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# Filaggrin expression in normal and pathological skin

## A marker of keratinocyte differentiation

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**Summary.** Filaggrin is a basic, histidine-rich protein synthesized by cells of keratinizing epithelia, that subserves major physiological functions in maturating epidermis. Its precursor, profilaggrin, constitutes a major component of keratohyalin granules. In this work the expression of profilaggrin/filaggrin was studied in 140 specimens of normal human adult and fetal skin, cultured epithelia before and after allo-and xeno-grafting, and several cases of keratinization disorders and tumours. An avidin-biotin-alkaline phosphatase technique was applied on deparaffinized tissue sections, by using a specific monoclonal antibody (AKH1) to profilaggrin/filaggrin. The results show that the expression of profilaggrin/filaggrin is variously altered in keratinization disorders, whereas in epithelial proliferations it seems to decrease with an increasing degree of dedifferentiation of the tumour. Therefore the expression of these antigens can be considered to be a sensitive marker of maturation of normal epidermis and provides a new tool for the study of differentiation of the surface epithelium in skin diseases.

**Key words:** Filaggrin – Profilaggrin – Epidermal differentiation

### Introduction

Filaggrin (FG) is a highly basic, histidine-rich protein of mammalian epidermis that was first isolated and characterized in rat epidermis in 1977 (Dale 1977). It is synthesized by differentiating epithelial cells as a large precursor, known as profilaggrin (PF), that subsequently undergoes proteolysis and dephosphorylation to yield FG (Meek et al. 1983). PF has been shown to constitute the major component of keratohyalin granules (KG) found in the stratum granulosum of epidermis and other keratinizing epithelia (Dale and Ling 1979; Lynley and Dale 1983; Smith and Dale 1986). Human FG has a molecular weight of 37 kd and a particular amino-acid composition, being rich in basic amino-acids like histidine, but completely lacking methionine, cysteine and tryptophan (Lynley and Dale 1983). Although it seems likely that not all aspects of the physiological role of PF/FG are presently understood, these proteins are already known to subserve major functions in maturating epidermis, such as aggregating keratin filaments (Steinert et al. 1981) and maintaining a normal hydrating state of the stratum corneum (Scott and Harding 1986). These proteins can therefore be considered as markers of keratinocyte maturation and differentiation, like other proteins such as keratins and involucrin. Up till now several antibodies have been raised to KG. While some of them react with as yet not well-defined components of keratohyalin (Laster and Haynes 1986; Zambruno et al. 1986), others, either polyclonal (Mamrak et al. 1984) or monoclonal (Hintner et al. 1983; Kubilus et al. 1985; Dale et al. 1987) react specifically with PF/FG, and have been used in the immunohistochemical investigation of the expression of the corresponding antigens in hyperproliferative or neoplastic skin lesions. In this work we further studied the expression of PF/FG in a variety of cutaneous diseases and in cultured epithelia before and after allo- and xeno-grafting, in order to assess better the potential usefulness of these differentiation markers in skin pathology.

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### Material and methods

Specimens studied included 12 specimens of normal adult skin, 3 skin specimens from a 19-week-old human fetus (obtained

**Table 1.** Specimens studied for profilaggrin/filaggrin expression

| Diagnosis | No. of specimens | No. of cases positive | Labelling intensity |
|---|---|---|---|
| Normal adult skin | 12 | 12 | ++ |
| Fetal skin (19th-week) | 3 | 3 | + |
| Cultured epithelial sheets | 4 | 1 | +/− |
| Epithelial allografts | 9 | 7 | + to ++ |
| Epithelial xenografts | 4 | 4 | + to ++ |
| Ichthyosis vulgaris | 1 | 1 | +/− |
| X-linked ichthyosis | 3 | 3 | +/− |
| Collodion baby | 1 | 1 | +/− |
| Confluent and reticulate papillomatosis | 2 | 2 | +/− to + |
| Congenital nonbullous ichthyosiform erythroderma | 3 | 3 | + or +++ |
| Epidermolytic hyperkeratosis | 3 | 3 | + or +++ |
| Erythrokeratodermia variabilis | 4 | 4 | +++ |
| Darier's disease | 4 | 4 | + to ++ |
| Psoriasis vulgaris | 6 | 6 | +/− |
| Pustular psoriasis | 3 | 0 | 0 |
| Lichen planus | 4 | 4 | ++ to +++ |
| Epidermal naevus | 4 | 4 | + to ++ |
| Epidermoid cysts | 7 | 7 | ++ to +++ |
| Trichilemmal cysts | 6 | 6 | +/− to + |
| Neuroendocrine skin carcinoma | 5 | 0 | 0 |
| Extramammary Paget's disease | 3 | 2 | +/− to + |
| Actinic keratosis | 11 | 11 | +/− |
| Bowen's disease | 12 | 9 | +/− to + |
| Basal-cell carcinoma | 10 | 1 | +/− |
| Squamous-cell carcinoma | 16 | 6 | +/− |
| Total | 140 | 107 | |

Labelling intensity: 0: negative; +/− or +: week; ++: normal; +++: increased

from the forearm, the ear and the finger), 4 specimens of epithelial sheets (CES) grown from human keratinocytes according to the method of Green et al. (1979), 13 specimens of CES grafted on humans and on nude mice, biopsied at various times after grafting, as well as a group of 108 cutaneous lesions representing keratinization disorders and tumours (Table 1). The afore-mentioned material, retrieved from the files of the Laboratory of Pathology (C. Hermier and B. Chouvet) of the Department of Dermatology, Ed. Herriot Hospital, France, had been collected during the last 4 years (1983–1987), fixed in formalin and embedded in paraffin. For normal skin, frozen sections were also used.

In immunohistochemistry 3 μ-thick sections were cut, allowed to dry overnight at 56° C, deparaffinized, rehydrated in alcohol and processed through an avidin-biotin-alkaline phosphatase technique, as detailed elsewhere (Kanitakis et al. 1987b). Briefly, after deparaffinization in xylene/toluene and rehydration in alcohols, the sections were incubated successively with the primary antibody (see below), biotinylated goat anti-mouse immunoglobulins and avidin-biotin-alkaline phosphatase complex. The reaction was revealed with a solution of fast-red TR salt and the sections were counterstained with Meyer's haematoxylin. Monoclonal antibody to PF/FG (AKH1) was obtained commercially (Biomedical Technologies Inc, Stoughton, USA) and used at a 1:50 dilution in tris-buffered saline. Control slides were obtained by omitting the first layer antibody and were consistently negative.

## Results

In deparaffinized sections of normal skin, PF/FG was detected in the cells of the stratum granulosum of the epidermis, in cells of the intraepidermal sweat duct (acrosyringium) and of the inner root sheath of the follicular infundibulum. The cytoplasmic labelling assumed a granular pattern that became diffuse wherever the staining intensity was strong. Cells of the horny layer were rarely labelled when deparaffinized sections were used; however, on frozen skin sections the horny layer was regularly positive. In some sections, a diffuse, cytoplasmic labelling was seen in cells of eccrine-secretory coils.

In fetal skin PF/FG were found expressed in the 1–2 uppermost cellular layers (including the periderm) of the 19-week-old fetal epidermis. A strong labelling was also observed in cells of the acrotrichium (intraepidermal part of hair follicles) (Fig. 1); in contrast, developing acrosyringia of finger skin were only rarely labelled.

Cultured epithelial sheets (CES), epithelial allografts (EAG) and xenografts (EXG). In 3 out of 4 CES labelling was virtually absent. In contrast, in one CES 26 days old, some cells were seen in the uppermost layers that exhibited a strong granular cytoplasmic labelling (Fig. 2).

In EAG, PG/FG reappeared progressively and were already detectable at day 7 after grafting in scattered cells of the granular layer. On later biop-

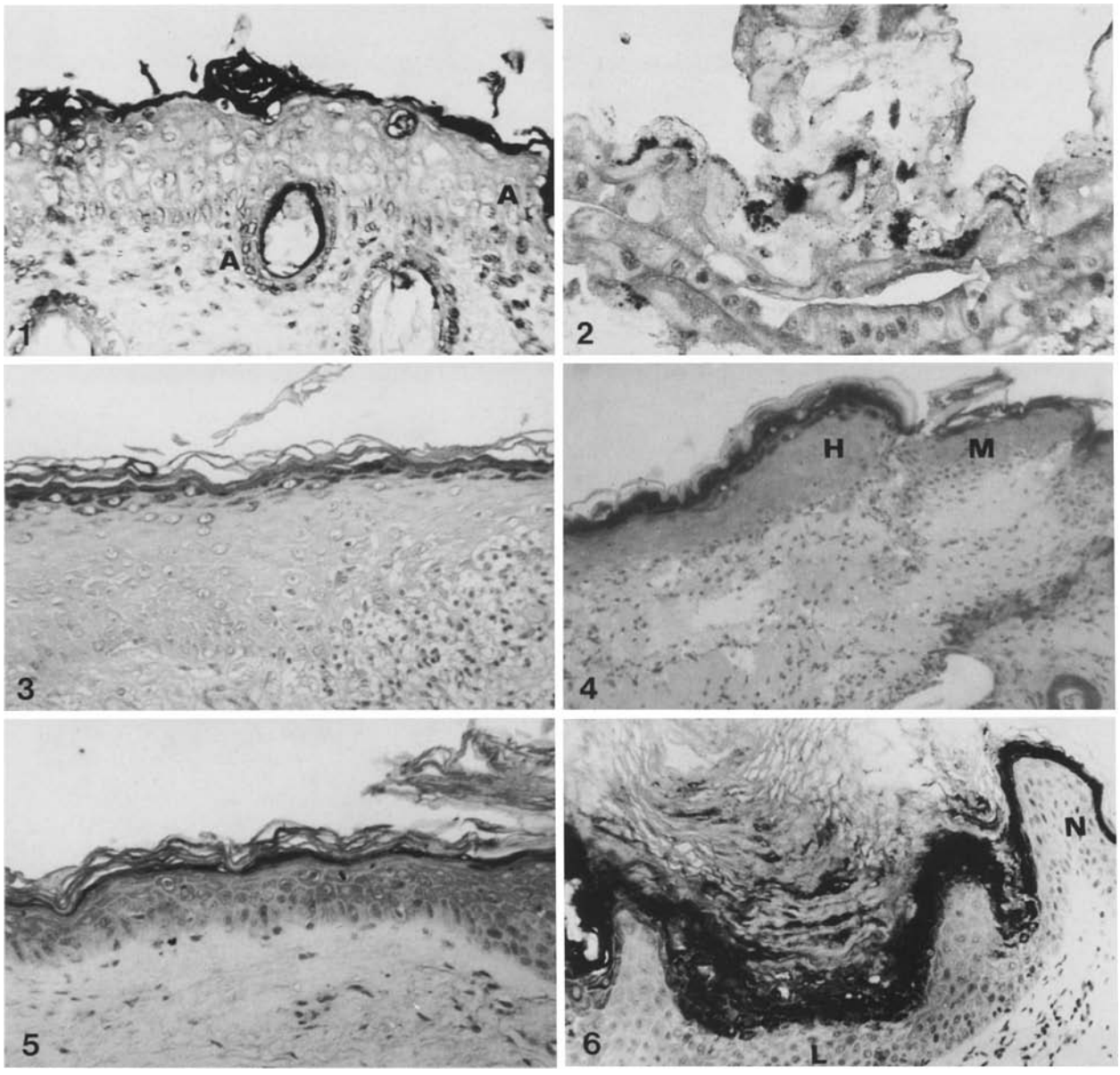


**Fig. 1.** On fetal skin (dorsum of the finger), PF/FG are expressed in the upper 1–2 epidermal cellular layers and in cells of the acrotrichium (*A*). (× 187.5)

**Fig. 2.** A cultured epithelial sheet comprises cells showing a granular staining pattern with AKH1 antibody (× 187.5)

**Fig. 3.** On day 14 after grafting a human epithelial allograft expresses PF/FG inside the granular layer (× 187.5)

**Fig. 4.** On day 7 after grafting, a human epithelial xenograft (H) is strongly reactive with AKH1 antibody; the murine epidermis (*M*) remains negative. (× 187.5)

**Fig. 5.** In ichthyosis vulgaris, staining for PF/FG is discontinuous (× 187.5)

**Fig. 6.** Epidermolytic hyperkeratosis. Staining for PF/FG in lesional epidermis (*L*) is increased as compared to normal (*N*) epidermis (× 187.5)

sies (from day 14 onwards) the PF/FG staining assumed the pattern observed in normal epidermis (Fig. 3). On the latest biopsy (day 155), the staining was completely normal, being also present in reconstituted acrosyringia. On EXG, PF/FG were already detectable at day 7 after grafting; the labelling was present solely on the human EXG but not on mouse epidermis (Fig. 4).

In the cases of Confluent and reticulate papillomatosis (Gougerot-Carteaud), Collodion baby,

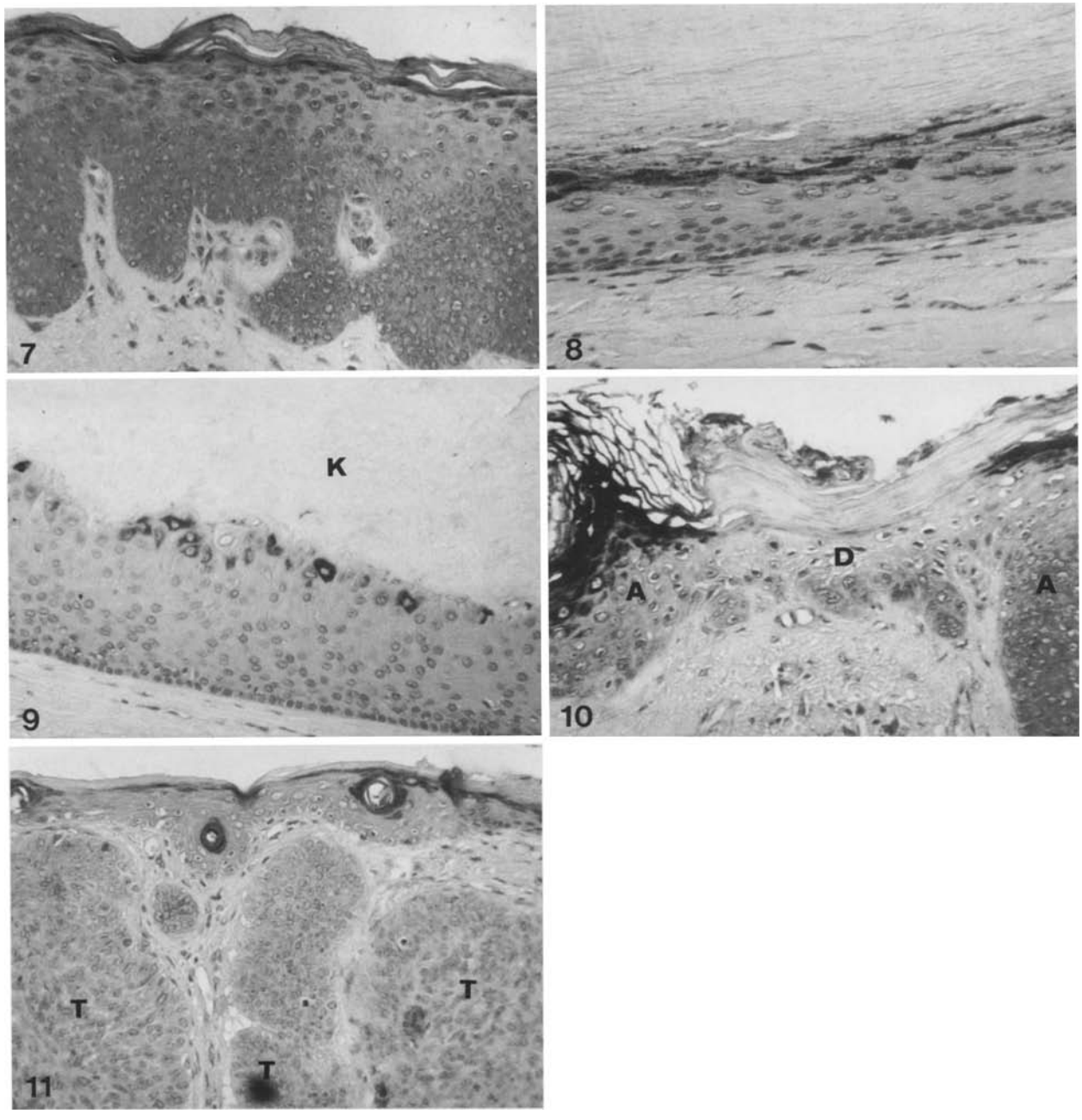


**Fig. 7.** Markedly reduced staining for PF/FG in a case of psoriasis (× 187.5)

**Fig. 8.** In epidermoid cysts PF/FG staining is granular and localizes at the level of the granular layer of the cyst wall (× 187.5)

**Fig. 9.** In trichilemmal cysts, scattered cells of the inner cellular layer of the cyst wall show a diffuse cytoplasmic labelling; the keratinous content (*K*) remains unstained (× 187.5)

**Fig. 10.** Actinic (preepitheliomatous) keratosis. PF/FG staining is detected at the level of two acrotrichia (*A*), but is abruptly interrupted in the interfollicular epidermis showing dysplastic changes (*D*). (× 187.5)

**Fig. 11.** Basal-cell carcinoma. PF/FG staining is seen in the epidermis overlying the tumour (*T*), that itself remains negative (× 187.5)

Ichthyosis vulgaris, X-linked ichthyosis the specific labelling was generally decreased; it was observed in a single row of cells (corresponding to the granular layer) but was often discontinuous (Fig. 5).

In two cases of congenital non-bullous ichthyosiform erythroderma (CNBIE) an intense labelling of the upper epidermal layers was seen, corresponding to the increased thickness of the granular and horny layers. In the third case, only moderate labelling of the upper horny layer was seen.

Epidermolytic hyperkeratosis (EH) showed generally decreased labelling in two cases when compared with normal epidermis; it was localized in part of the horny layer, the granular layer being negative. In contrast, in one case of EH, the labelling was increased and observed both in the granular and horny layers (Fig. 6).

In all cases of erythrokeratodermia variabilis, strong labelling was seen within both the hyperplastic granular and the horny-cell layers.

In Darier's disease (DD) the PF/FG labelling assumed an irregular pattern when compared with normal epidermis. In all samples studied, in areas adjacent to lesions of dyskeratosis, the horny layer was strongly stained, whereas the granular layer was negative. In areas overlying dyskeratotic cells and/or clefts, the granular layer was labelled and the horny layer tended to be negative.

Cases of psoriasis (PS) showed markedly reduced labelling for PF/FG, especially in areas of parakeratosis (Fig. 7). The most dramatic decrease in staining intensity was noted in cases of pustular PS, where the whole length of the biopsy specimen was negative.

In areas of lichen planus papules, many layers of the upper viable epidermis were positive; this correlated well with the thickness of the stratum granulosum.

In all cases of epidermal naevus studied, an intense labelling was observed inside the thickened, orthokeratotic horny layer. The granular layer was also weakly stained.

Epidermoid (EC) and trichilemmal (TC) cysts showed varying patterns. In EC, a cytoplasmic staining of cells of the granular layer of the cyst wall was observed in all cases. However, the labelling was not continuous throughout the whole circumference of the cyst wall, but was interrupted, namely at places where no identifiable granular layer was observed. The keratin filling the cyst also comprised consistently areas of positivity (Fig. 8). In the wall of TC, isolated cells of the innermost viable layers were occasionally labelled, in a diffuse rather than a granular pattern. Noticeably, the keratin content of TC remained in all cases unlabelled (Fig. 9).

In all cases of neuroendocrine skin carcinoma the tumour cells proved completely negative.

In two of three cases of extramammary Paget's disease (EMP) diffuse cytoplasmic PF/FG reactivity of Paget's cells was observed. The third case proved negative.

In Actinic keratosis (AK) and Bowen's disease (BD) the lesions exhibited a similar staining pattern, i.e. an irregular, discontinuous PF/FG labelling. This was interrupted in areas where the more severe dysplastic alterations in the epidermis were noted. The horny layer was more frequently labelled in BD than in AK. Noticeably, the intraepidermal portion of cutaneous appendages in AK and scattered dyskeratotic cells in BD were positive (Fig. 10).

In Basal (BCC) and Squamous (SCC) cell carcinomas the epidermis overlying tumour masses generally showed a less intense labelling when compared with normal epidermis at the level of the granular layer. In 7 out of 26 tumours, weak labelling was detectable in horny pearls but the majority of them, as well as the remaining parts of the tumours, were negative (Fig. 11).

## Discussion

Our knowledge of the biology of human epidermis has witnessed considerable progress, reflected in the discovery of new differentiation antigens. Filaggrin is one of the such recently characterized proteins of keratinizing epithelia. The investigation of its expression is greatly facilitated by the production of specific (monoclonal or polyclonal) antibodies. Among the former, AKH1 appears to be a sensitive probe for the detection of PF/FG, since it gives reliable and reproducible results on formalin-fixed, paraffin-embedded tissues. In fact, when frozen skin sections were used, AKH1 is regularly reactive also with the horny layer of the epidermis. This reactivity may be partly lost after standard fixation, as happens with involucrin or keratins. The reactivity of AKH1 with both PF and FG is presumably due to the presence of similar antigenic sites in these proteins (Dale et al. 1987). AKH1 staining usually parallels the presence of KG, but is sometimes observed even in the absence of visible KG. Indeed, in trichilemmal cysts that undergo the so-called trichilemmal keratinization and therefore synthesize only few KG (Kimura 1978), scattered cells were found to exhibit a diffuse cytoplasmic labelling. It seems likely that PF is not necessarily associated to morphologically visible KG but can also be detected in the cytoplasm before its aggregation in mature KG. Immuno-electronmicroscopic studies should clarify this point by demonstrating the precise ultrastructural localization of PF/FG.

AKH1 antibody was reported to react poorly with rodent tissues. Our results, obtained on cultured human epithelia xenografted onto nude mice, confirm this fact, by showing a clear-cut appearence of reactivity on human-derived epithelium.

Thus, next to involucrin (Zambruno et al. 1987) an additional immunohistochemical marker is provided for the confirmation of the origin of human material (cultured epithelium or diseased epidermis) grafted onto athymic rodents, a technique used with an increasing frequency.

The expression of PF in cultured human and rodent keratinocytes has already been documented (Kubilus et al. 1985; Fleckman et al. 1984) and proven to be regulated by various factors, such as calcium concentration (Dale et al. 1983) or exposure to 12-o-tetradecanoylphorbol-13-acetate (Kim and Bernstein 1987). In our material, consisting of secondary cultures obtained according to the method of Green et al. (1979), PF/FG were detectable in few cells of only one specimen. Submerged epithelia produced in this way are known to exhibit a moderate degree of differentiation and generally show few KG as revealed by electron-microscopy (Kanitakis et al. 1987a). The low AKH1 reactivity in these cultured epithelia is undoubtedly correlated with the paucity of KG in this material. Interestingly, however a re-expression of PF/FG in both allo- and xeno-grafts was observed rapidly (at day 7) after grafting, and this became completely normal on later dates. This fact parallels the reappearance of morphological features of differentiation of the grafted epithelium (Kanitakis et al. 1987a) and provides additional evidence in favour of the key-role of dermally-conveyed, human or murine factors in the normal maturation of epidermis. As far as the appearance of PG/FG during embryogenesis is concerned, our results show that these molecules are already expressed in fetal skin at the 19th week of intrauterine life. At that date PF/FG seem to be more strongly expressed by acrotrichial cells than by the surrounding epidermal keratinocytes. This fact is in accordance with previous immunohistochemical (Dale et al. 1985) and ultrastructural data, showing the presence of granular and keratinized cells at the level of the acrotrichium, but not in the interfollicular epidermis (Holbrook and Odland 1975; Holbrook et al. 1983).

With regard to keratinization disorders, our findings confirm earlier results and provide some new data. Thus, in diseases such as psoriasis and ichthyosis vulgaris we observed decreased PG/FG reactivity, as has been deduced by using either specific antibodies (Sybert et al. 1985; Dale et al. 1987; Fleckman et al. 1987), the incorporation of 3H-histidine or the Cox-Reaven staining of histidine (Baden et al. 1974). In our material, it seemed that the decreased expression or the complete absence of PF/FG correlated well with the absence of a visible granular layer. This was observed both in the case of a orthokeratotic (such as in ichthyosis vulgaris) or a parakeratotic (such as in psoriasis) keratinization process. Thus, the absence of KG that (along with the presence of nucleated horny cells) constitutes the histological hallmark of parakeratosis and reflects an abnormal keratinization pathway is shown immunochemically by a decreased or absent PF/FG synthesis. In epidermolytic hyperkeratosis, a keratinization disorder characterized by abnormal tonofilament clumping, an increased AKH1 reactivity was observed in only one out of three cases studied. This heterogeneity may be due to a variable severity of the pathological process of each individual case. In lichen planus, congenital non-bullous ichthyosiform erythroderma and erythrokeratodermia variabilis, the increased PF/FG staining parallels the orthokeratotic hyperkeratosis and hypergranulosis seen in these diseases (Civatte 1982). The irregular staining pattern observed in Darier's disease reflects the abnormal maturation of keratinocytes (known as dyskeratosis), characterized by tonofilament clumping, individual cell keratinization and acantholysis with cleft formation (Civatte 1982). Our survey of benign, premalignant and malignant epithelial tumours shows that PF/FG expression decreases with an increasing severity of dysplastic or anaplastic changes. This is clearly evidenced in the case of actinic (pre-epitheliomatous) keratoses, where PF/FG staining was interrupted abruptly in areas of dysplastic epidermis, usually corresponding to a parakeratotic keratinization. In addition, the intraepidermal parts of cutaneous appendages (hair-follicles and sweat-glands), known to be spared of dysplastic changes (Civatte 1982), maintained a normal PF/FG staining pattern that contrasted with that of surrounding dysplastic keratinocytes. These findings are in accordance with those of earlier works comparing keratoacanthoma vs squamous-cell carcinoma (Klein-Szanto et al. 1984; Vigneswaran et al. 1987), preneoplastic vs neoplastic lesions of the oral mucosa (Itoiz et al. 1985) and chemically-induced papillomas and carcinomas of the mouse (Mamrak et al. 1984).

The AKH1 reactivity we observed in cases of extramammary Paget's disease is puzzling. It is known that Paget's cells share immunohistochemical characteristics such as the expression of carcinoembryonic antigen (Mazoujian et al. (1984)) with sweat-gland cells, and it is interesting to note that in some skin biopsies we detected PG/FG in eccrine-secretory cells. More cases need to be studied

both by immunohistological and biochemical methods before definite conclusions can be drawn. Finally, the negativity of many cases of basal and squamous-cell carcinomas and of all cases of neuroendocrine carcinomas, known to be epithelial in origin by virtue of expression of keratins (Moll et al. 1984) suggests that PF/FG are not appropriate probes for the immunohistochemical discrimination between epithelial and non-epithelial tumours, but rather represent sensitive markers of terminal epidermal differentiation. Another point that awaits confirmation is the absolute specificity of PF/FG as markers of epithelial tissues. Indeed, despite the fact that these molecules were undetectable in non-epithelial tissues (Dale et al. 1987), it has been shown that PF/FG are able to aggregate in vitro other types of intermediate filaments, such as vimentin and desmin (Steinert et al. 1981). Therefore, the possibility of PF/FG being expressed by mesenchymal or muscular tumours cannot be completely ruled out on a theoretical basis and this point is currently under investigation in our laboratory.

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