We investigated the expression of both epidermal fatty acid-binding protein (FABP5), a marker of transit amplifying cells, and nestin, a putative marker of epidermal stem cells, in psoriatic epidermis and in normal human cultured keratinocytes. In lesional psoriatic epidermis, immunostaining showed that the suprabasal layer was positive for nestin, with some cells co-expressing FABP5. Flow cytometric analysis revealed that the expression of both nestin and FABP5 were increased in keratinocytes cultured in a low concentration of calcium relative to those cultured in a high concentration of calcium. These results suggest that nestin and FABP5 are expressed in actively proliferating keratinocytes in vitro and in the suprabasal layer in lesional psoriatic epidermis, and that double-positive cells may identify transit amplifying cells in the epidermis. Key words: calcium concentration; epidermal fatty acid-binding protein (FABP5); nestin; psoriasis; stem cell; transit amplifying cell.

(Accepted May 8, 2012.)


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Stem cells and transit amplifying (TA) cells are involved in cell division in the epidermis (1–3). In psoriasis, epidermal hyperproliferation occurs and is associated with an increase in TA cells. Epidermal fatty acid-binding protein (FABP5), first cloned from the epidermis, is a novel marker of epidermal TA cells (4), but is also expressed in tissues throughout the body, including the brain and liver (5–7). Ogawa et al. (8) reported that FABP5 regulates keratinocyte differentiation; but the underlying mechanism is not entirely clear.

Several markers for epidermal stem cells have been proposed (9), including the neural stem cell marker, nestin. Nestin is expressed in the bulge area of the mouse hair follicle. Nestin-expressing cells can differentiate in vitro into neurones, glia, keratinocytes, smooth muscle cells, and melanocytes. Nestin-expressing cells are positive for the stem cell marker CD34 and negative for K15, suggesting that they are in a relatively undifferentiated, pluripotent state (10–15). In the present study, we examined the expression of nestin and FABP5 in normal human keratinocytes cultured in different calcium concentrations and in skin samples of psoriasis vulgaris.

MATERIALS AND METHODS

Subjects and skin specimens

This study was performed in accordance with the Declaration of Helsinki and complied with Japanese regulations for the experimental use of human material. A total of 28 patients with psoriasis vulgaris were recruited and informed consent was obtained from all patients. Sex, age and disease duration were recorded, as well as an assessment of disease severity using the Psoriasis Area and Severity Index (PASI) (16). The group consisted of 19 males and 9 females, with a mean age of 51.5 years and an age range of 16–83 years. Patients had not been treated with any systemic drugs and did not apply any topical drugs to the biopsy area for several weeks. Skin specimens comprised lesional skin together with non-lesional skin surgically excised from the extremities, which were fixed in 10% formalin and embedded in paraffin. Both immunohistochemical and immunofluorescent staining were performed on serial sections of the skin samples.

Immunohistochemical and immunofluorescent staining

Immunohistochemical staining of paraffin-embedded, 4-μm thick tissue sections was performed. As a control, normal human skin was obtained during surgery for an epidermal cyst. For immunohistochemical staining, paraffin sections were treated with 0.3% hydrogen peroxidase and then heated for 5 min in 10 mM citrate buffer (pH 6.0) in a microwave oven for antigen retrieval. Sections were incubated with anti-nestin rabbit polyclonal IgG antibody (1:40; IBl, Gunma, Japan) for 60 min, and then sections were incubated with ChemMate Envision (Dako) according to the manufacturer’s instructions. Counterstaining was performed using Mayer’s haematoxylin (Wako Pure Chemical, Osaka, Japan).

Immunofluorescence double-staining of paraffin-embedded tissue sections was performed using the indirect method. Antigen retrieval was performed in the same way as immunohistochemical staining, except for nestin/involucrin double staining. Nestin/involucrin double staining was treated with proteinase K (Dako) for 5 min. After antigen retrieval, the following primary antibodies were used: anti-nestin rabbit polyclonal IgG antibody (1:40; IBL, Gunma, Japan), anti-involucrin mouse monoclonal (CY5) antibody (1:100; Thermo Fisher Scientific, CA, USA), anti-FABP5 goat polyclonal IgG antibody (1:20; R&D Systems, Minneapolis, MN, USA) and anti-Ki-67 mouse monoclonal (CY5) antibody (1:100; Daco, Tokyo, Japan). The respective secondary antibodies for nestin, involucrin, FABP5 and Ki-67 (all from Invitrogen, Carlsbad, CA, USA) were as follows: Alexa Fluor 488-labelled anti-rabbit IgG antibody (1:100), Alexa Fluor 568-labelled anti-mouse IgG antibody (1:500), Alexa Fluor 568-labelled anti-goat IgG antibody (1:500), Alexa Fluor 568-labelled anti-mouse
IgG antibody (1:500, nestin/Ki-67 double staining) and Alexa Fluor 488-labelled anti-mouse IgG antibody (1:100, FABP5/Ki-67 double staining). Cell nuclei were counterstained with 4’-6-Diamidino-2-phenylindole (Invitrogen).

The strength of nestin expression in the epidermis was compared with the staining of the cutaneous vessels and graded: −, no staining; +, weaker staining than vessels; ++, staining equal to that of vessels; ++++, stronger staining than vessels. Three dermatologists reviewed all immunohistochemical slides under a light microscope (Olympus BX51, Tokyo, Japan) at ×20 magnification.

**Keratinocyte culture and flow cytometric analysis**

Normal human epidermal keratinocytes from neonatal foreskin (Kurabo, Osaka, Japan) were grown in Epilife Calcium-Free MEPICF cell culture medium (Cascade Biologicals, Carlsbad, CA, USA) supplemented with calcium chloride at 0.06 mM. Keratinocytes were cultured in 0.03 mM or 2.8 mM calcium to induce terminal differentiation for 48 or 72 h and flow cytometry was subsequently performed. Cells were fixed in 4% paraformaldehyde, permeabilized using phosphate-buffered saline (PBS) with 0.1% saponin, and incubated with the following antibodies in sequence: anti-human FABP5, Alexa Fluor 568 anti-goat IgG, and anti-human nestin fluorescein-conjugated monoclonal antibody (R&D Systems). Flow cytometric analysis was performed with FACScan (Becton Dickinson).

**RESULTS**

**Expression of nestin, FABP5 and Ki-67 in non-lesional and lesional skin of psoriasis vulgaris and normal skin**

Nestin was positive in the epidermis of all samples of psoriatic skin (Fig. 1). No significant differences in the strength of nestin expression according to sex, age, disease duration and PASI were noted (Table I).

Immunofluorescence double staining for nestin and involucrin in the lesional psoriatic epidermis revealed that the first few suprabasal layers were positive for nestin, while involucrin was positive above the nestin-positive layers (see Fig. 1). Immunofluorescence double staining for nestin and FABP5 revealed that most of the FABP5-positive cells express nestin in the lesional psoriatic epidermis (Fig. 2). On the other hand, non-lesional psoriatic epidermis showed little staining for nestin and FABP5 (Fig. 3). Immunofluorescence double staining for nestin and Ki-67 in the lesional psoriatic epidermis revealed that Ki-67-positive keratinocytes were located in the basal and the first suprabasal layers, and some cells were positive for both nestin and Ki-67 (Fig. 4). The staining behaviour of immunohistochemical staining for nestin was similar to immunofluorescence staining for nestin (data not shown). The results of immunofluorescence double staining for FABP5 and Ki-67 in lesional psoriatic epidermis were similar to those of nestin and Ki-67 staining (data not shown). Immunofluorescence staining of normal skin was similar to that of non-lesional psoriatic skin (data not shown).

**Influence of extracellular calcium concentration on nestin and FABP5 expression in cultured epidermal keratinocytes**

Normal human keratinocytes were cultured in a specific medium that promotes keratinocyte growth containing...
calcium at a low concentration (0.03 mM). Keratinocytes were subsequently cultured in media containing either 0.03 mM or 2.8 mM calcium. Flow cytometry showed that nestin (Fig. 5a) and FABP5 (Fig. 5b) were increased in keratinocytes cultured at a low concentration of calcium relative to those in a high concentration of calcium. After 72 h in a low calcium environment, 67.9% of keratinocytes were double positive for nestin and FABP5 (Fig. 5c).

DISCUSSION
Psoriasis is a chronic, inflammatory skin disorder. In psoriasis, the turnover time of the epidermis is markedly shortened, with incomplete keratinization, as evidenced by parakeratosis and the lack of a granular layer. The shortened cell cycle time is 36 h, compared with 311 h in normal skin, and the turnover time of the psoriatic epidermis is 4 days, compared with 27 days in normal skin.

Cell division occurs in the stem cells and TA cells in the epidermis (1–3). Stem cells are characterized by a slow growth rate, a long cell cycle and the ability to differentiate into cells of different lineages. TA cells divide rapidly after their exit from the stem cell compartment, and are associated with a faster cell cycle compared with stem cells. Epidermal stem cells are located in the basal cell layer at the base of the rete ridges, while TA cells are located in both the basal and suprabasal layers (1). The proliferation of stem cells and TA cells are tightly regulated to maintain homeostasis in the epidermis; thus, it is important to understand the conditions and mechanisms that control their proliferation and differentiation. In vitro, the keratinocyte growth and differentiation switch is generally associated with decreased proliferation, cell cycle arrest in Go/G1 phase and expression of epidermal differentiation markers, such as keratin 1, keratin 10, involucrin and transglutaminase (17–19).

Although several markers for epidermal stem cells have been proposed (9), the marker profile of the keratinocyte stem cell remains to be fully defined. Nestin, a marker of neural stem cells, is expressed by hair follicle stem cells. We reported that nestin-expressing murine hair follicle stem cells are relatively undifferentiated and pluripotent (10–15). In normal human skin, the cells in the epidermal basal layer were positive for K15 and negative for nestin and CD34.

In the present study, we showed that nestin- and FABP5-expressing cells are located in the first few suprabasal layers in lesional psoriatic epidermis, while involucrin-expressing cells are located above the nestin-positive keratinocytes. Although co-expression of nestin and FABP5 has been reported in the hippocampal neurogenic niche of post-ischaemic monkeys (20), the co-expression of nestin and FABP5 in the human epidermis, as well as the presence of nestin-positive cells in the psoriatic epidermis, have not been reported.
In addition, we investigated the effect of the calcium-induced switch from proliferation to differentiation in normal human keratinocytes on nestin and FABP5 expression. Using flow cytometry, we found that both nestin and FABP5 expression were relatively increased in cultured keratinocytes in a low calcium concentration, while involucrin expression was decreased. Cytokines, such as epidermal growth factor and transforming growth factor (21), and inorganic ions, such as calcium, influence the differentiation of epidermal keratinocytes. In a low-calcium medium, cultured murine epidermal keratinocytes remain undifferentiated and proliferate (22), whereas keratinocytes differentiate relatively rapidly in a high-calcium medium (23). Moreover, an increased calcium concentration induces the differentiation of FABP5-positive keratinocytes to a greater extent than FABP5-negative keratinocytes (8). Our study showed that low calcium concentration led to the expression of nestin and FABP5 in cultured epidermal keratinocytes.

Using mathematical models, typical psoriasis histology, such as acanthosis with elongated rete ridges, thin suprapapillary epidermal plates and parakeratosis, was explained as a result of a markedly increased proliferative compartment (2, 24). It is interesting to note that nestin expression was seen in proliferating epidermal keratinocytes in a low calcium concentration with a similar phenomenon observed in the psoriatic epidermis. Our findings suggest that nestin- and FABP5-expressing keratinocytes may have an important role in the pathogenesis of psoriasis vulgaris.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of the Japan Government (Assistance for Strategic Creation of Research Basis, 2009–2013), SRL Inc. and the Uehara Memorial Foundation.

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