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Immuno-Ultrastructural Localization of Involucrin in Squamous Epithelium and Cultured Keratinocytes¹

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Involucrin immunoreactivity was localized ultrastructurally with protein A-gold in epidermis and cultured keratinocytes embedded in Lowicryl K4M. In the skin, immunoreactivity was found predominantly in cells of the granular layer and inner stratum corneum. The label was associated primarily with amorphous cytoplasmic material and especially keratohyaline granules. Some labeling was observed at the cell periphery, but little with keratin filaments. Tissue samples examined without aldehyde fixation showed relatively greater labeling in the outer stratum corneum than fixated tissue. In cultured cells, the labeling was also associated primarily with cytoplasmic granular material and to a lesser extent with the cell periphery. Upon treatment with the ionophore X537A, keratin filaments were found in aggregated arrays and the plasma membranes became convoluted. That involucrin immunoreactivity persisted in the cytoplasm in cultured cells and in vivo after cross-linking occurs could account for considerable isopeptide bonding detected in epidermal keratin fractions and indicates that not all the involucrin participates in envelope formation.

Key Words: Squamous epithelium; Involucrin; Lowicryl K4M; Protein A-colloidal gold.

Introduction

The epidermis serves as a barrier protecting the organism from the environment. As squamous epithelium matures, structural proteins are synthesized that ultimately perform this protective function. The final product of squamous maturation is the stratum corneum, which consists of a network of condensed proteins and cellular debris. The keratin class of proteins is the principal component of this integument and is complemented by another structural element, the cornified envelope (8, 11, 20).

Previous biochemical studies have identified the protein involucrin as a precursor of the highly cross-linked material referred to as envelopes (13, 15). Visualized in paraffin-embedded tissue sections by immunocytochemical procedures, involucrin is first detected several cell layers above the basal layer and reaches maximal intensity at the granular layer. Plasma membranes of the granular layer become permeable to calcium, thus activating the cellular transglutaminase. This enzyme forms stabilized structures by ε-(γ-glutamyl) lysine cross-linking (4, 14). To help understand the fate of involucrin during this process, we have employed a low temperature embedding technique using Lowicryl K4M to precisely localize involucrin immunoreactivity subcellularly both in vivo and in cultured cells.

Materials and Methods

Normal human skin was obtained from surgical specimens: mastectomies, amputations, and wide resections of tumors. Promptly after surgical removal, the epidermis was separated from the dermis by crude blunt dissection and minced into 1 mm cubes. The epidermis was fixed with four different fixation protocols: 1) 1% glutaraldehyde for 2 hr; 2) 3% paraformaldehyde-0.1% glutaraldehyde for 1 hr; 3) direct immersion in 30% ethanol; and 4) an overnight fixation in half-strength Karnovsky’s fixative. Following fixation, both the glutaraldehyde- and paraformaldehyde-fixed skin was washed three times in phosphate buffered saline, pH 7.4 (PBS), once in 0.5 M NH₄Cl to quench unreacted aldehyde groups, and finally in PBS. The material was fixed initially in half-strength Karnovsky’s fixative and postfixed for 4 hr in osmium tetroxide, and dehydrated and embedded in Epon for conventional electron microscopy.

Human epidermal keratinocytes were cultivated from circumcision...
Figure 1. Skin, stratum spinosum. (A) Involucrin immunoreactivity of granular, amorphous material in the cytoplasm. Desmosomes are unlabeled. (B) The granular material between the tonofibrils is labeled (small arrow), while dense tonofibril bundles show little labeling by the gold particles (large arrow). Original magnification × 15,000. Bar = 200 nm.

Figure 2. Skin, stratum granulosum and stratum corneum. (A) Keratohyaline granules labeled with anti-involucrin (arrows). Original magnification ×25,000. (B) Keratohyaline granules labeled with gold appearing to coalesce into a dense network (arrow). Original magnification × 20,000. (C) Granules labeled with anti-involucrin in the stratum corneum, visualized in unfixed skin embedded in Lowicryl (arrow). Original magnification × 30,000. Bar = 100 nm.

Both the aldehyde-fixed skin specimens and the skin placed directly in 30% ethanol were embedded in Lowicryl K1M as previously described (18). The tissues were dehydrated in 1 hr incubations with 30, 50, 70, 100, and 100% ethanol at 35°C and then incubated with ethanol-Lowicryl mixtures in the ratio of 1:1 and 1:2 each for 1 hr at −40°C. Following a 1 hr incubation with 100% Lowicryl K1M at −40°C, the tissue was again incubated in Lowicryl K1M overnight at −40°C. The tissue samples were then transferred to fresh Lowicryl in gelatin capsules and polymerization begun by exposing the capsules to ultraviolet (UV) light for 2 hr at −40°C. The capsules were then brought to room temperature and polymerization was completed by further exposure to UV light for 72 hr. Following polymerization, 1 micron sections were cut and stained with toluidine blue to select appropriate areas for thin sectioning. Thin (600–800 Å) sections were picked up on nickel grids for immunocytochemical studies.

All samples, from both skin and cell culture, were subjected to identical immunocytochemical procedures. The grids were initially incubated with 0.5% egg albumin for 5 min at room temperature. They were then incubated with anti-involucrin at dilutions of 1:1000 or 1:1500 for 1 hr at room temperature. The anti-involucrin antiserum was that previously described (13) and showed immunoreactivity only toward involucrin upon Western blotting of human skin extracts (16). Controls included preimmune serum and antisera absorbed with purified envelopes.

Following incubation with antiserum, the grids were washed twice in PBS, and then incubated with colloidal gold-Staphylococcal protein A complexes. These complexes were prepared as previously described (17), yielding gold particles 14 nm in diameter. Following this incubation, the grids were again washed in PBS followed by distilled water, stained with uranyl acetate for 5 min followed by lead citrate for 45 sec, and examined in a JEOLCO JEMS 100 electron microscope.

Tissue postosmicated and embedded in Epon was sectioned and stained with uranyl acetate followed by lead citrate.

Results

Immunocytochemical studies of skin sections revealed no involucrin immunoreactivity in the stratum basale. As visualized with colloidal gold, involucrin was detected in the stratum spinosum and became most intense in the stratum granulosum and inner stratum corneum. Within the stratum spinosum, the colloidal gold predominantly labeled the amorphous, faintly electron-dense material between tonofilament bundles, with little staining of tonofilaments or desmosomes (Figure 1). In the granular layer, not only was the amorphous material labeled but also the keratohyaline granules to a striking degree (Figures 2, 3). In the transition layer of the inner stratum corneum, there was marked general staining of the cytoplasmic remnant (Figures 3, 4). Keratohyaline granules within this transitional layer became coalescent structures that were also intensely labeled. Labeling of the outer layers of the stratum corneum was considerably less intense than the innermost layer (Figure 5). In these experiments, there was essentially no difference in labeling between 3% paraformaldehyde–0.1% glutaraldehyde fixation and 1% glutaraldehyde fixation.

The involucrin staining pattern was also examined in unfixed tissue placed directly in 30% ethanol with subsequent dehydration. Involucrin immunoreactivity was retained, and both the amorphous granular material and keratohyaline granules...
Figure 1. Skin, transitional layer. The network of dense fibrillar material in the transitional layer (T) is heavily labeled with the anti-involucrin antiserum. The neutrophilic leukocyte within this layer (N) is essentially unlabeled. Original magnification × 20,000. Bar = 0.75 μ.

Figure 5. Skin stratum corneum. (A) Intense labeling of the stratum corneum in unfixed skin with some labeling at the periphery of the cellular remnants (arrowheads). (B) In osmicated and Epon-embedded tissue, the stratum corneum exhibits a peripheral electron-dense band (arrowheads). This feature is not observed in osmicated tissue. Original magnification × 20,000. Bar = 500 nm.

Figure 6. Control samples. (A) Skin treated with preimmune serum revealing only weak background staining within the stratum granulosum (G). The keratohyaline granules within this layer (arrows) are unlabeled. Original magnification × 10,000. Bar = 100 nm. (B) Skin treated with antiserum absorbed with purified "envelopes." Both the granular layer (G) and the transition zone (T) are unlabeled. Remnants of keratohyaline granules are seen in the transition layer (arrow). Original magnification × 5,000. Bar = 200 nm.
ULTRASTRUCTURAL LOCALIZATION OF INVOLUCRIN

Figures 5 and 6
ULTRASTRUCTURAL LOCALIZATION OF INVOLUCRIN

Figure 7. Cell cultures. (A) Cultured cell fixed in glutaraldehyde and osmium exhibiting abundant cytoplasmic filaments. Original magnification × 2,000. (B) Cultured cell with randomly oriented filaments (arrowheads) and a cell border with few interdigitations. Original magnification × 10,000. Bar = 300 nm. (C) Ionophore-treated cell showing exaggerated interdigitations and cytoplasmic collections of granular material (higher magnification in E) (arrows). Original magnification × 8,000. (D) Ionophore-treated cell with tonofilaments organized into thick bundles (arrowheads) and desmosome-tonofilaments complexes (arrow). Original magnification × 8,000. (E) Granular material in the cytoplasm of ionophore-treated cell coalescing into larger granular structure (arrow). Original magnification × 5,000. Bar = 300 nm.

dramatically different appearance. The intermediate filaments become organized into thick interwoven bundles, many of which insert into desmosomes, and granular material is visible between them. In some areas this granular material appears to coalesce into larger granules. Cell borders show extreme interdigitations, and desmosomes are more numerous.

Labeling of cultured cells with anti-involucrin reveals relatively little immunoreactivity within the cells not treated with ionophore. The involucrin appears to be present both at the periphery as well as within the cytoplasm of the cells (Figure 8). The ionophore-treated cells have a marked increase in cytoplasmic immunoreactivity. There also appears to be coalescence of the involucrin into granules. In contrast to the untreated cells, labeling at the cell periphery is difficult to discern.

Discussion

The distribution of involucrin we observed in normal skin closely parallels that previously described by light microscopy of fixed sections (2). Involucrin is first detectable in the stratum spinosum and reaches maximal levels in the stratum granulosum and stratum corneum. The staining appears cytoplasmic and distinct from intermediate filaments, consistent with biochemical observations that involucrin behaves as a water-soluble protein when purified from cultured keratinocytes (15).

Within the stratum granulosum, the anti-involucrin antis-erum stains not only amorphous material in the cytoplasm but also keratohyaline granules. These granules are known to contain a high molecular weight precursor of filaggrin (9), a basic protein proposed to induce keratin aggregation during terminal differentiation (3). The pattern of filaggrin immunoreactivity in human skin (10) is rather similar to that of involucrin, although these proteins are biochemically and immunochemically distinct. This similarity in temporal expression and localization emphasizes the profound change in character of the epithelium between the stratum spinosum and corneum and raises the possibility that involucrin, as a constituent of the granules, has some functional significance in the cytoplasm. The insertion of keratin tonofibrils into keratohyaline granules, part of a dense meshwork in the transition layer between the granular and cornified layers, suggests that the granules may serve as a scaffolding for the interlacing keratin network in the stratum corneum. Formation of keratohyaline granules appears to be an element of the maturation

Figure 8. Cultured cells treated with anti-involucrin; both the cytoplasm and the cell periphery are labeled. Original magnification × 8,000. Bar = 350 nm.
program yielding stratum corneum. Involucrin is expressed in normal esophageal and vaginal epithelia, however, which lack both strata cornea and keratoxyaline granules.

The results with cultured keratinocytes mimic those with epidermal tissue in certain respects. The cells not treated with ionophore resemble basaloid epithelium in expression of randomly oriented individual intermediate filaments. After treatment with ionophore, the effect of altered ionic conditions is manifest in the organization of keratin into tonofibril bundles, an increase in desmosomes and tonofilament-desmosome complexes, and some coalescence of involucrin into granular cytoplasmic structures. Although introduced divalent ions may stimulate fibril aggregation (5), involvement of granular structures in consolidating the keratin network is possible. In any case, the persistence of involucrin in the cytoplasm after cross-linking takes place in cultured cells and in vivo could account for a considerable fraction of the isopeptide bonding reported in epidermal keratin (11), which occurs at a low level in comparison with that in envelopes (13).

While evidence for some localization of involucrin at the cell border was obtained in epidermis and cultured epidermal cells, the observed involucrin immunoreactivity was considerably more striking in the cytoplasm of maturing and even mature squames as well as the cultured cells. This finding indicates that a substantial amount of involucrin does not become incorporated into cross-linked envelopes adjacent to the plasma membrane, even though (in culture) it is not extractable after the cross-linking process takes place (15). The data do suggest some chemical alteration of involucrin between the transition cell layer and the stratum corneum. Compared with tissue placed directly in ethanol, the aldehyde-fixed tissue exhibits diminishing staining of the outer stratum corneum. In the outer cells, continued transglutaminase cross-linking could make the reduction of immunoreactivity by fixation more pronounced.

Quantitative evaluation of relative involucrin disposition is not possible at present in view of reduced antigenicity in aldehyde-fixed tissue (2) and uncertain access of the antibody to highly cross-linked envelope structures in skin. The envelopes visible in mature squares of skin were not evident in the cultured cells after ionophore treatment, although the cell borders comprised detergent-resistant structures of diameter comparable to the plasma membrane. Thus under the artificial condition of ionophore treatment, these structures are stabilized rapidly, whereas the much slower spontaneous cross-linking process in suspension culture can yield the thicker envelopes seen in skin (6). The biochemical events leading to formation of these latter structures remain to be elucidated but appear to involve several membrane-associated proteins in addition to involucrin (19).
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