Ultrastructural and immunocytochemical features of the epidermis of the lizard *Heloderma suspectum* indicate richness in lipids and lack of a specialized shedding complex

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**Keywords:** Gila monster, lizard, epidermis, keratin immunocytochemistry, ultrastructure

**Accepted for publication:** 19 August 2011

**Abstract**


The epidermis of the venomous lizard *Heloderma suspectum* has been studied for detecting generalized and desert adaptations. A thick and non-completely syncytial beta-keratin layer is followed by 60–90 layers of mesos-cells. Non-lamel-lated or sparse lamellated lipid material is seen among mesos-cells where lipids form the main barrier against water loss. The alpha-layer is made of interlocking cells with irregular perimeter that are connected through desmosomal remnants. Immunocytochemistry shows that beta-keratin is present in beta-cells, disappears in mesos-cells but is diffuse in alpha-cells. Alpha-keratin is seen in mesos-cells but lowers in alpha-cells where alpha-keratin probably mixes with beta-keratin. Although the sequence of layers formed during the renewal stage of the epidermis was not available, a specialized shedding layer with an outer oberhautchen faced to an inner clear layer appears absent in this species. This condition suggests that shedding occurs at the boundary between the outer (old) alpha-layer and the inner (new) beta-layer formed underneath the alpha-layer. The thick mesos layer is likely an efficient adaptation to limit water loss in desert conditions while the poorly specialized shedding complex may suggest a primitive stage in the evolution of the shedding layer in this lizard or a special adaptation to water shortage.

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**Introduction**

The epidermis of squamate reptiles is formed by a sequence of different layers of keratinocytes that accumulate hard (beta-) or soft (alpha-) keratin (Maderson 1985; Maderson et al. 1998). The formation of these layers occurs during the renewal phase of the shedding cycle terminating in a molt and is followed by a resting phase where little proliferation occurs, mainly to complete the maturation of the alpha-keratin layer. Numerous studies in the past 10 years have indicated that the main difference between alpha-keratogenic and beta-keratogenic layers is the amount of accumulation of hard proteins, previously indicated as beta-keratins (βK, Fraser and Parry 1996), during the renewal phase of the shedding cycle (Alibardi et al. 2009). The hard proteins of the outer part of lizard scales are now indicated as keratin-associated beta-proteins (KAβPs) because they are analogous to the mammalian keratin-associated proteins. These relatively small proteins (12–28 kDa) possess a central beta-pleated sheet of amino acids that is absent in the mammalian keratin-associated proteins.

The detailed ultrastructural analysis of some species of lizards and snakes has indicated that a mature (outer) epidermis contains a syncytial layer made by merged oberhautchen and beta-cells. The more external layer, the oberhautchen, determines the characteristics species-specific microornamentation of lizards and snakes (Irish et al. 1988). Largely merged with the oberhautchen at maturity, the underlying beta-layer constitutes the mechanical barrier of scales, imparting resistance and stiffness. This superficial layer is followed by a mesos layer made of 5–10 layers of thin cells or by a mesos region where
narrow cells gradually turn into 5–8 layers of alpha-cells. The latter form the basalmost part of the corneous layer of the mature epidermis (Maderson 1985; Irish et al. 1988; Alibardi and Maderson 2003). Some variations to this general scheme have been found, especially in the differentiation of the shedding complex, the region where the splitting of the old (outer) and the new (inner) epidermis takes place during shedding (see Discussion).

Past and present studies have shown that, based on the accumulation of KAβPs, a transition between the beta-layer and mesos layer or the alpha-layer of the lower part of the epidermis can vary among species. Also the new oberhautchen-beta layer produced with the new epidermal generation can vary in morphology and number of layers in different species of squamates (Maderson 1985; Irish et al. 1988; Alibardi 1999, 2000). Therefore, it is of interest for further insights into these aspects of the evolution of the squamate shedding complex to analyze the fine morphology of the epidermis of a peculiar and desert-adapted species such as the Gila monster, representing the small family of the venomous lizards Helodermatidae (Beck 2005). The study on this species will contribute to the identification of special characteristic adaptations to dry conditions as well as to the nature of layer stratification in the epidermis of lepidosaurian reptiles.

Materials and Methods

The present study was conducted on four specimens of the Arizona Gila monster (Heloderma suspectum) kept in cages at the School of Life Sciences, AZ State University. Two individuals were juveniles (165 and 195 mm snout-vent lengths, SVL) while the other two were adults of 308 and 310 mm SVL. Each animal was anesthetized using 5% isoflurane and then two small skin samples (2 x 4 mm), which included both dark and orange-pale scales, were collected from the dorsal region of the animals. One skin sample was immediately fixed for 5–8 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, and then the tissues were post-fixed for 90 min in 2% OsO₄, dehydrated in ethanol, then acetone, and finally infiltrated with acetone and then embedded in Durcupan Resin (Sigma, St Louis, MO, USA). The tissues included in this Resin were sectioned with an ultramicrotome LKB-Nova at 1–3 μm thickness, collected over glass slides and then stained with 0.5% toluidine blue for the histological examination. Thin sections for the following electron microscopic analysis were collected on 150–200 mesh copper grids, counterstained with uranyl acetate and lead citrate according to routine procedures, and then studied under a transmission electron microscope (CM-100 Philips).

The other skin sample was instead fixed at 0–4 °C in 4% paraformaldehyde in phosphate buffer for 4–5 h, dehydrated in ethanol, and embedded in Lowryl Resin under ultraviolet lights at 0–4 °C. For immunocytochemistry, some sections collected with the ultramicrotome at 3–5 μm in thickness were immunolabeled with a beta-1 keratin antibody (kindly supplied by Dr. R. Sawyer, University of South Carolina, Columbia, USA). This rabbit polyclonal antibody recognizes avian and reptilian beta-keratins (Sawyer et al., 2000). The primary antibody was diluted (1 : 100) in 0.05 M Tris buffer with 2% BSA at pH 7.6, and sections were incubated overnight at 0–4 °C. In controls, the primary antibody was omitted or sections were incubated with a pre-immune rabbit serum (diluted 1 : 100). After rinsing a FITC-conjugated or a TRITC-conjugated anti-rabbit-IgG, secondary antibody was applied on the sections for 1 h at room temperature. The dilution of the secondary antibody for both tested sections and their controls was 1 : 100. The immunolabeling for alpha-keratins was carried out using a chicken anti-alpha antibody (G-30, kindly supplied by L. Knapp and R. Sawyer, Univ of South Carolina, US) at a dilution of 1 : 100 in buffer as mentioned earlier. Sections immunolabeled with FITC (fluorescein isothiocyanate) or TRITC (tetracyclhistidine isothiocyanate) were rinsed and mounted in Fluormount (Electron Microscopic Sciences, Hatfield, PA, USA) for the following microscopic examination under an epifluorescence microscope (Euromex, Arnhem, the Netherlands).

For ultrastructural immunocytochemistry, thin sections of the epidermis were collected with an ultramicrotome on Nickel grids of 200 mesh, incubated with the beta-1 or the alpha-keratin antibody as mentioned earlier (dilution 1 : 100), and rinsed in the buffer. The dilution of the secondary antibodies (anti-rabbit or anti-goat 10 nm Gold Conjugated IgG, Sigma) in the buffer utilized in the immunoreactions was 1 : 80 in buffer. In controls, the primary antibodies (Beta-1 or G-30) were omitted in the incubating solution. Sections were lightly stained (for 6 min) at room temperature with 2% uranyl acetate, rinsed in buffer and distilled water, and left to dry. The grids were observed under a CM-100 Philips transmission electron microscope operating at 60–80 kV.

In addition to the skin samples, we also collected molt samples from each of these individuals.

Results

Light microscopy and immunocytochemistry

The skin of H. suspectum consists of dome-like elevation of corneous, hard material located in the central part of rhomboid-shaped scales (inset of Fig. 1A). Shed skin preserves the general shape of the original scale although the hinge region is expanded, so that the central dome occupies less surface than in the original scales. Scales therefore consists of a hard, dome-shaped central area surrounded by a thinner epidermis.

The available skin of the four specimens here analyzed showed an epidermis featuring an external beta-layer of variable thickness followed by an incomplete alpha-layer along most of the outer scale surface (Fig. 1A–C). In the central dome, the thickness of the beta-layer could reach 60 μm, representing more than 50% of the entire epidermal thickness.
Outside the dome region, the beta-layer was thinner and tapered into the hinge region where it was much reduced in thickness. The available epidermis was in a post-shedding condition, when the alpha-layer is slowly forming. The thickness of the external beta-layer varied according to the region of scales: it was thicker in the central part of the outer surface of scales in adult individuals and thinner in the hinge region (Fig. 1A, B). The beta-layer was generally thinner in juvenile individuals where the following mesos and alpha-layers were composed of several layers, often artifactually detached from the remaining epidermis after sectioning (Fig. 1C). No difference in thickness of the mesos and alpha-layers was noticed between juvenile and adult epidermis.

At the light microscope level, it was not easy to separate the limits of the mesos layer, present underneath the beta-layer, and the following alpha-layer as a continuous stratum of thinner cells was seen (but see the ultrastructural description later). The beta-layer and less intensely the mesos alpha-layer appeared pigmented in the dark areas of the skin. In addition to the relevant number of epidermal melanocytes, a dense band of dermal melanophores was present underneath the epidermis in the dark areas of scales.

The molts comprised three main layers when studied under the light microscope: an external pigmented beta-layer with a finely irregular surface (microornamentations, see ultrastructure), a layer of mesos-cells beneath the beta-layer that could
not be discerned from the remaining, and a more compact alpha-layer (Fig. 1D, but see the ultrastructural description later).

Immunofluorescence using the beta-1 antibody clearly showed that the external, compact layer was immunofluorescent while the mesos alpha-layers were not stained (Fig. 1E,F). In some cases, a perimeter of beta-cells was observed in the beta-layer, suggesting the cells were not completely merged into a compact, syncytial layer (but see ultrastructure). Negative controls appeared immunonegative while the use of the alpha-keratin antibody stained weakly the entire corneous layers or isolated molts (Fig. 1H).

The dermis appeared composed by a loose superficial part of 40–60 μm in thickness, containing numerous but irregular collagen fibers non-organized in large bundles while in the deeper dermis, large bundles of collagen fibrils were present (see ultrastructural description later).

Ultrastructure and immunocytochemistry

The ultrastructural examination of the corneous layer showed that mainly a smooth surface or only small incisions not organized in a regular pattern were present on the surface of the beta-layer, the presumed oberhautchen (Fig. 2A). The corneous material of oberhautchen-beta cells was prevalently of low electron density but denser areas were also seen within the pale corneous material. Within the beta-layer, cells appeared still delimited by boundaries (tile-like pattern) where the plasma membrane was generally not visible and instead replaced by a limiting membrane revealed by the deposition of an electron-dense material along it (Fig. 2B,C). Junctional or even desmosomal remnants were often seen along the limiting membrane, especially in the connected extremities of beta-cells (Fig. 2B,C).

An amorphous, electron-dense material resembling cell fragments was often seen with irregular distribution on the outer part of the corneous layer. Using the beta-1 antibody, the beta-layer appeared more or less intensely labeled, more than the superficial oberhautchen (Fig. 1A,D,E). Embedded melanosomes within the beta-layer were immunonegative (Fig. 1E) while denser area within the beta-layer were less intensely labeled than the prevalent pale areas (Fig. 1D,E). Although spinulated microornamentation was rarely observed on most surfaces of scales, some denticles were present in the hinge region (Fig. 2F). The entire beta-layer was very thin, 1–3 μm, in hinge regions and few layers of mesos-like cells were present over a thin epidermis made of flat epidermal cells.

Beneath the beta-layer, a very thick stratum of 10–30 μm in thickness, and made of 60–90 of extremely narrow mesos-cells, was present (Fig. 3A–D). Therefore, what initially appeared at the light microscope as an alpha-layer was instead mainly constituted by mesos-cells. The latter were completely beta-1 immunonegative (Fig. 1C) and often showed a paler central material surrounded by irregularly developed dense periphery (Fig. 1B). Lamellated structures, reminiscent of mesos granules (Menon et al. 1996), or extracellular lamellae were rarely seen in our material both inside and outside mesos-cells. The plasma membrane of mesos-cells sometimes showed an electron-dense marginal layer (equivalent to the cell corneous envelope of mammalian corneocytes).

Differentiated alpha-cells formed a stratum of 2–8 layers of thicker and more irregular dense cells resting upon the living keratinocytes (Fig. 3D–F). These cells derived from the packing of the numerous tonofilaments with vesicles or lipid droplets seen in the cytoplasm of pre-corneous (transitional) cells (Fig. 3E,F). Nuclei of transitional cells became condensed and embedded in the dense corneous material of mature alpha-cells (data not shown). An electron-dense marginal layer was slightly evident within the mature electron-dense mass of corneous material of mature alpha-cells (arrow in Fig. 3F). The presence of electron-pale material among ribosomes and pale vesicles of extracted lipids indicated that alpha-cells store elevated amount of lipids.

Because of the insufficient, delayed, or inappropriate fixation of the samples, the detection of lipids structures and droplets was not optimal in our samples. Despite this short- age, lipid vesicles were commonly seen in suprabasal and pre-corneous cells of the epidermis, down to the basal layer (Fig. 3G). Basal cells generally rested upon a folded basement membrane, and contained numerous tonofilaments.

Differentiated alpha-cells in both juvenile and adult epidermis as well as in molts showed an unexpected diffuse labeling using the beta-1 antibody (Fig. 4A). These cells showed an irregular thickness, a compact or fibrous texture with alpha-filaments still visible (k in Fig. 4A) and a tortuous surface where numerous desmosomal remnants were connecting these cells one to another. This same aspect was also seen in molts, where the lowermost layer that represents the shedding line (where molts have detached from the remaining epidermis at shedding) appeared linear and devoid of microornamentation (Fig. 4B). The rare denticles occasionally seen along this layer (double arrowhead in Fig. 4B) never showed a specific pattern reminiscent of the indented clear layer seen in many other lizards and snakes (Irish et al. 1988; Maderson et al. 1998).

The ultrastructural examination of the dermis confirmed the random organization of collagen fibrils in the superficial, loose dermis with sparse bundles among an amorphous extracellular matrix (Fig. 4C). Conversely, the deep dermis was formed by large (6–12 μm) bundles of hundreds of collagen fibrils (Fig. 4D). These large bundles were wrapped around the cytoplasmic elongation of sparse fibrocytes, a structure reminiscent of the dense connective tissue of tendons.

Finally, the use of the alpha-keratin antibody (G30) allowed for detailing of the light microscopic observations. No labeling was seen in the beta-layer (Fig. 5A) while sparse or clustered gold particles were decorating regions of the mesos-cells (Fig. 5B), more frequently and intensely than in alpha-cells where the labeling was more both less intense and uneven than in the mesos layer (Fig. 5C). Therefore, although these two layers appeared to contain alpha-keratin,
the labeling observed was low and not uniform to indicate a difficult access to the antigenic sites. Negative controls were however completely immunonegative in these layers (data not shown).

Discussion

The present study has shown that the epidermis of the desert-adapted lizard *H. suspectum* presents not only the usual characteristics of the complex and multilayered squamate epidermis but also some unique features. Among the latter, two peculiar characteristics of *Heloderma* epidermis stand out. The first is the lack of marked oberhautchen microornamentation and of an indented clear layer in the central dome and along most of the outer scale surface, aside from some regions of the hinge region. This aspect strongly suggests that the surface of *Heloderma* is relatively smooth, suggesting a ‘lamellate pattern’ (Peterson 1984). The presence of sparse small depressions or indentations on the oberhautchen surface may instead correspond to the incisions of the oberhautchen surface that form the ‘pit and groove dentate pattern’ previously observed by Scanning Electron Microscopy (SEM, see Stewart and Daniel 1975). In fact, the incisions have dimension corresponding to the pits described on the oberhautchen.
surface, probably derived from the process of detaching of the irregular boundaries of the alpha-layer in the previous epidermal generation from the new oberhautchen when shedding occurred. This could be further studied on samples of the skin during the renewal phase in this species. However, in other areas of the scales, namely near the hinge region, some oberhautchen spinulae can be truly present, indicating some heterogeneity in surface features of the oberhautchen. The observations indicate that the shedding layer of the epidermis of *Heloderma* has a poorly specialized shedding complex if compared with other species. Whether this is a primitive or a specialized condition derived from the desert adaptation is not known. In most species of lizards and snakes studied so far, the shedding complex consists in only two layers: the clear layer of the outer epidermal generation that is interfaced with the oberhautchen of the inner epidermal generation (Mader-son 1985; Maderson et al. 1998). This specialized shedding complex ensures a rapid and efficient shedding line for the loss of the outer (old) epidermis, the molt, in large patches (lizards) or in a single piece (few lizard species and most snakes).

Numerous SEM studies have indicated that along the epidermis of scales in various squamates the oberhautchen surface can present some variations from the hinge region to more central or apical areas of the same scale, including the appearance of spinulae (Peterson and Bezy 1985). Another difference between the beta-layer of *Heloderma* with the
beta-layer of many lizards and snakes is the presence of individual cells partially merged and not completely fused to form a syncytial beta-layer. This aspect resembles the morphology of the beta-layer in turtles, tortoises and crocodilians (Alibardi 2003), and in Sphenodon (Alibardi and Maderson 2003).

Recent studies have however shown that a non-syncytial beta-layer is also present in some terrestrial and marine snakes (Tu et al. 2002; Lillywhite et al. 2009) and in some agamid lizards (Alibardi 2000). Whether a syncytial beta-layer represents an evolution of a non-syncytial beta-layer remains unexplained because of the detection of different conditions in both lizards and snakes.

The second peculiar characteristic of Heloderma is the exceptionally thick mesos region of the epidermis, both in juveniles and in adult scales. The mesos region represents the transitional area of the epidermis between beta-cells and alpha-cells and is indicated as the mesos layer in most snakes and lizards so far analyzed (Maderson 1985; Maderson et al. 1998). The mesos layer consists of 3–5 thin cells loosely associated because desmosomes are relatively infrequent in these cells in the epidermis of most species so far analyzed. Other ultrastructural studies have however indicated that this region consists of more layers, 8–10 in the normal epidermis of Sphenodon punctatus (Alibardi and Maderson 2003), and up to 20 in the normal epidermis of the agamid lizard Physignatus lesueurii (Alibardi 2000).

In the present study, a specific staining for lamellar bodies (e.g. using the ruthenium tetroxide dye instead of the osmium tetroxide, see Menon et al. 1996; Tu et al. 2002) was not feasible. Also, some tissues appeared difficult to be rapidly fixed so that some not optimal preservation was obtained. Despite these shortages, discrete cytological details of epidermal cells were obtained for most cell organelles, and this indicates that the fixation was sufficiently good for revealing the essential
The mesos region observed in all samples of Heloderma suspectum relays more on the exceptionally thick stratification of its mesos region more than on the extrusion of specific lipids to limit water loss. Previous studies on snake epidermis have correlated the increase in mesos layer stratification with improved skin resistance to evaporative water efflux (Tu et al. 2002; Lillyhite et al. 2009). Therefore, the stratification of the mesos layer appears correlated with the ecological adaptation and has no phylogenetic significance.

Water balance in Heloderma suspectum has received considerable attention in recent years, and, interestingly, this species uses other water balance related strategies that are atypical of squamates in general. During hot, dry conditions, Heloderma suspectum is primarily nocturnal (Davis and DeNardo 2009) and supports hydric needs through the use of the urinary bladder as a substantial water reservoir (Davis and DeNardo 2007), a trait undescribed in any other adult lizard. Furthermore, Heloderma suspectum uses cloacal evaporative water loss to counter against extreme temperatures (DeNardo et al. 2004). Despite this assemblage of adaptations to a hot dry environment, Heloderma suspectum distribution is predominantly limited to the Sonoran Desert, which has a reliable late summer rainy season, and is absent from adjacent deserts where summer rainfall is inconsistent. Physiological (i.e., transcutaneous water loss) and morphological (i.e., mesos layer thickness) comparisons of Heloderma skin during the wet and dry seasons may provide further insight into the role of the thickened mesos layer in water balance in this species.

The immunolabeling has indicated that the alpha-layer of Heloderma suspectum seems to contain a small amount of beta-keratin together with alpha-keratin, a condition that reflects the intermediate region of Sphenodon (Alibardi 1999; Alibardi and Maderson 2003). This result strengthens the idea that the main difference between beta- and alpha-layers in squamate epidermis is not the presence of two types of keratins, beta-keratin versus alpha-keratin, but instead the much high production of beta-keratin (KαPs) in the beta-layer relative to the alpha-layer. This synthetic capability varies during the shedding cycle, suggesting that some limited amount of beta-keratin may actually be produced also in alpha-cells.

In conclusion, the present ultrastructural description of the skin of Heloderma suspectum has shown that the pigmentation of the beta-layer and the presence of a band of dermal melanophores underneath the epidermis are both responsible for the dark pigmentation areas in the skin in this species. The firm dermis resembles that of crocodilians as it is made of a very dense and organized meshwork of collagen bundles and forms a type of plywood pattern that determines the high resistance and toughness of the skin. The more peculiar characteristics in comparison with other squamates are present in the epidermis
where a specialized shedding layer composed of a clear and oberhautchen layers is absent. The exceptionally developed mesos region appears a unique specialization of the epidermis that is effective in limiting the loss of water through the skin in the desert environment inhabited by this unique lizard.

Acknowledgements

The study was largely self-supported (Comparative Histolab) and in part from the University of Bologna. Animal care and maintenance were supported through funds from an Arizona State University (ASU) Foundation account. Surgeries were performed under the oversight of the ASU Institutional Animal Care and Use Committee (protocol #09-1044R).

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