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Expression of Trichohyalin in Dermatological Disorders: a Comparative Study with Involucrin and Filaggrin by Immunohistochemical Staining

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To investigate the function of trichohyalin during terminal differentiation of the skin, immunohistochemical studies were performed on trichohyalin and its related proteins, filaggrin and involucrin, the components of the cornified cell envelope. In skin disorders unrelated to tumours, weak trichohyalin expression was found in a few granular cells or in the horny layer of psoriasis, ichthyosis, keratosis pilaris, porokeratosis, chronic dermatitis and callus. Similar trichohyalin expression was found in epidermal tumours, such as seborrheic keratosis, actinic keratosis, Bowen's disease and well-differentiated squamous cell carcinoma. In follicular tumours, trichohyalin expression was positive in trichoepithelioma, keratotic basal cell epithelioma, proliferating trichilemmal tumour, trichilemmoma, pilomatricoma and keratoacanthoma. From comparative studies with filaggrin and involucrin, trichohyalin expression was co-localized with them in molluscum contagiosum, keratoacanthoma and pilomatricoma. From this study, trichohyalin is revealed to have close functional relationship with other markers of terminal differentiation as a precursor of the cornified cell envelope of the skin. ***Key words: terminal differentiation; cornified cell envelope; precursor.***

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During terminal differentiation of the skin, a characteristic structure called the cornified cell envelope (CE) is formed in the inner surface of the cell periphery of the granular layer. It is a rigid, complex structure composed of many precursor proteins of transglutaminase (TGase) which catalyses ε-(γ-glutamyl)lysine cross-linking (1 – 7). Trichohyalin is one of substrate proteins of TGase, and it can bind with keratin filaments as intermediate filament associated protein (IFAP) (8, 9). Trichohyalin is found mainly in the inner root sheath (IRS) and medulla of the hair follicle where it gives a structural rigidity, but its possible function as a precursor of the CE has been proposed previously (8). In a recent report (10), trichohyalin is found as a cross-linked component of mouse forestomach CE, and it is thought to modulate the biomechanical properties of the CE. In the skin, however, trichohyalin is fleetingly expressed in the neonatal foreskin, and it is not detected in the adult epidermis (11, 12). A few scattered observations reported that it is aberrantly expressed only in a number of dermatoses, such as psoriasis, molluscum contagiosum, actinic keratosis, trichofolliculoma, epidermolytic hyperkeratosis and pilomatricoma (13 – 15).

In the present study, an attempt was made to delineate the role of trichohyalin as a component of the CE during terminal differentiation by screening its expression in various skin disorders. We also evaluated the expression of trichohyalin in comparison with that of filaggrin and involucrin to clarify the functional relationship among them as precursor proteins of the CE.

MATERIALS AND METHODS

Materials

Formalin-fixed, paraffin-embedded samples were selected after evaluation of their histopathological features with haematoxylin and eosin staining. In the present study, 49 samples of skin disorders unrelated to tumours, 36 samples of epidermal tumours and 25 samples of follicular tumours were examined.

Antibodies

A polyclonal anti-trichohyalin antibody was elicited from rabbits with recombinant human trichohyalin of 1,250 – 1,849 residues (domain 8) located in the carboxy-terminus (16). Antibodies to involucrin and filaggrin were purchased from Biomedical Technologies Inc. (MA, USA). The polyclonal anti-involucrin antibody was raised from rabbits with purified cultured human epidermal cells (17). The monoclonal anti-filaggrin antibody was elicited from mice with partially purified filaggrin of the newborn human epidermis (18).

Immunohistochemical staining of trichohyalin, involucrin and filaggrin

Immunohistochemical procedures were carried out according to the protocol described in the LSAB kit (DAKO, CA, USA) with minor modifications. Samples 5 μm thick were deparaffinized, rehydrated and pre-treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, followed by incubation in the blocking reagent for 10 min. Then the sections were incubated with the primary antibodies under the following conditions: anti-trichohyalin at 1 : 50 dilution for 1 h; anti-involucrin at 1 : 10 dilution for 30 min; and anti-filaggrin at 1 : 150 dilution for 30 min at room temperature. For trichohyalin staining, sectioned samples were pre-treated in the autoclave at 121°C, 15 lbs/inch^2 for 5 min before reaction with the primary antibody to retrieve antigenicity. Then the sections were incubated with a biotin-labelled anti-immunoglobulin antiserum for 15 min, followed by peroxidase-conjugated streptoavidin for 15 min. As the chromogen, 3-amino-9-ethylcarbazole, was applied, red-coloured precipitates appeared at the antigen site. During immunohistochemical staining, each sample was washed with phosphate-buffered saline (pH 7.4). Sections were counterstained with Meyer's haematoxylin and mounted with Universal Mount (Research Genetics, AL, USA). As a negative control, sections were incubated with non-immunized serum instead of the primary antibodies.

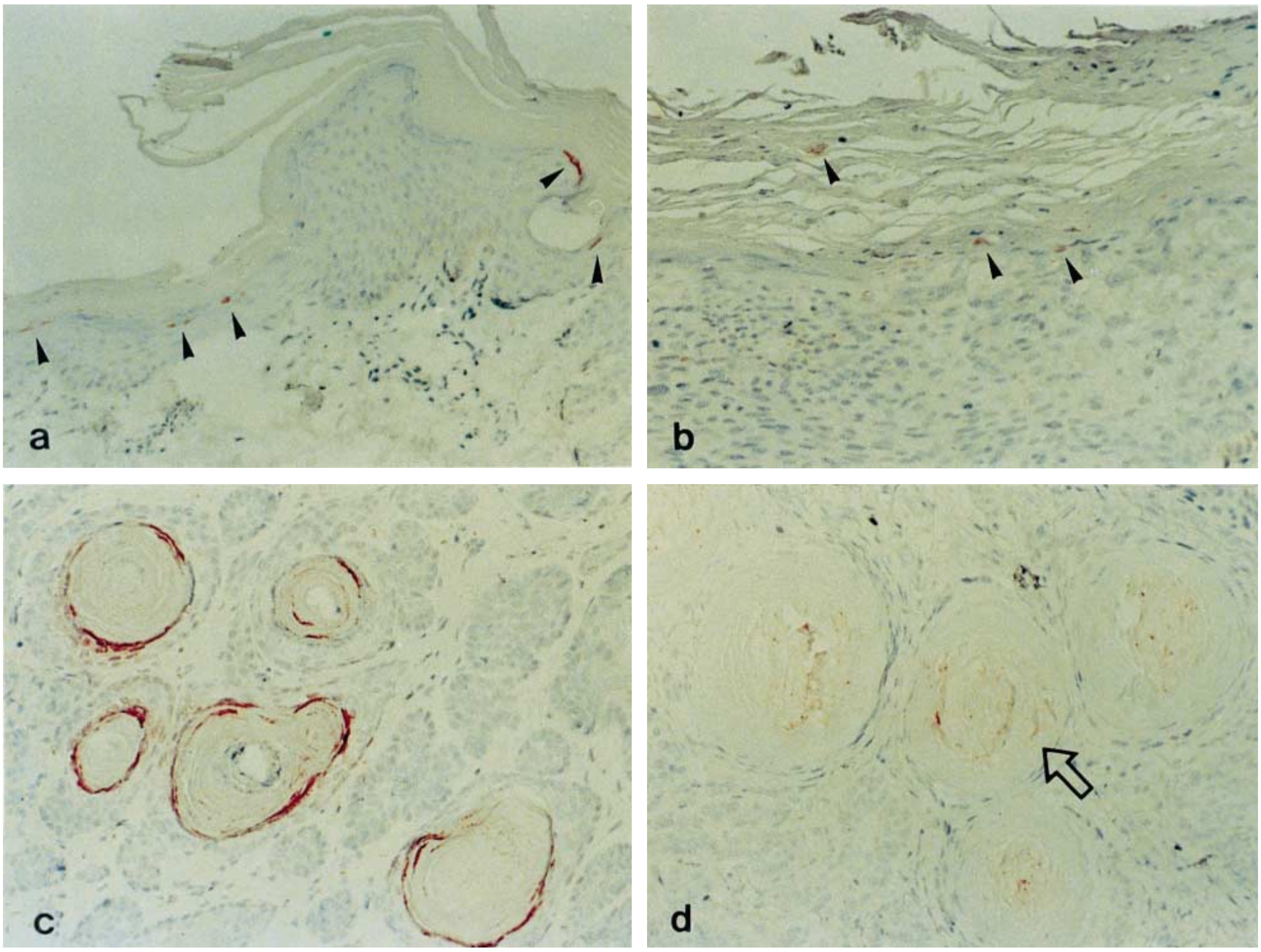

Fig. 1. Localization of trichohyalin in (a) ichthyosis vulgaris (× 160), (b) Bowen's disease (× 200), (c) trichoepithelioma (× 200) and (d) keratotic basal cell epithelioma (× 200). Arrows and arrowheads indicate positive expression of trichohyalin.

RESULTS

Trichohyalin expression in skin disorders unrelated to tumours

In deparaffinized autoclaved sections, trichohyalin expression was not detected in the interfollicular epidermis of normal adult skin, but it was strongly expressed in the IRS and medulla of hair follicles (data not shown). Such immunoreactivity was not detected in the sections without pre-treatment by autoclaving. Among samples, weak immunoreactivity of trichohyalin was localized to a few granular cells or to the horny layer of ichthyosis (Fig. 1a), psoriasis, keratosis pilaris, porokeratosis, chronic dermatitis and callus. Strong immunreactivity of trichohyalin was found from the horny to spinous layers of molluscum contagiosum (Fig. 2b).

Trichohyalin expression in epidermal tumours

The expression pattern in several epidermal tumours, such as seborrheic keratosis, actinic keratosis, Bowen's disease (Fig. 1b) and squamous cell carcinoma (SCC), was similar to that of skin disorders unrelated to tumours, as described before. In particular, all samples of seborrheic keratosis showed positive expression of trichohyalin in the granular and horny layers, and in the lining cells of horn cysts. In actinic keratosis and SCC, several dyskeratotic cells were also positive in trichohyalin expression. No immunoreactivity was found in malignant tumours of non-keratotic basal cell carcinoma (BCC).

Trichohyalin expression in follicular tumours

Immunoreactivity of trichohyalin was found in trichoepithelioma (Fig. 1c), keratotic BCC (Fig. 1d), proliferating trichilemmal tumour, trichilemmoma, pilomatricoma and keratoacanthoma. In trichoepithelioma and keratotic BCC, immunoreactivity was found around horn cysts, but there was contrast between them; major immunoreactivity was localized around cysts showing a palisading pattern in trichoepithelioma, while positive immunoreactivity was localized to cystic contents in keratotic BCC. In pilomatricoma, immunoreactivity of trichohyalin was localized mainly to the junction between basophilic and shadow cells (Fig. 2h).

Sequential expression of trichohyalin, filaggrin and involucrin in molluscum contagiosum, keratoacanthoma and pilomatricoma

In a comparative study of trichohyalin with filaggrin and involucrin, we found a sequential order among them in 3

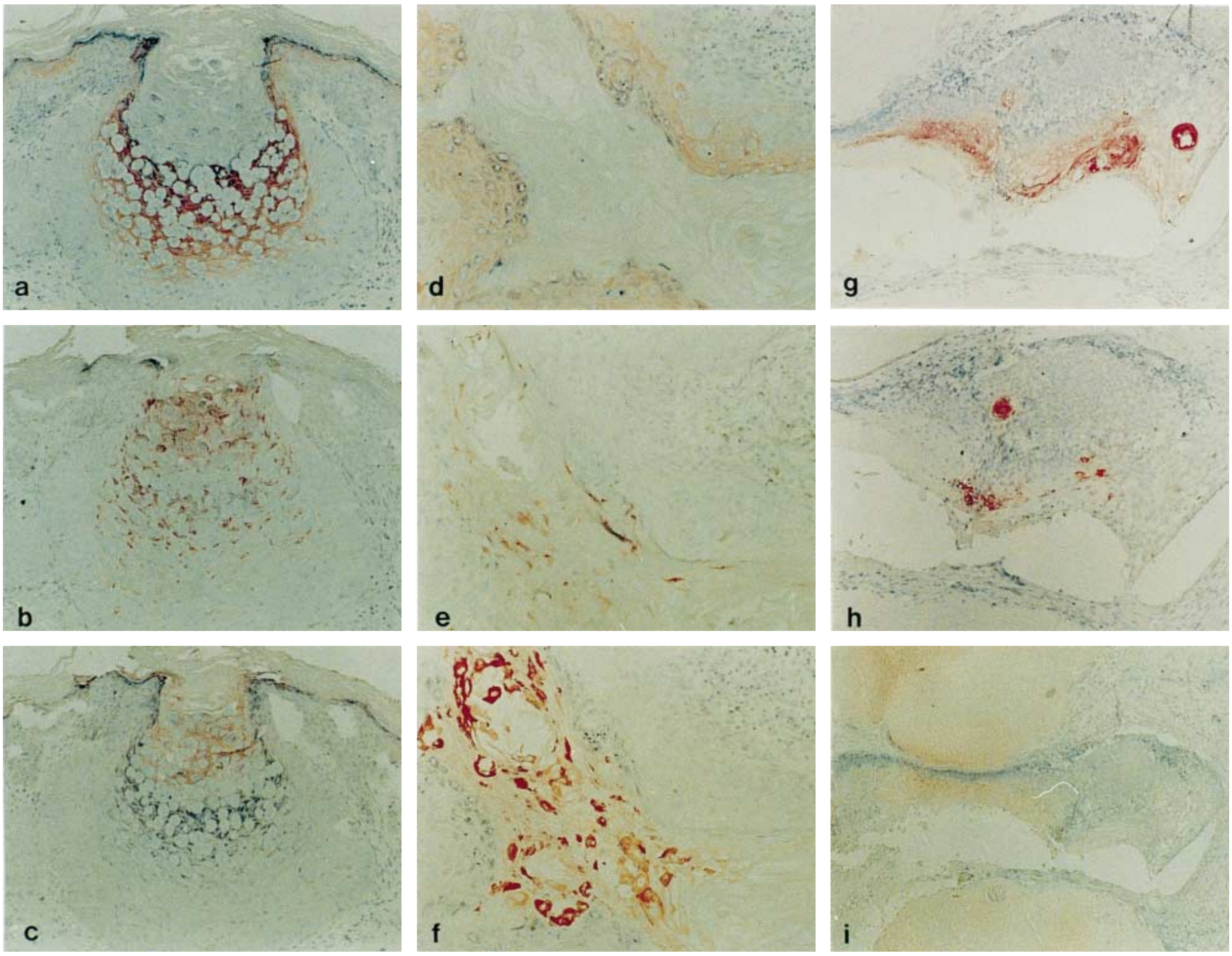

Fig. 2. Co-localization of (b, e, h) trichohyalin with (c, f, i) filaggrin and (a, d, g) involucrin in molluscum contagiosum (a – c, ×100), keratoacanthoma (d – f, ×132) and pilomatricoma (g – i, ×80).

dermatoses. In molluscum contagiosum, major immunoreactivity of involucrin was localized to the upper or mid-spinous layer, while that of filaggrin was confined to the horny layer of molluscum body (Fig. 2a, c). Immunoreactivity of trichohyalin was scattered to whole layers, from the horny to upper spinous layers, overlapping with the other precursors (Fig. 2b). A similar pattern of immunoreactivity was found in the follicular-originating tumours of keratoacanthoma and pilomatricoma. In keratoacanthoma, major immunoreactivity of involucrin was localized to the upper spinous layer, while that of filaggrin was localized to the horny layer (Fig. 2d, f). Immunoreactivity of trichohyalin was mostly observed in the granular and horny layers localized between filaggrin and involucrin expression (Fig. 2e). In pilomatricoma, immunoreactivity of involucrin was localized to the basophilic cells and the junction between basophilic and shadow cells (Fig. 2g), and that of filaggrin was localized mainly to shadow cells (Fig. 2i). Immunoreactivity of trichohyalin was localized to the junction and shadow cells, which overlapped with the other precursors (Fig. 2h).

DISCUSSION

Our study shows that trichohyalin expression is evident in the epidermis of pathological conditions irrespective of epidermal and follicular origins. Such morphological evidence strongly suggests a functional role for trichohyalin as a precursor of the CE in the keratinizing process of the skin. Notably, trichohyalin was co-localized with the other precursors of the CE, involucrin and filaggrin, in molluscum contagiosum and in follicular-originating tumours, such as keratoacanthoma and pilomatricoma. The overlapped expression suggests the presence of an intimate functional relationship among the precursors and of their sequential deposition to form the CE during terminal differentiation. An immunoelectron microscopic study showed that the co-expression of trichohyalin and filaggrin was present in the form of hybrid granules in several disorders, such as psoriasis, molluscum contagiosum and epidermolytic hyperkeratosis (13 – 15).

It is intriguing to consider how the CE is assembled with its many candidate precursor proteins during terminal differentiation. The most attractive model suggests that CE assembly starts with involucrin and cystatin-α cross-linking, acting as a scaffold, followed by accumulation of loricrin and small proline-rich proteins (SPRR) (17, 18). Ishida-Yamamoto et al. (19) demonstrated sequential synthesis of involucrin followed by SPRR in cultured human keratinocytes. Therefore, involucrin is thought to act as a frame for the cross-linking of other components among precursors of the CE, qualifying it as a

Table I. *Trichohyalin expression in various skin disorders*

Disease	Number of samples	Number of positive samples	Intensity of staining[a]
Psoriasis	8	4	+/−
Lichen planus	3	0	−
PRP	4	0	−
Ichthyosis vulgaris	6	2	+/−
EHK	2	2	+/−
Keratosis pilaris	4	2	+/−
Porokeratosis	6	3	+/−
Molluscum contagiosa	6	6	+/++
Chronic dermatitis	8	4	+/−
Callus	2	2	+/−
Seborrheic keratosis	4	4	+ or +/−
Actinic keratosis	6	5	+ or +/−
Bowen's disease	7	2	+/−
Basal cell carcinoma[b]	5	0	−
Basal cell carcinoma[c]	4	2	+
Squamous cell carcinoma	10	2	+/−
Trichoepithelioma	4	2	+
PTT	3	2	+
Trichilemmoma	3	1	+/−
Pilomatricoma	9	9	+ or +/−
Keratoacanthoma	6	4	+ or +/−
Total	110	58	

[a] Intensity of staining: − negative, +/− weakly positive, + moderately positive, ++ strongly positive.
[b] Basal cell carcinoma of non-keratotic type.
[c] Basal cell epithelioma of keratotic type.
PRP, pityriasis rubra pilaris; EHK, epidermolytic hyperkeratosis; PTT, proliferating trichilemmal tumour.

distinctive marker for a stage of keratinocyte maturation just before the final events of terminal differentiation (3, 20). Filaggrin is thought to participate in the final stage of CE formation, combining with keratin filaments as an IFAP (7, 21). From our study, the orchestrated sequence in forming the CE was clearly seen in the epidermis of molluscum contagiosum; involucrin deposits in the early stage followed by trichohyalin, and filaggrin deposits in the final stage (Fig. 2a–c). A similar pattern of sequential deposition in pilomatricoma or keratoacanthoma suggests that a spatial relationship between the precursors can be applied to the keratinizing process of follicular differentiation. Our present observation showing co-localization of their expression in several keratinizing disorders with similarities in their structural properties (8), suggests an intimate functional relationship among them in forming the CE. Interestingly, all 3 precursors are members of the "epidermal differentiation complex" comprising a number of genes involved in epidermal differentiation and they are present in this complex in 1q21 (22).

Except for a few dermatoses, which show positive expression of trichohyalin in all tested samples, such as molluscum contagiosum and seborrheic keratosis, there was considerable variation in the positive immunoreactivity of trichohyalin even within the same disorder (Table I). To account for this variation, the following possibilities may be taken into consideration. First, there is an intrinsic variation in expression of precursor proteins forming the CE in the same disorder. Second, the expression level of trichohyalin is too low to be detected with conventional immunostaining in some samples. Third, antigenicity of trichohyalin can be abolished during the fixation procedure in some samples. The first possibility was suggested earlier by demonstration of intra- and inter-individual variations in CE composition of normal and psoriatic epidermis (23).

In our comparative studies with the 3 markers, involucrin expression was positive in all squamous epithelia of both orthokeratotic and parakeratotic process, suggesting its availability as a marker of terminal differentiation (data not shown). The results were consistent with a previous report which suggests that involucrin is a sensitive marker of terminal differentiation, but that it is not suitable for differentiating between mature and premature differentiation (24). On the other hand, filaggrin expression matched well with expression of the granular layer, enabling one to differentiate between orthokeratotic and parakeratotic differentiation in the epidermis (data not shown), supporting the previous report (25).

Trichohyalin expression in the interfollicular epidermis starts at the same stage, when the rigid mature type CE is formed for the first time during foetal skin development in humans (unpublished). Such coincidental appearance of trichohyalin expression and CE formation is further supportive evidence to suggest its function during terminal differentiation of the skin. Recently, amino acids analysis of forestomach CE has provided direct evidence that trichohyalin is a component of the CE (10). Further biochemical studies need to be carried out to confirm our results by analysis of the CE in the skin.

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