room temperature containing 1 : 50 of anti-mouse-IgG (for antikeratin, involucrin, and iso-peptide)

or anti-rabbit-IgG (for filaggrin, loricrin, beta-1, transglutaminase, and trichohyalin) -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 immunoelectron microscopy, 40-90 nm thick sections were collected on nickel grids, and immunostained with the primary antibody against loricrine and transglutaminase as above (in controls the primary antibody was omitted). As secondary antibody an anti-mouse or anti-rabbit IgG conjugated to 10 nm large gold particles were used (Sigma, USA, or Biocell, UK). Sections were observed under the electron microscope either unstained or lightly stained with uranyl acetate, under a CM-100 Philips electron microscope operating at 60-80 kV.

RESULTS

Crocodilian scales

Crocodilian scales formed from narrow hinge regions among expanding outer scale surfaces (Fig. 1 A). By the end of development, as well as in adult scales, hinge regions remained as narrow areas between the outer surface of scales (Fig. 1 B). The outer scale surface will progressively expand into larger scales during the growth of the animal.

The epidermis of the young crocodilian scales consisted in a basal layer made of polygonal cells, 3-6 suprabasal layers made of flat cells, a transitional pre-corneous layer, a variably thick stratum corneum (for details see ALIBARDI, 2003a). Most of epidermal surface was formed by the dorsal part of scales which were delimited by narrow hinge regions. The corneous layer of alligator and crocodilian scales appeared thinner in hinge regions, where a higher AE2-immunoreactivity for alpha-keratin was present (Figs 1 C-D). As opposed, the immunoreactivity of the thicker epidermis of the dorsal portion of scales was immuno-negative for AE2-alpha-keratin, but immunopositive for beta-1 keratin which tended to disappear in the hinge regions (Figs 1 D-E). Corneocytes (mature keratinocyets of the stratum corneum) in the dorsal part of the scale were 0.5 μm or thicker, and featured a spiny or tortuous surface (Fig. 1 F). Bundles of beta-keratin accumulated onto the plasma membrane in the transitional layer. The mature cells of the beta-keratin corneous layer consisted in compact corneocytes. Melanosomes were mixed to beta-keratin, especially in dorsal scales in both alligator and crocodile.

In the hinge region, suprabasal keratinocyets accumulated numerous lipid vesicles and smooth endoplasmic reticulum, mitochondria often showed tubular cristae and tonofilaments were sparse (Fig. 1 G). In pre-corneous keratinocytes bundles of tonofilaments tended to accumulate along the thickened plasma membrane, and the core contained lipids and dense, mucus-like, granules (Fig. 1 H). Occasional beta-keratin bundles were present in these cells.

Corneocytes of hinge regions were very narrow (0.05-0.2 μm), and showed a smooth surface (inset in Fig. 1 H). The latter morphology resembled that of sebokeratinocytes in avian apteric epidermis (see later).

Apteric epidermis

In the zebrafinch embryo few large apteric regions formed between pterylae tracts (Fig. 2 A). The epidermis of ratites and zebrafinch was very similar, although variations in thickness were seen (Figs 2 B-C). Above a cubic to flat basal layer, 2-4 layers of flat intermediate cells preceded the straum corneum, which was made by several layers of thin cells. The basement membrane followed the tortuous indentation sometimes present along the basal cytoplasm of germinal cells. The latter, among the usual organelles, contained sparse bundles of tonofilaments which increased in diameter, density and dimension in suprabasal and pre-keratinized cells of the transitional layer (Figs 2 D-E). Bundles of electron-dense alpha-keratin were accumulated along the plasma membrane. The transitional or pre-corneous layer marked the passage from upper intermediate cells to completely keratinized cells of the stratum corneum (sebokeratinocytes). No keratohyalin granules could be differentiated from the irregularly sectioned tonofilament bundles that were present in transitional cells. Lipid vacuoles tended to concentrate in the middle of transitional and corneous cells, the plasmalemma became thicker and electrondense forming a cornified cell envelope (Figs 2 D-E). Lamellar figures were often seen extracellularly among corneous cells.

In the epidermis of the zebrafinch, 4 hours after tritiated histidine injection, the labelling appeared evenly distributed over all layers of the epidermis, and absent in the dermis (Fig. 2 F). The ultrastructural details of the labelling showed that silver grains were generally associated with the diffuse or condensed keratin filaments of transitional or mature sebokeratinocytes (Figs 2 G-H).

The AE1 immunofluorescence was mainly or exclusively seen in the germinal and first suprabasal layers of ratite epidermis (Figs 2 I-J), the AE2 antibody mainly stained the corneous and transitional layers (Figs 2 K-I, 3 A). Finally, the AE3 stained mostly living layers while the corneous layer (made of sebokeratinocytes) was generally less stained or not stained at all (Figs 3 B-C). The AE2 antibody sometimes also produced a nuclear staining, which was seen in many but not all nuclei of ratite epidermis.

In some apteric areas the anti-filaggrin antibody reacted over the transitional layer, while a large part of the corneous layer remained unstained (Figs 3 D-E). The anti-loricrin antibody produced a stronger and more consistent reaction than that against filaggrin, and the staining was more evenly distributed over all areas of apteric epidermis, especially in the transitional and lowermost part of the corneous layer (Figs 3 F-H). The transglutaminase antibody immunostained the transitional and lowermost corneous layer of the epidermis, but the immunofluorescence was patchy or completely absent in the corneous layer (Fig. 3 I). No immunolabelling was observed using antibodies directed against iso-peptide, trichohyalin and involucrin.

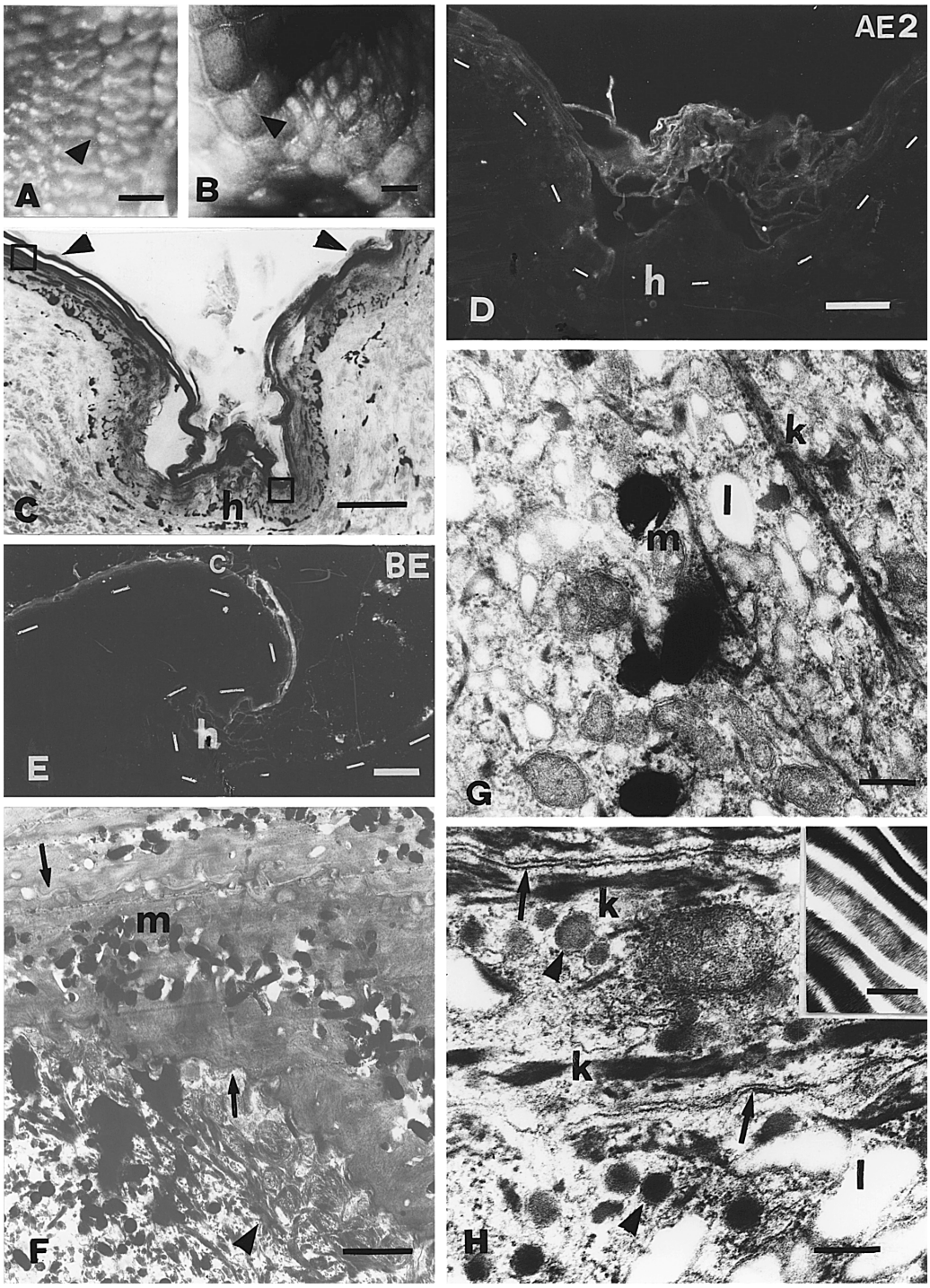

Fig. 1. – **A**, latero-ventral forming hinge regions (arrowhead) among the outer scale surface of scales of late alligator embryo. Bar, 0.5 mm. **B**, narrow hinge regions (arrowhead) among neck scales of late alligator embryo. Bar, 0.5 mm. **C**, hinge region of dorsal scale of alligator showing the disappearence of the pale beta-layer of the stratum corneum of scales (arrowheads). Bar, 40 µm. **D**, AE2-immunopositive corneous layer in hinge region of alligator dorsal scale. Bar, 30 µm. **E**, Beta-1 immunopositive corneous layer of outer surface of alligator dorsal scale, which disappears in the hinge region. Bar, 50 µm. **F**, accumulation of beta-keratin bundles (arrowhead) in pre-corneous cells of neck scale in crocodile. Numerous melanosomes are accumulated inside mature corneocytes which show an irregular surface (arrows). Bar, 1 µm. **G**, cytoplasm of differentiating keratinocyte in the hinge region of alligator dorsal scale where numerous lipid vesicles and sparce keratin bundles and mitochondria are seen. Bar, 0.25 µm. **H**, pre-corneous keratinocyte in the hinge region of alligator dorsal scale showing parallel alpha-keratin bundles, lipid vesicles, mucous-like granules (arrowheads), and thickened plasma membrane (arrows). Bar, 0.25 µm. Inset, narrow corneocytes in hinge region of alligator scale. Bar, 0.2 µm. **Legends** : AE2, AE2-immunoreaction for alpha-keratins; BE, beta-1 immunoreaction for beta-keratin; c, corneous layer; h, hinge region; k, keratin bundles; l, lipid material. m, melanosomes. Dashes underline the basal layer.

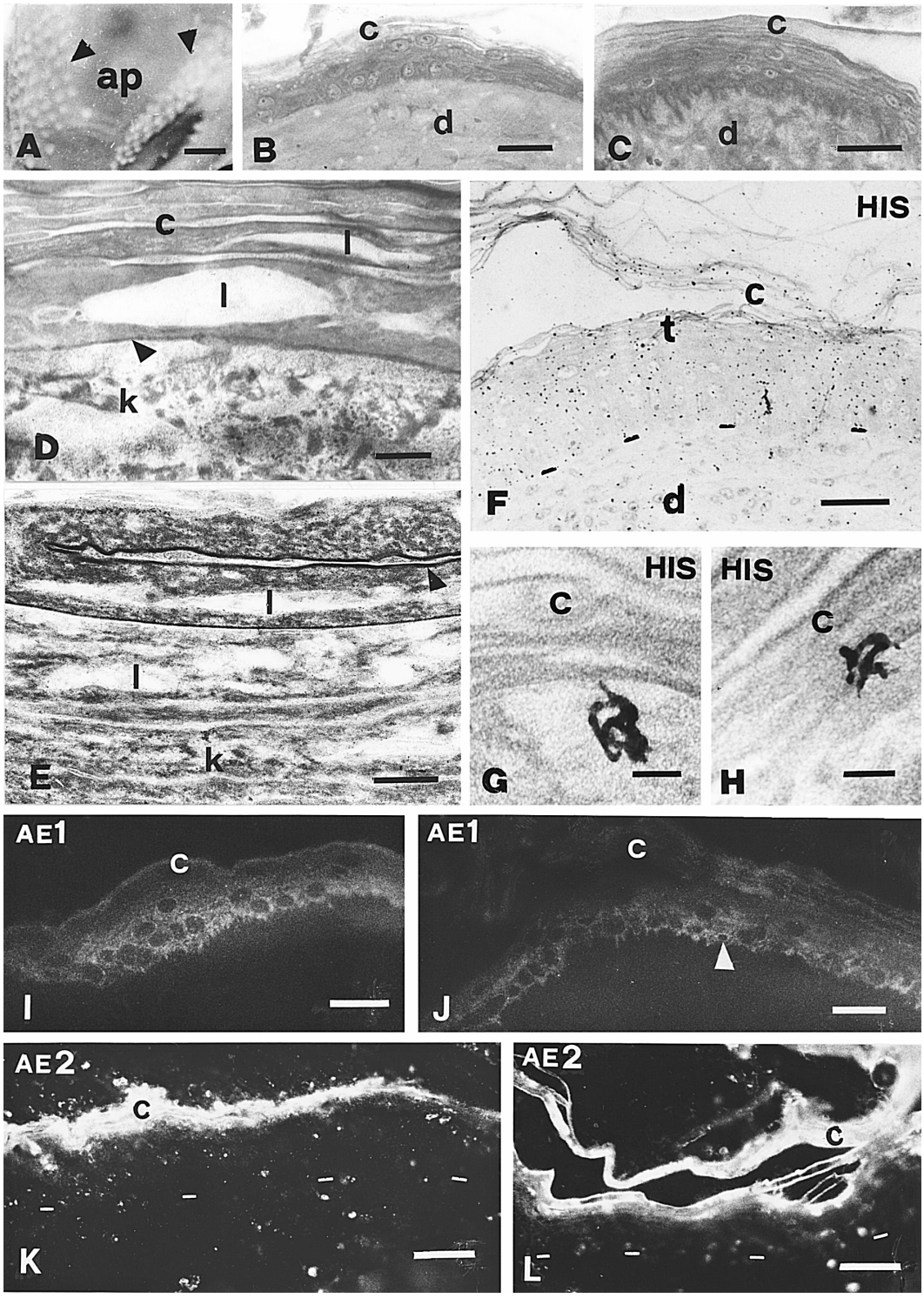

Fig. 2. – **A**, overview of zebrafinch embryo showing the smooth, apteric region between rows of feather germs (arrowheads) in the lumbar region. Bar, 1.5 mm. **B**, thin epidermis of emu. Bar, 20 µm. **C**, epidermis of ostrich. Bar, 20 µm. **D**, sparce keratin bundles and dense cornified cell envelope (arrowhead) in cell of the transitional layer of emu epidermis. Bar, 0.25 µm. **E**, merging lipid vacuoles among keratin bundles in cells of the transitional layer of ostrich epidermis (arrowhead on cornified cell envelope). Bar, 0.25 µm. **F**, evenly labeled zebrafinch epidermis 4 hours after tritiated histidine injection. Bar 15 µm. **G**, ultrastructural detail of autoradiographic silver grain over pale cytoplasm of transitional cell in zebrafinch epidermis. Bar, 0.2 µm. **H**, ultrastructural detail of autoradiographic silver grain over keratin filaments in sebokeratinocytes. Bar, 0.2 µm. **I**, AE1 immunofluorescence in emu epidermis. Bar, 15 µm. **J**, AE1 immunofluorescence in ostrich epidermis (arrowhead on the basal layer). Bar, 15 µm. **K**, AE2 immunofluorescence in emu epidermis. Bar, 20 µm. **L**, AE2 immunofluorescence of ostrich epidermis. Bar, 20 µm. **Legends** : ap, apteric region; c, corneous layer; d, dermis; k, keratin bundles; l, lipid material. Dashes underline the basal layer.

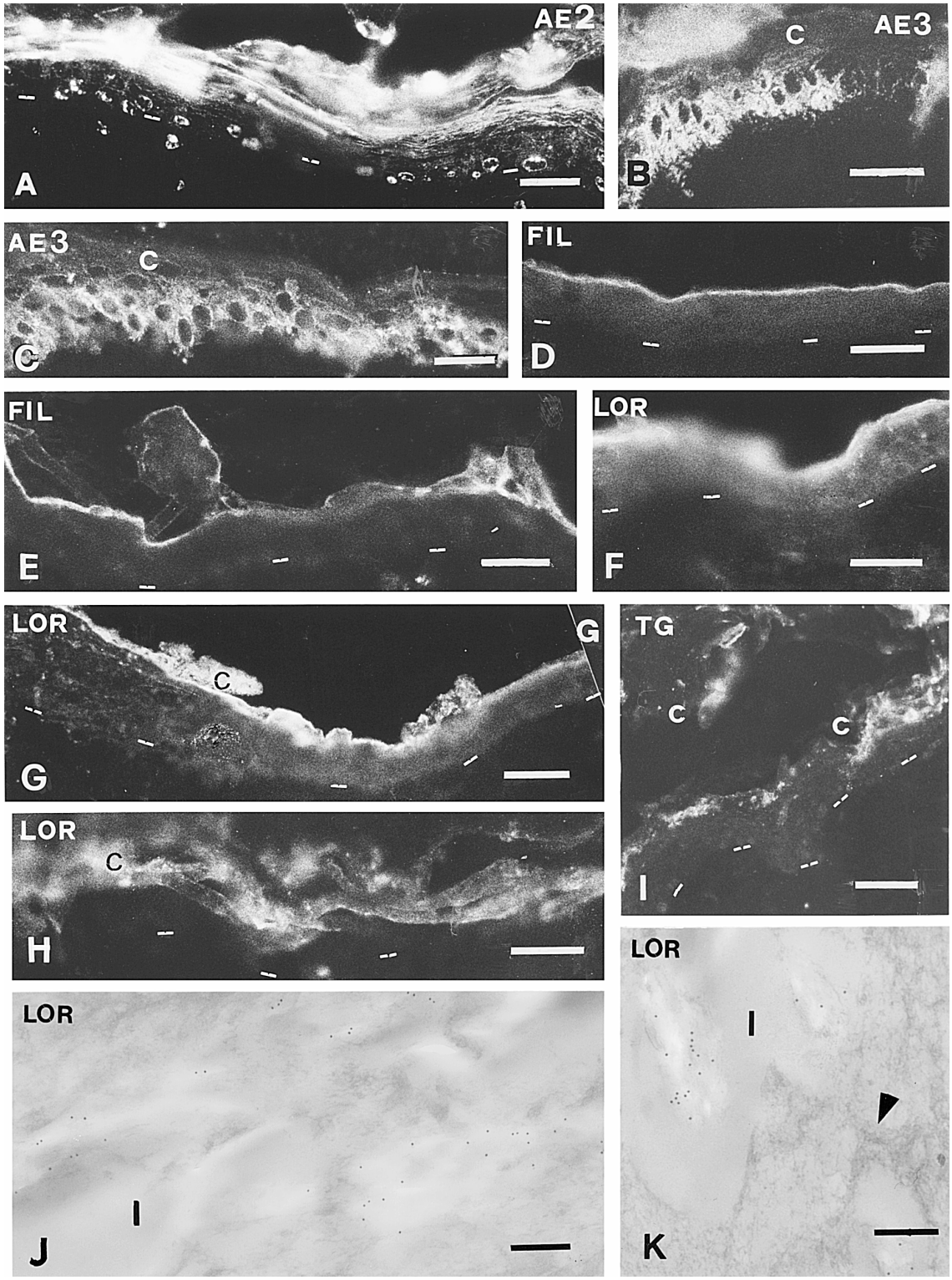

Fig. 3. – **A**, AE2 positive thick corneous layer of ostrich epidermis. Bar, 10 µm. **B**, AE3 immunofluorescence in emu epidermis. Bar, 15 µm. **C**, AE3 fluorescence in emu epidermis. Bar, 15 µm. **D**, thin filaggrin-like immunofluorescent transitional layer of emu. Bar, 20 µm. **E**, filaggrin-like immunofluorescent transitional layer of ostrich. Bar, 20 µm. **F**, loricrine immunoreactive transitional and corneous layer of ostrich. Bar, 20 µm. **G**, loricrine immunoreactive transitional and corneous layer of emu. Bar, 20 µm. **H**, thick loricrine immunoreactive corneous layer of ostrich. Bar, 20 µm. **I**, tranglutaminase immunofluorescence in the transitional and patchy corneous layer (here artefactually fragmented during sectioning). Bar, 20 µm. **J**, loricrine gold immunolabeled pale vesicles within upper spinosus cell of emu epidermis. Bar, 0.25 µm. **K**, loricrine gold labelling within pale vesicle of upper spinosus cell in ostrich epidermis. The arrowhead points to keratin filaments. Bar, 0.25 µm. **Legends** : c, corneous layer; k, keratin bundles; l, lipid material. Dashes underline the basal layer.

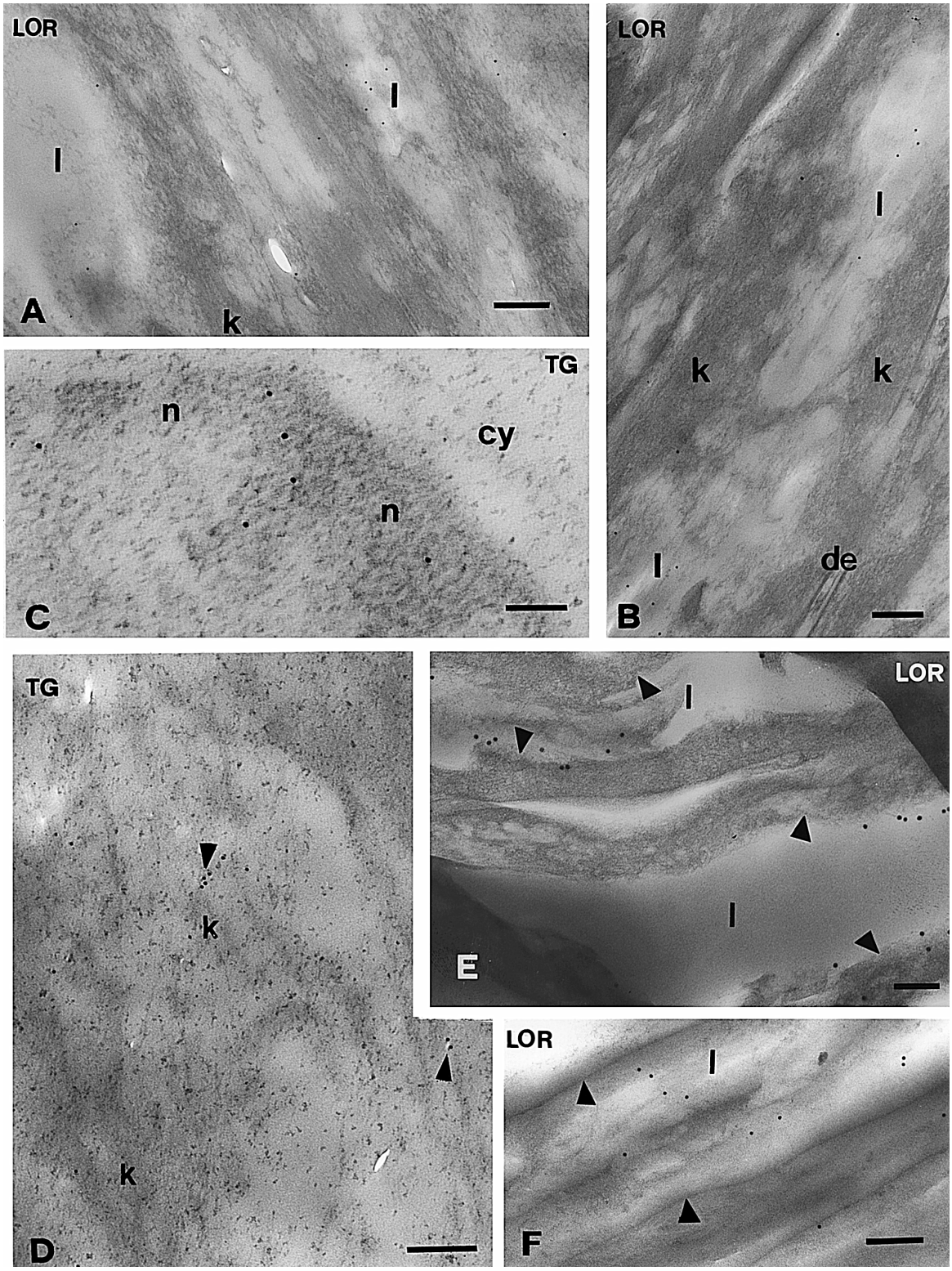

Fig. 4. – **A**, diffuse loricrine immunolabelling among keratin bundles of upper intermediate cell of ostrich. Bar, 0.25 µm. **B**, weak loricrine labelling within the central, lipidic-like spaces of transitional cell of emu epidermis. Bar, 0.25 µm. **C**, transglutaminase labeled nuclear chromatin of upper spinosus cell of ostrich epidermis. Bar, 0.1 µm. **D**, diffuse transglutaminase labelling (arrowheads) among keratin filaments in upper intermediate cell of ostrich epidermis. Bar, 0.2 µm. **E**, loricrine labelling along the external keratin bundles (arrowheads) within sebokeratinocytes of ostrich stratum corneum. Bar, 0.1 µm. **F**, diffuse loricrine immunolabelling within ostrich sebokeratinocytes but not along the cornified cell envelope (arrowheads). Bar, 0.1 µm. **Legends** : c, corneous layer; cy, cytoplasm; de, desmosomes; k, keratin bundles; l, lipid material. n, nucleus. Dashes underline the basal layer.

The ultrastructural localization for loricrin immunoreactivity showed a light but specific labelling associated with lipid-like or pale vesicles in cells of the upper spinosus or in the transitional layer (Figs 3 J-K). A diffuse labelling was seen among keratin bundles or in the core of the flattening sebokeratinocytes among the central lipid material (Figs 4 A-B). The transglutaminase labelling was similar to that for loricrine but present only in transitional cells and more diffuse than the loricrin labelling. It was diffuse over lipid or vacuoles among keratin filaments in transitional cells, and some weak labelling was seen in the condensing perinuclear chromatin of nuclei in upper intermediate cells and in transitional cells (Figs 4 C-D).

The immunolabelling for loricrin in transitional cells contacting mature sebokeratinocytes, was most frequently seen than that for transglutaminase, especially within the central lipid core, but not in the peripheral keratin material beneath the plasmalemma (Fig. 4 E). The immunolabelling was however diffuse or completely absent in most areas of the stratum corneum. Finally, the patchy labelling observed in mature sebokeratinocytes was generally absent along the plasma membrane (Fig. 4 F).

DISCUSSION

Keratins

The present study confirms that distribution of beta-keratin is found only in the dorsal surface of crocodilian scales and is absent in apteric epidermis of ratite, as in the remaining neognate birds (SAWYER et al., 2000; ALIBARDI & SAWYER, 2002; ALIBARDI, 2003a, 2004a). This type of keratin (beta) forms the hard epidermis of reptilian and scutate-scutellate scales of birds.

The present study also shows that alpha-keratin bundles are present in the germinative and intermediate epidermal cells, and include both acidic and basic keratins. The pattern of immunolocalization therefore resembles that of the other amniotes, with acidic and basic keratins associated in most epidermal layers but decreasing in the corneous layer where their reactive epitopes becomes masked or modified (SUN et al., 1983). Mature keratinocytes of avian epidermis, termed sebokeratinocytes, resemble mesos or thin alpha-cells of reptilian epidermis (MENON & MENON, 2000). These lipid-rich cells do not show an alpha-keratin patter under electron microscopic analysis (MATOLTSY, 1969; MADERSON et al., 1972; MADERSON, 1985; LANDMANN, 1979, 1980). This has been correlated with the absence or with the extremely reduced amount of interkeratin (matrix) material present in sebokeratinocytes.

The AE1 immunolabeling, limited to the basal layer in human epidermis (SUN et al., 1983), is less precisely localized in that of crocodilian and ratite epidermis, where not only the basal layer but also some suprabasal cells are labeled, although less intensely (ALIBARDI & THOMPSON, 2002; ALIBARDI, 2003a). Acidic keratins (AE1-positive) are also present in the intermediate and transitional layer of crocodilian and avian epidermis, where they are coupled to basic keratins according to the pair-rule (one acidic keratin molecule is bound to one basic keratin molecule, see SUN et al., 1983; O'GUIN et al., 1987).

The immunocytochemical results suggest that reactive epitopes of acidic keratin becomes somehow masked in the uppermost epidermal layers. The AE2 immunolabelling (for 56.5 and 66-67 kDa mammalian keratins) often shows a similar pattern as that seen using antibodies against filaggrin. It is however known that the AE2 antibody can also cross-reacts with common epitopes between keratins and filaggrin (DALE & SUN, 1983), and therefore the presence of filaggrin-like proteins in avian epidermis requires a biochemical study. The immunolabelling with AE2 in apteric sebokeratinocytes indicates the presence of some high molecular weight keratins involved in cornification (K1, 66-67 kDa and K10, 56.5 kDa; O'GUIN et al., 1987). Basic keratins (AE3 positive) in ratite epidermis appear more abundant, and present in transitional and also the corneous layer (O' GUIN et al., 1987; ALIBARDI, 2004a, b). The AE1, AE2 and AE3 immunoreactive patterns observed in crocodile and ratite epidermis are in general similar to that of the epidermis of other amniotes (SUN et al., 1983; O'GUIN et al., 1987; ALIBARDI, 2002b, c, d, e, 2003a).

The AE2 nuclear staining seen over many but not all of the nuclei in some sections, appears either unspecific or may be due to the recognition of this antibody to nuclear intermediate filaments other then keratin, but with a common antigenic determinant (PRUSS et al., 1981).

Keratin-associated matrix proteins

The dorsal surface of crocodilian scales, which represents most of epidermal surface, is made of relatively larger corneocytes with a rough or spiny surface, and which contain beta-keratin (MADERSON, 1985; SAWYER et al., 2000; ALIBARDI & THOMPSON, 2002; ALIBARDI, 2003a). As opposed, in the hinge regions corneocytes are very narrow, have a smooth surface, contain little or no beta-keratin, but instead lipids, and sparse tonofilaments bundles with prevalent parallel orientation with that of the epidermis. Keratohyalin granules are also absent in these cells in crocodilians, and this condition resembles that present in apteric epidermis of birds.

As in zebrafinch and chick epidermis, filaggrin-like immunoreactivity is weak and uneven over ratite sebokeratinocytes (ALIBARDI, 2004a). This result further suggests that, aside the poor cross-reactivity of this antibody with heterologous species (RESING & DALE, 1991), not much interkeratin (matrix) molecules rich in histidine are present in avian sebokeratinocytes.

The low labelling in the transitional layer of the zebrafinch 4-24 hours after injection of tritiated histidine has indicated that no keratohyaline or histidine-rich molecules are present among keratin bundles of sebokeratinocytes. The filament bundles present in transitional cells of avian epidermis previously indicated as avian keratohyalin (MATOLTSY, 1969; SPEARMAN & HARDY, 1985; PELTONEN et al., 1998, 2000), are not equivalent to mammalian keratohyalin. In fact, these bundles incorporate little or no tritiated histidine, and therefore very little histidine-rich and other proteins are present in these cells.

In conclusion, the present observations suggest that no or little matrix material is present in sebokeratinocytes of

apteric epidermis, as in reptilian mesos and alpha-cells (LANDMANN, 1979, 1980), and in hinge regions among reptilian and avian scales. The negative results using antibodies against trichohyalin, iso-peptide bonds, and involucrin indicate that these proteins are species-specific, and that no cross-reactivity between avian and mammalian epidermal proteins is possible after fixation of cells.

Cornified cell envelope proteins

A very weak loricrine-immunoreactivity is present in hinge regions of crocodilian epidermis (ALIBARDI, 2003a). X-ray microanalysis of the corneous and transitional layer of the chick have indicated the presence of sulfur (ALIBARDI & KAPLIN, unpublished observations) but not of phosphorous (the latter is typical in mammalian keratohyalin; see RESING & DALE, 1991). This may explain the detection of a weak loricrin-like immunoreactivity in the transitional and corneous layers of avian epidermis, as loricrin in mammalian epidermis is a sulfur-rich protein (MEHREL et al., 1990; LEAPMAN et al., 1997). A sulfur-containing protein with some immunocross-reactivity with mammalian loricrin seems to be present in small amount in avian sebokeratinocytes, although it is not concentrated along the cornified cell envelope.

The low amount of both keratin bundles and loricrin-like molecules in avian alpha-layer is confirmed by the poor reactivity to sulfydryl groups (WESSELS, 1961; CANE & SPEARMAN, 1964; SPEARMAN, 1966). Loricrin-like molecules are sparse among lipids and keratin bundles, and the immunolabelling is not localized into specifc granules (L-granules) as in corneocytes of mammals (STEVEN et al., 1990; LEAPMAN et al., 1997; HARDMANN et al., 1998; ISHIDA-YAMAMOTO et al., 2000). Also in human corneocytes a large amount of loricrin is initially distributed within the cytoplasm, but it concentrates along the cornified cell envelope at maturation (ISHIDA-YAMAMOTO et al., 2000). This is not seen in avian, lizard, snake, turtle or in corneocytes of monotremes (ALIBARDI, 2002b, c, d, e; ALIBARDI & MADERSON, 2003), where loricrin-like labelling remains cytoplasmic. This suggests that the antibody does not cross-react with reptilian-avian sulfur-rich proteins after fixation, or that these proteins are under the detection sensitivity of the employed immunocytochemical procedures.

It may also be possible that other proteins more than loricrin form the softer cornified cell envelope of avian sebokeratinocytes, but further biochemical studies are needed. The presence of transglutaminase in the same areas where loricrin-like immunoreactivity is present (loricrin is one of the main substrates of transglutaminase) further suggests that the two proteins are functionally linked during cornification of avian epidermis. The lack of isopeptide-bond detection (which derives from the action of transglutaminase on protein substrate such as loricrin) indicates that so little cross-linking is present in the apteric sebokeratinocytes to be under the detection or accessibility of antibodies.

Transglutaminase appears linked to condensing nuclear chromatine, as it is typical of nuclear membrane cross-linking during apoptosis in mammalian epidermis (HAAKE & POLAKOWSKA, 1993). During terminal differentiation in the transitional layer, nuclei become pycnotic, and are incorporated among the mature corneous layer (MENON & MENON, 2000, present observations). Transglutaminase labelling appears in the condensed perinuclear chromatin already in upper intermediate and precorneous, cells of the epidermis. Keratinization of apteric epidermis is, like that of mammals, probably an apoptotic-driven process.

Avian soft keratinization and evolutive speculations

The present observations in ratite birds confirm the immunolocalization of keratins, associated proteins, and tritiated histidine in the epidermis of neognates birds (ALIBARDI, 2003b, 2004a, b). These results probably indicate that the pattern of keratinization of apteric epidermis is similar in all extant birds. Complex lipids and waxes are synthesized in sebokeratinocytes, while the avian corneous cell envelope is probably simpler in comparison to that of mammalian corneocytes (MENON & MENON, 2000; KALININ et al., 2002). Only future biochemical work will allow identification of specific avian proteins involved in sebokeratinocytes differentiation.

The similarity between corneocytes in crocodilian hinge regions and sebokeratinocytes of apteric epidermis allows formulating some speculations about the evolution of apteric epidermis from pro-avian ancestors (Fig. 5 A). Apteric areas perhaps derived from the progressive expansion of the surface of hinge regions and the concomitant reduction of the dorsal surface of scales to bumps (Fig. 5 B). The selection and the progressive spreading of interfollicular/apteric epidermis to broader areas among scales of proto-avian reptiles might have been a major trend toward the origin of the avian integument. The columnar epidermal cells of the basal layer of the bumbs (resembling a placode) might have been connected to a group of mesenchymal cells forming a dermo-epidermal complex (Figs 5 A-B). The molecular basis of this dermo-epidermal association in reptilian skin is unknown (ALIBARDI, 2004b).

The contraction of the basal cells of the epidermis within progressively smaller bumps or cones (located toward the tip of the original scales, see sequence in Figs 5 B-C) might have determined an apparent condensation of dermal cells connected to the epithelium. The interfollicular and the apteric epidermis located around the cones and protofeathers (Figs 5 C-D) maintained the characteristics of hinge regions epidermis, in particular lack of keratohyalin and formation of thin alpha-keratin cells. This may explain the absence of keratohyalin and of a granular layer in the derived apteric epidermis.

The lengthening of the bumps into cones (Figs 5 C-D), together their associated dermis was at the origin of protofeathers (CHUONG et al., 2000; PRUM, 2002). The epidermis of the cones was formed by beta-keratin synthesizing cells organized in a circular region that might have functioned similarly to a collar region (the beta-keratin producing region at the base of a feather follicle in extant birds, see Fig. 5 E). The progressive restriction in the localization of the collar region and associated mesenchyme at the base of proto-feathers and the downward

proliferation of the collar might have produced the follicle (Figs 5 D-E).

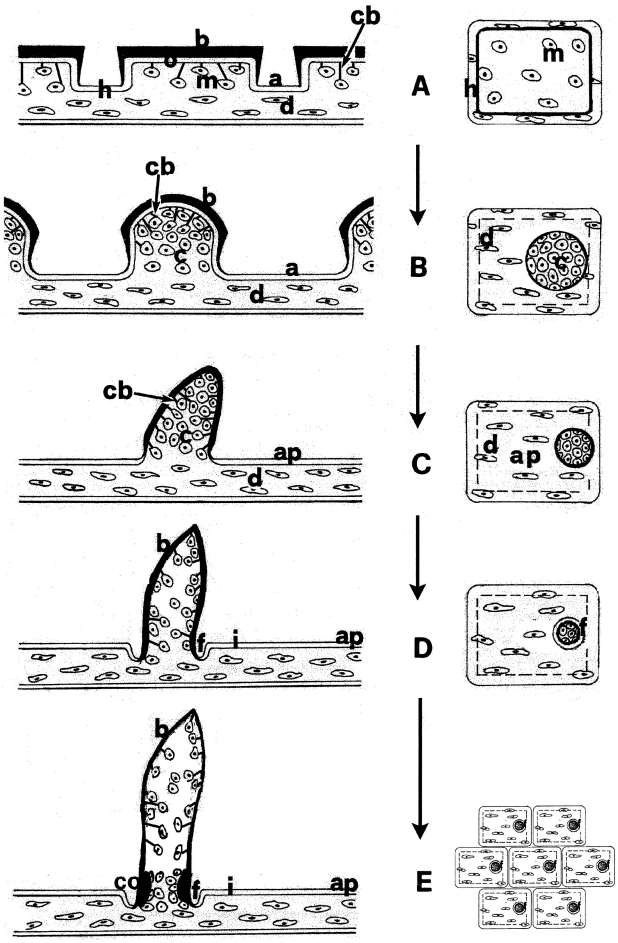

Fig. 5. – Schematic drowing illustrating five hypothetical stages (**A**-**E**) for the origin of the apteric epidermis and protofeathers from an archosaurian scaled epidermis (on the left are the histological sections; on the right are their dorsal view showing the progressive localization of the evolving protofeather toward the original scale tip). **A**, scales of an hypothetical archosaurian progenitor where mesenchymal fibroblasts are joined to the epidermis by cytoplasmic bridges; **B**, tuberculate scale of a protoavian archosaur; **C**, conical scale of a protoavian archosaur; **D**, elongating conical scale with forming follicle. Mesenchymal fibroblasts associated to the collar progressively reduce in the lower part of the cone; **E**, protofeather within a follicle (the dorsal view of 7 protofeathers together the area originally occupied by 7 scales shows the exagonal arranging present in pterylae tracts). Mesenchymal fibroblast are connected to the collar at the base of the protofeather. **Legends** : a, alpha-keratin layer; ap, apteric epidermis (outside interfollicular epidermis of pterylae epidermis); b, beta-keratin layer; c, dermal condensation of mesenchyme; cb, cytoplasmic bridges between mesenchyme and epidermis; d, fusiform fibroblasts of the dense/normal dermis; f, follicle; h, hinge region; i, interfollicular epidermis; m, mesenchymal fibroblasts of the loose dermis; o, outer scale surface. The dashes lines represent epidermal areas of the original scales.

The soft and wrinkly apteric epidermis seems to be associated with the need to maintain a very elastic and flexible tissue among feathers that favors their movement (Homberger & De Silva, 2000). Due to the limited importance of apteric epidermis in the mechanical protection of the body, cornification is reduced in this type of epidermis. The plumage takes up most of the mechanical protection (covering by flattening the feathers over the apteric epidermis), and acts as thermal insulation, leaving to the apteric epidermis a major role in the control of water loss (Lucas & Stettenheim, 1972; Menon et al., 1996; Menon & Menon, 2000).

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REFERENCES

Alibardi, L. (2002a). Keratinization and lipogenesis in embryonic derivatives of the zebrafinch, *Taeniopygia guttata castanotis* (Aves, Passeriformes, Ploecidae) during embryonic development. *J. Morphol.*, 251 : 294-308.

Alibardi, L. (2002b). Loricrin-like immunoreactivity during keratinization in lizard epidermis. *J. Morphol.*, 254 : 132-138.

Alibardi, L. (2002c). Immunocytochermistry of alpha- and beta-layers in lizard epidermis. *Belg. J. Zool.*, 132 : 71-81.

Alibardi, L. (2002d). Immunocytochemical observations on the cornification of soft and hard epidermis in the turtle *Chrysemys picta*. *Zoology*, 105 : 31-44.

Alibardi, L. (2002e). Immunocytochemical analysis of the process of keratinization of the epidermis of snakes. *J. Zool.* (London), 248 : 541-552.

Alibardi, L. (2003a). Immunocytochemistry and keratinization in the epidermis of crocodilians. *Zool. Stud.*, 42 : 346-356.

Alibardi, L. (2003b). Adaptation to land : the skin of reptiles in comparison to that of amphibians and endotherm Amniotes. *J. Exp. Zool.,* 298B : 12-41.

Alibardi, L. (2004a). Immunocytochemical and autoradiographic studies on the process of keratinization in avian epidermis suggest absence of keratohyalin. *J. Morphol.*, 259 : 238-253.

Alibardi, L. (2004b). Dermo-epidermal interactions in reptilian scales : speculations on the evolution of scales, feathers, and hairs. *J. Exp. Zool.*, 302B : 365-383.

Alibardi, L. & P.F.A. Maderson (2003). Distribution of keratin and associated proteins in the epidermis of monotreme, marsupial, and placental epidermis. *J. Morphol.,* 258 : 49-66.

Alibardi, L. & R.H. Sawyer (2002). Immunocytochemical analysis of beta-keratins in the epidermis of anapsids, lepidosaurians and archosaurians. *J. Exp. Zool.*, 293 : 27-38.

Alibardi, L. & M.B. Thompson (2000). Scale morphogenesis and ultrastructure of dermis during embryonic development in the alligator (*Alligator missippippiensis*, Crocodilia, Reptile). *Acta Zool.*, 81 : 325-338.

ALIBARDI, L. & M.B. THOMPSON (2001). Fine structure of the developing epidermis in the embryo of the american alligator (*Alligator missippippiensis*, Crocodilia, Reptilia). *J. Anat.*, 198 : 265-282.

ALIBARDI, L. & M.B. THOMPSON (2002). Keratinization and ultrastructure of the epidermis of late embryonic stages in the alligator (*Alligator mississippiensis*). *J. Anat.,* 201 : 71-84.

BRUSH, A.H. (1993). The origin of feathers : a novel approach. In : Farner D., J.A. King & K.C. Parker (eds), *Avian Biol. IX*. New York, Academic Press Ltd. : 121-162.

BRUSH, A.H. & J.A. WYLD (1980). Molecular correlates of morphological differentiation : avian scutes and scales. *J. Exp. Zool*., 212 : 153-157.

CANE, A.K. & R.I.C. SPEARMAN (1964). A histochemical study of keratinization in domestic fowl (*Gallus gallus*). *J. Zool.,* 153 : 337-352.

CHUONG, C.M. (1993). The making of a feather : homeoproteins, retinoids and adhesion molecules. *BioEssays,* 15 : 513-521.

CHUONG, C.M., R. CHODANKAR & R.B. WIDELITZ (2000). Evo-Devo of feathers and scales : building complex epithelial appendages. *Curr. Opin. Genet. Developm.,* 10 : 449-456.

DALE, B.A. & T.T. SUN (1983). Filaggrin, 56.5 kd and 65-67 kd keratins share an antigenic determinant as definied by the AE2 monoclonal antibody. *J. Cell. Biol.,* 97 : 228a (Abstract).

ELIAS, P.M., G.K. MENON, S. GRAYSON, B.E. BROWN & S.J. REHFELD (1987). Avian sebokeratinocytes and marine lipokeratinocytes : structural, lipid biochemical, and functional considerations. *Amer. J. Anat.,* 180 : 161-177.

FUCHS, E. (1990). Epidermal differentiation : the bare essentials. *J. Cell. Biol.,* 111 : 2807-2814.

GREGG, K. & G.E. ROGERS (1986). Feather keratin : composition, structure and biogenesis. In : Bereiter-Hahn J., A.G. Matoltsy & K. Sylvia-Richards (eds), *Biology of the integument, vol 2*. Berlin : Springer-Verlag : 666-694.

HAAKE, A.R. & R.R. POLAKOWSKA (1993). Cell death by apoptosis in epidermal biology. *J. Inv. Dermatol*., 101 : 107-112.

HARDMANN, M.J., P. SISI, D.N. BANBURY & C. BYRNE (1998). Patterned acquisition of skin barrier function during the development. *Development,* 125 : 1541-1552.

HOMBERGER, D.G. & K.N. DE SILVA (2000). Functional microanatomy of the feather-bearing integument : implications for the evolution of birds and avian flight. *Amer. Zool.,* 40 : 553-574.

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

KALININ, A.E., A.V. KAJAVA & P.M. STEINERT (2002). Epithelial barrier function : assembly and structural features of the cornified cell envelope. *BioEssays,* 24 : 789-800.

LANDMANN, L. (1979). Keratin formation and barrier mechanisms in the epidermis of *Natrix natrix* (Reptilia, Serpentes) : an ultrastructural study. *J. Morphol.,* 162 : 93-126.

LANDMANN, L. (1980). Lamellar granules in mammalian, avian, and reptilian epidermis. *J. Ultrastr. Res.,* 72 : 245-263.

LAVKER, R.M. (1975). Lipid synthesis in chick epidermis. *J. Inv. Dermatol.,* 65 : 93-101.

LEAPMAN, R.D., M. JARNIK & C.A STEVENS (1997). Spatial distribution of sulfur-rich proteins in cornifying epithelia. *J. Struct. Biol.,* 120 : 168-179.

LUCAS, A.M. & P.R. STETTENHEIM (1972). Growth of follicles and feathers. Color of feathers and integument. In : *Avian anatomy. Integument*. Agriculture Handbook 362. US Department of Agriculture. Chapt 7 : 341-419.

MADERSON, P.F.A. (1972). On how an archosaurian scale might have given rise to an avian feather. *Amer. Nat.,* 176 : 424-428.

MADERSON, P.F.A. (1985). Some developmental problems of the reptilian integument. In : Gans C., F. Billett & P.F.A. Maderson (eds), *Biology of Reptilia*, vol. 14 B, New York : John Wiley & Sons : 525-598.

MADERSON, P.F.A. & L. ALIBARDI (2000). The development of the sauropsid integument : a contribution to the problem of the origin and evolution of feathers. *Amer. Zool.,* 40 : 513-529.

MADERSON, P.F.A., A.B. FLAXMAN, S.I. ROTH & G. SZABO (1972). Ultrastructural contributions to the identification of cell types in the lizard epidermal generation. *J. Morphol.,* 136 : 191-210.

MATOLTSY, A.G. (1969). Keratinization of the avian epidermis. An ultrastructural study of the newborn chick. *J. Ultrastruct. Res*., 29 : 438-458.

MATULIONIS, D.H. (1970). Morphology of the developing down feathers of chick embryos. A descriptive study at the ultrastructural level of differentiation and keratinization. *Z. Anat. Entw. Gesch.*, 132 : 107-157.

MEHREL, T., D. HOHL, J.A.ROTHNAGEL, M.A. LONGLEY, D. NUNDMAN, C. CHENG, U. LICHTI, M.E. BISHER, A.C. STEVEN, P.M. STEINERT, S.H. YUSPA & D.R. ROOP (1990). Identification of major keratinocyte cell envelope protein, loricrin. *Cell,* 61 : 1103-1112.

MENON G.K. & J. MENON (2000). Avian epidermal lipids : functional considerations in relation to feathering. *Amer. Zool.,* 40 : 540-552.

MENON, G.K., B.E. BROWN & P.M. ELIAS (1986). Avian epidermal differentiation : role of lipids in permeability barrier formation. *Tiss. Cell.,* 18 : 71-82.

MENON, G.K., P.F.A. MADERSON, R.C. DREWES, L.F. BAPTISTA, L.F. PRICE & P.M. ELIAS (1996). Ultrastructural organization of avian stratum corneum lipids as the basis for facultative cutaneous waterproofing. *J. Morphol.,* 227 : 1-13.

O'GUIN, M.W., S. GALVIN, A. SHERMER & T.T. SUN (1987). Pattern of keratin espression define distinct pathways of epithelial development and differentiation. *Curr. Top. Dev. Biol.,* 22 : 97-125.

PELTONEN, L., Y. ARIELI, A. PYORNILA & J. MARDER (1998). Adaptive changes in the epidermal structure of the heat-acclimated rock pigeon (*Columbia livia*) : a comparative electron microscopic study. *J. Morphol.,* 235 : 17-29.

PELTONEN, L., Y. ARIELI, R. HARJULA, A. PYORNILA & J. MARDER (2000). Local cutaneous water barrier in cold- and heat-acclimated pigeons (*Columbia livia*) in relation to cutaneous water evaporation. *J. Morphol.,* 246 : 118-130.

PRUSS, R.M., R. MIRSKY, M.C. RAFF, R. THORPE, A.J. DOWNING & B.H. ANDERTON (1981). All classes of intermediate filaments share a common antigenic determinant definied by a monoclonal antibody. *Cell,* 27 : 419-428.

PRUM, R.O. (2002). Why ornithologists should care about the thropod origin of birds. *The Auck* 119 : 1-117.

RAWLINGS, A.V., I.R. SCOTT, C.R. HARDING & P.A. BOWSER (1994). Stratum corneum moisturization at the molecular level. *J. Inv. Dermatol.,* 103 : 731-740.

RESING, K.A. & B.A. DALE (1991). Proteins of keratohyalin. In : Goldsmith L.A. (ed), *Physiology, Biochemistry, and Molecular Biology of the Skin* Vol 1. New York and Oxford : Oxford Uniersity Press : 148-167.

ROTHNAGEL, J. & G.E. ROGERS (1996). Trichohyalin, an intermediate filaments associated protein of the hair follicle. *J. Cell Biol.,* 102 : 1419-1429.

SAWYER, R.H., L.W. KNAPP & M.W. O'GUIN (1986). The skin of Birds. Epidermis dermis and appendages. In : Bereiter-Hahn J., A.G. Matoltsy & K. Sylvia-Richards (eds), *Biology of the*

integument, Vol 2, Vertebrates, Berlin : Springer-Verlag : 374-408.

SAWYER, R.H., T. GLENN, B. FRENCH, B. MAYS, R.B. SHAMES, G.L. BARNES & Y. ISHIKAWA (2000). The expression of beta-keratins in the epidermal appendages of reptiles and birds. *Amer. Zool*., 40 : 530-539.

SPEARMAN, R.I.C. (1966). The keratinization of epidermal scales, feathers and hairs. *Biol. Rev.,* 41 : 59-96.

SPEARMAN, R.I.C. & J.A. HARDY (1985). Integument. In : King A.S. & J. McLelland (eds), *Form and function of birds*, Vol. 3, London : Academic Press Inc. : 1-56.

STEVEN, A.C., M.E. BISHER, D.R. ROOP & P.M. STEINERT (1990). Biosynthetic pathways of filaggrin and loricrin- Two major proteins expressed by terminally differentiated epidermal keratinocytes. *J. Struct. Biol*., 104 : 150-162.

SUN, T.T., R. EICHER, W.G. NELSON, S.G.C. TSENG, R.A. WEISS, M. JARVINEN & J. WOODCOCK-MITCHELL (1983). Keratin classes : molecular markers for different types of epithelial differentiation. *J. Inv. Dermatol*., 81 : 109-115.

WESSEL, N.K. (1961). An analysis of chick epidermal differentiation in situ and in vitro in chemically definied media. *Dev. Biol*., 3 : 355-389.

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