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Immunohistochemical Identification of Thymosin $\beta 4$ in Langerhans Cells in Human and Rat Skin

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Summary. **Localization of thymosin $\beta 4$(T$\beta 4$) in human and rat skin was examined by immunohistochemistry using a monoclonal antibody against T$\beta 4$. The specificity of the monoclonal antibody to T$\beta 4$ was verified by Western blot analysis, where the antibody reacted to recombinant T$\beta 4$ but not to thymosin $\beta 10$(T$\beta 10$), and stained a band corresponding to T$\beta 4$ in the homogenate of human skin. By double immunostaining for T$\beta 4$ and S-100 protein or by immunohistochemical staining for T$\beta 4$ and CD1a or HLA-DR on sequential sections, the T$\beta 4^+$ cells were identified in the epidermis as cells which also expressed S-100 protein, CD1a or HLA-DR. The findings indicate that T$\beta 4^+$ cells in the epidermis are Langerhans cells (LC), and propose T$\beta 4$ to be a marker of LC in the epidermis. Actin-filaments in the LC may be implicated in the migration of their progenitors from the circulation to the epidermis and in their intimate adhesion to keratinocytes. T$\beta 4$ may play an important role in the polymerization and depolymerization of actin during these processes.**

Key words—Thymosin $\beta 4$, Langerhans cell, skin, development, laser-microscopy.

INTRODUCTION

The thymosins are a family of polypeptides originally isolated from the mammalian thymus.[1-3] Thymosin $\beta 4$(T$\beta 4$), a 43 amino acid polypeptide, is a well-characterized member of this family.[4] This peptide was recently identified as the major monomeric globular actin (G-actin)-binding protein which interferes with polymerization of G-actin to filamentaous actin (F-actin) by the sequestration of G-actin, suggesting a role for this peptide in cell movement, proliferation, and differentiation.[4-10] Previous studies have demonstrated the presence of T$\beta 4$ in platelets,[11] neutrophils, monocytes,[12-14] and an inhibitory function of T$\beta 4$ for the proliferation of hematopoietic progenitor cells.[15] Likewise, T$\beta 4$ was found in Langerhans cells (LC) in the skin by immunohistochemistry using an antibody against T$\beta 4$.[16] This finding may be comparable to the concept that LC are originated from bone marrow-derived progenitor cells.[17] However, the identification of LC in the skin and T$\beta 4$ in the previous study was incomplete since all dendritic cells were regarded as LC and the anti-T$\beta 4$ antibody was not well-characterized. Recent studies have demonstrated that LC are identified by phenotypic markers such as CD1a-, Ia-, and S-100 protein and that dendritic cells are not always LC in the skin.[18-21] In addition, several β-thymosin isoforms have also been identified in a variety of animals; $\beta 9$,[22,23] $\beta 10$,[24-27] $\beta 11$,[28] $\beta 12$,[29] $\beta 13$,[30] $\beta 14$,[31] and $\beta 15$.[32] In the β-thymosin family, T$\beta 4$ and T$\beta 10$ are major thymosins in normal mammalian cells, and the similarities of amino acid sequence between T$\beta 4$ and T$\beta 10$ are shown to be 74%.[29] The amino acid sequences of these thymosins are well conserved in different species and are 100% homologous in the human, rat, and mouse.[33] Since both a possible cross-reactivity of the anti-T$\beta 4$ antibody to T$\beta 10$ and a phenotypic identification of LC were not examined in the previous study, a monoclonal antibody against T$\beta 4$ which does not cross-react to T$\beta 10$ was prepared in the present study to examine whether LC contain T$\beta 4$ in the human and rat skin, where LC are also identified by the presence of CD1a, HLA-DR, and S-100 protein.

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MATERIALS AND METHODS

Human and rat skin samples

Normal human skin samples were obtained from patients who visited the outpatient department of Niigata University Medical Hospital or Niigata Cancer Center Hospital for skin biopsy examination. Normal rat skin was obtained from newborns, 1-week, 2-month, and 5-month-old WKY rats. These samples were divided into two parts. One part was fixed in the 4% paraformaldehyde in PBS, pH-7.4 overnight and embedded in paraffin for immunohistochemistry. The other part was incubated in 2M BrNa for 30 min at room temperature for separation of the epidermis from the dermis; the sheet of epidermis was homogenized in modified guanidinium thiocyanate buffer (TRIZOL Reagent, GIBCO BRL., Maryland, USA) for isolation of the protein fraction according to the manufacture's protocol.

Western blot analysis

Recombinant Tβ4 and Tβ10 polypeptides were prepared as follows: E. coli (DE3, Novagen, Inc. Madison, WI) were transformed by an expression vector (pET3a, Novagen) inserted with Tβ4 cDNA (~700bp) or Tβ10 cDNA (~500bp), cultured in TB medium and stimulated with 1mM IPTG when the OD600 of the culture medium reached 0.6~0.8. After 5 h, the E. coli were lysed by freezing and thawing three times and centrifuged at 18,000g for 10 min.

BALB/c mice were immunized with ~50μg of the supernatants emulsified with complete Freund's adjuvant several times. Then their spleen cells were fused with a myeloma cell line- NS1 - by a polyethyleneglycol method, and monoclonal antibodies secreted from the hybridomas were screened by Western blot analysis.

For Western blot analysis, the supernatants containing recombinant Tβ4 or Tβ10 and the human epidermis protein fraction were mixed with Laemmli's buffer, boiled for 5 min at 95℃ and electrophoresed on 20% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE: IWAKI GLASS Co. Ltd., Tokyo, Japan). The gel was incubated in PBS containing 10% glutalaldehyde for 1 h, washed three times in PBS for 10 min, incubated in a blotting buffer for 30 min at room temperature, and then the protein bands were transferred to a polyvinylidene difluoside membrane (Immobiron-P, Japan Millipore, Ltd., Tokyo, Japan). The blots were sequentially incubated with 5% skim milk/PBS containing 0.05% Tween 20 (PBST) and anti-Tβ4 monoclonal antibodies or normal mouse serum (~1μg IgG/ml). The blots were

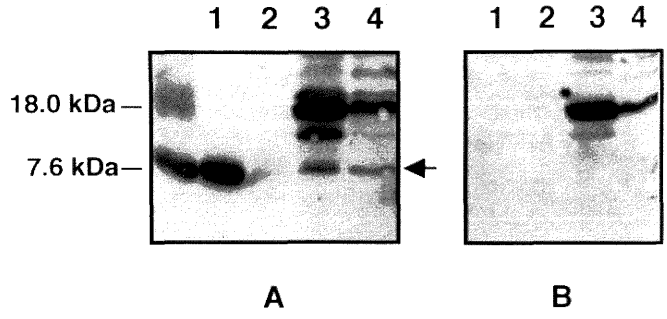

Fig. 1. Western blot analysis. **A.** Monoclonal antibody against Tβ4 detects the recombinant Tβ4 (lane 1) but not Tβ10 (lane 2) and also atains specific bands corresponding to Tβ4 in the protein fractions of human epidermis and dermis (lane 3, 4: *arrow*). **B.** No Tβ4 bands are stained with normal mouse serum.

washed three times with PBST, incubated with peroxidase-conjugated rabbit antibody against mouse immunoglobulins (1:2000 dilution, DAKO Japan, Kyoto, Japan) for 2 h, and washed three times with PBST. The immunoreacted bands were visualized by ECL (Enhanced chemiluminescence regent, Amersham, Buckinghamshire, UK) using Hyper-film (Amersham).

Immunohistochemistry

The paraffin-embedded tissues were sectioned at a 2 ~4 μm thickness. After dewaxing and hydration through graded ethanol, the sections were incubated in: a bleaching solution (100% methanol contained 0. 3% H_2O_2) for 5 min to block endogenous peroxidase, 10% normal rabbit serum for 20 min at room temperature, and with monoclonal antibodies against Tβ4(10μg/ml), human CD1a (Immunotech, Marseilles, France), HLA-DR (Nichirei Corp., Tokyo, Japan), rabbit antibody against human S-100 protein (DAKO Japan) or normal mouse serum (~10μg IgG/ml) as a control at 4℃ overnight, followed with biotinylated rabbit antibody to mouse immunoglobulins or biotinylated goat antibody to rabbit immunoglobulins (10μg/ml, Nichirei Corp.) and peroxidase-conjugated streptavidin (100μg/ml, Nichirei Corp.). The staining was visualized with the metal-enhanced DAB reaction (Nichirei Corp.), and the sections were counterstained with hematoxyline. Alternatively, fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse immunoglobulins (DAKO Japan) or rhodamine isothiocyanate (RITC)-labeled goat anti-rabbit immunoglobulins (1: 20 dilutions, DAKO Japan) was used as a second antibody and the sections were examined under a Zeiss laser microscope (Carl Zeiss Inc., Jene, Germany).

RESULTS

Western blot analysis

Monoclonal antibodies were selected by Western blot analysis using recombinant Tβ4 and Tβ10, and a monoclonal antibody was demonstrated to react to the recombinant Tβ4 but not to the recombinant Tβ10(Fig 1A: Lanes 1,2). The presence of Tβ4 protein in human epidermis and dermis was also examined by Western blot analysis. The monoclonal antibody stained specific bands corresponding to Tβ4 in the protein fractions of human epidermis and dermis (Fig 1A: Lanes 3, 4: arrow). No bands of recombinant Tβ4 and Tβ10 or those corresponding to the native Tβ4 in the human skin samples were blotted with normal mouse serum (Fig 1B), although non-specific bands of larger molecular mass were shown in both blots.

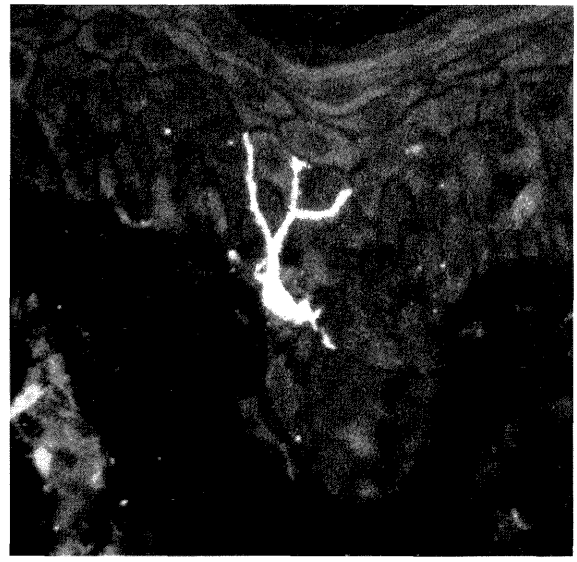

Fig. 2. Tβ4 expression in dendritic cells in human epidermis by laser-microscopy. Tβ4 is intensely expressed in star-shaped cells in human epidermis when examined under a laser scanning confocal microscope (Zeiss). ×640

Immunodetection of Tβ4-positive (Tβ4$^+$) cells in human skin

In the human skin, anti-Tβ4 antibody intensely stained the dendritic or star-shaped cells sparsely present in the epidermis as observed by laser microscopy (Fig 2). The dendrites were expanded into the spinal layer of the epidermis.

To examine whether the Tβ4$^+$ cells were LC or not, serial sections were stained either with anti-CD1a, anti-HLA-DR, anti-S-100 protein or anti-Tβ4 antibody. Tβ4$^+$ cells and CD1a$^+$ cells were present in the epidermis as solitary cells which were discriminated from keratinocytes (Fig 3a, b). By observing the same sites in the sequential sections, the Tβ4$^+$ cells were also found to be labeled with anti-CD1a antibody (Fig 3a, b: arrows); however, not all the CD1a$^+$ cells were always Tβ4$^+$.

The expression of HLA-DR on Tβ4$^+$ cells was similarly examined by immunostaining for HLA-DR or Tβ4 on sequential sections of human skin. Again, the immunohistochemistry demonstrated that the Tβ4$^+$ cells were all HLA-DR$^+$ (Fig 3c, d).

Double staining using anti-Tβ4 antibody colored with FITC (Fig 4a, d) and anti-S-100 protein antibody

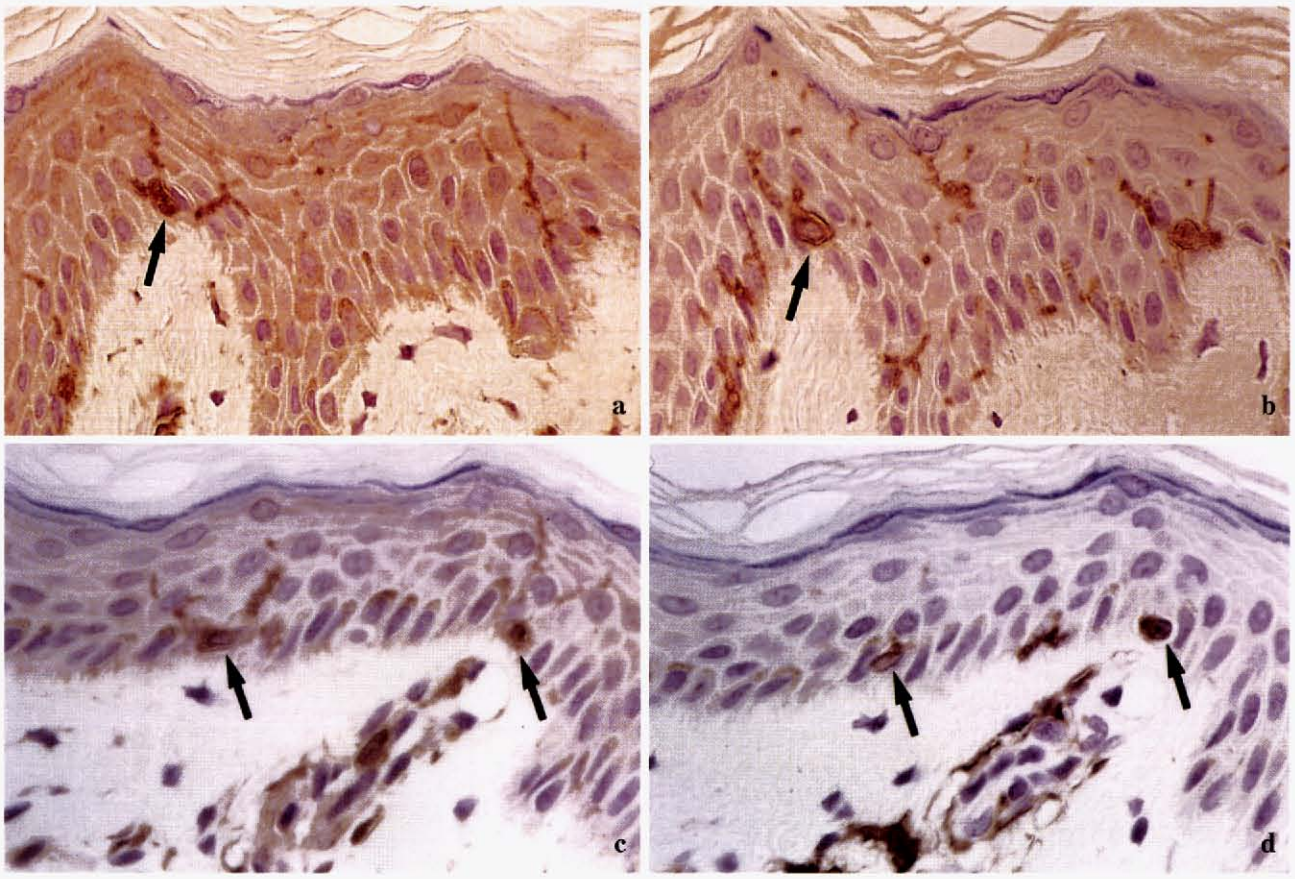

Fig. 3. Immunocytochemical localization of thymosin $\beta 4$, CD1a, and HLA-DR staining in normal human skin. Anti-T$\beta 4$(**a**, **c**) and anti-CD1a (**b**) or anti-HLA-DR (**d**) staining on sequential sections with hematoxyline counter-staining on paraffin sections. A T$\beta 4^+$ cell (a: *arrow*) is labeled with anti-CD1a antibody (b: *arrow*). T$\beta 4^+$ cells are also stained with anti-HLA-DR antibody. (c, d: *arrows*) $\times 520$

colored with RITC (Fig 4b, e) depicted the presence of T$\beta 4^+$/S-100$^+$ double-positive cells in the skin as yellow, T$\beta 4^+$/S-100$^-$ single-positive cells as green, and T$\beta 4^-$/S-100$^+$ single-positive cells as red (Fig 4c, f). T$\beta 4^-$/S-100$^+$ cells were localized in the basal layer of epidermis (Fig 4a-f: arrowheads). On the other hand, T$\beta 4^+$/S-100$^-$ cells were negligible in the epidermis, and endothelial cell were T$\beta 4^+$/S-100$^-$ in dermis. The T$\beta 4^+$ cells in the epidermis always possessed S-100 protein and were imaged as yellow cells (Fig 4c, f: arrows). Cells with a round cell body and dendrites expanding into the spinal layer were intensely stained with both anti-T$\beta 4$ and anti-S-100 protein antibodies in the epidermis (Fig 4a-f: arrows). The T$\beta 4^+$/S-100$^+$ cell was also found in close association with dermal capillaries.

Localization of T$\beta 4$ in the rat skin during development

In the newborn rat skin, anti-T$\beta 4$ antibody intensely stained solitary cells which extended their short processes between keratinocytes in the epidermis (Fig 5a). Their processes tended to elongate in the epidermis with age (Fig 5C). In contrast, the density of T$\beta 4$ in these cells seemed to weaken with age. (Fig 5a, c).

DISCUSSION

The present study demonstrated the presence of T$\beta 4$-bearing cells in the epidermis of human and rat skin. The T$\beta 4$ was morphologically identified in a population of epidermal cells which were characterized by elongated dendritic processes projecting from their cell bodies between keratinocytes. This feature

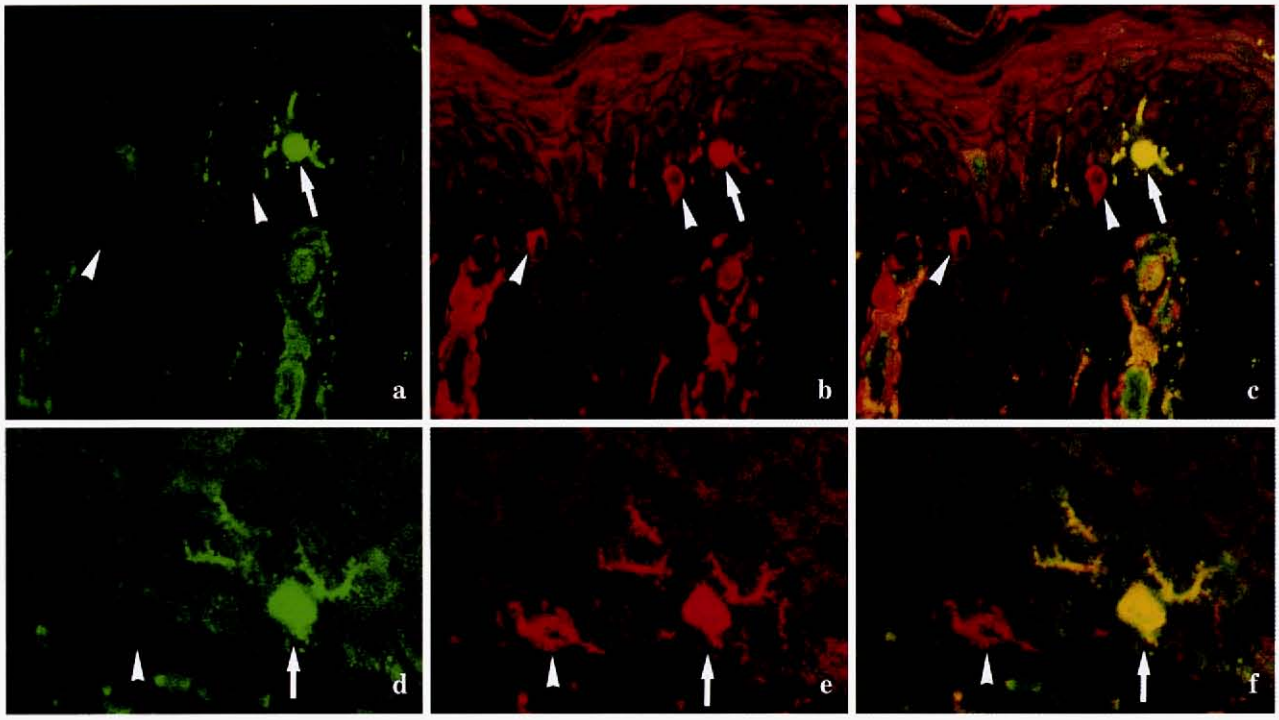

Fig. 4. Double-labeled indirect immunofluorescence of human skin. Tβ4 is colored green (**a**, **d**) and S-100 *red* (**b**, **e**). Overlayed images are shown in Figs c and d. Tβ4$^+$ cells (*green*) in epidermis always express S-100$^+$(*red*), resulting in yellow cells (a-f: *arrow*). Melanocytes in the basal layer of epidermis are detected as Tβ4$^-$/S-100$^+$ cells (red: *arrowheads*). a, b, c: ×640 d, e, f: ×1280

suggests that they are LC since the cells are histologically recognized by their localization in the mid portion of the stratum spinosum, nuclei of reniform to cleaved contours, and delicate dendrites often extending to the level of the stratum corneum. However, LC have been identified more definitely by their phenotypic markers such as CD1a, HLA-DR, and S-100 protein.[18-21] By double immunostaining for Tβ4 and S-100 protein or by immunohistochemical staining for Tβ4 and CD1a or HLA-DR on sequential sections, the Tβ4$^+$ cells were identified as cells which also expressed CD1a, HLA-DR or S-100 protein. The findings indicate that Tβ4$^+$/CD1a$^+$/HLA-DR$^+$/S-100$^+$ cells in the epidermis are LC. Tβ4$^-$/S-100$^+$ single-positive cells in the epidermis are considered melanocytes.

The presence of Tβ4 in LC in human, monkey and rat skin was reported previously;[16] however, the specificity of the antibody against Tβ4 remained to be fully verified since a possible cross-reactivity of the antibody to Tβ10 had not been examined in the study.[34,35] The monoclonal antibody used in the current study was demonstrated to react specifically to Tβ4 but not to Tβ10, indicating that this antibody is a good tool for differentiating Tβ4 from Tβ10 without a sequencing of their amino acids. In addition, the identification of LC was based on their morphologic characteristics from the previous study[16]. In contrast, LC were critically identified by the phenotypic markers recently defined: the CD1a, HLA-DR, and S-100 protein of the present study. Since cells other than LC or keratinocytes in epidermis, such as melanocytes or indeterminate cells, have been demonstrated to express one of these markers, all these markers should be examined for the identification of LC, as was done in this study. As a consequence, the present study apparently demonstrated that Tβ4 was intensely and exclusively expressed in LC in the epidermis and confirmed the previous study. Additionally, Tβ4 can be proposed as a marker of LC in the epidermis.

LC are bone marrow-derived cells that begin to appear in the epidermis by 7 weeks of gestation in humans and are immunologically related to the monocyte-macrophage-histiocyte series,[36,37] functioning as antigen-presenting cells in the skin.[38] They are present not only in the skin but also in the lymph-nodes, thymus, and occasionally in the dermis.[39] The Tβ4$^+$/S-100$^+$ cells seen in association with dermal capillaries may be progenitors of LC or histiocytes.

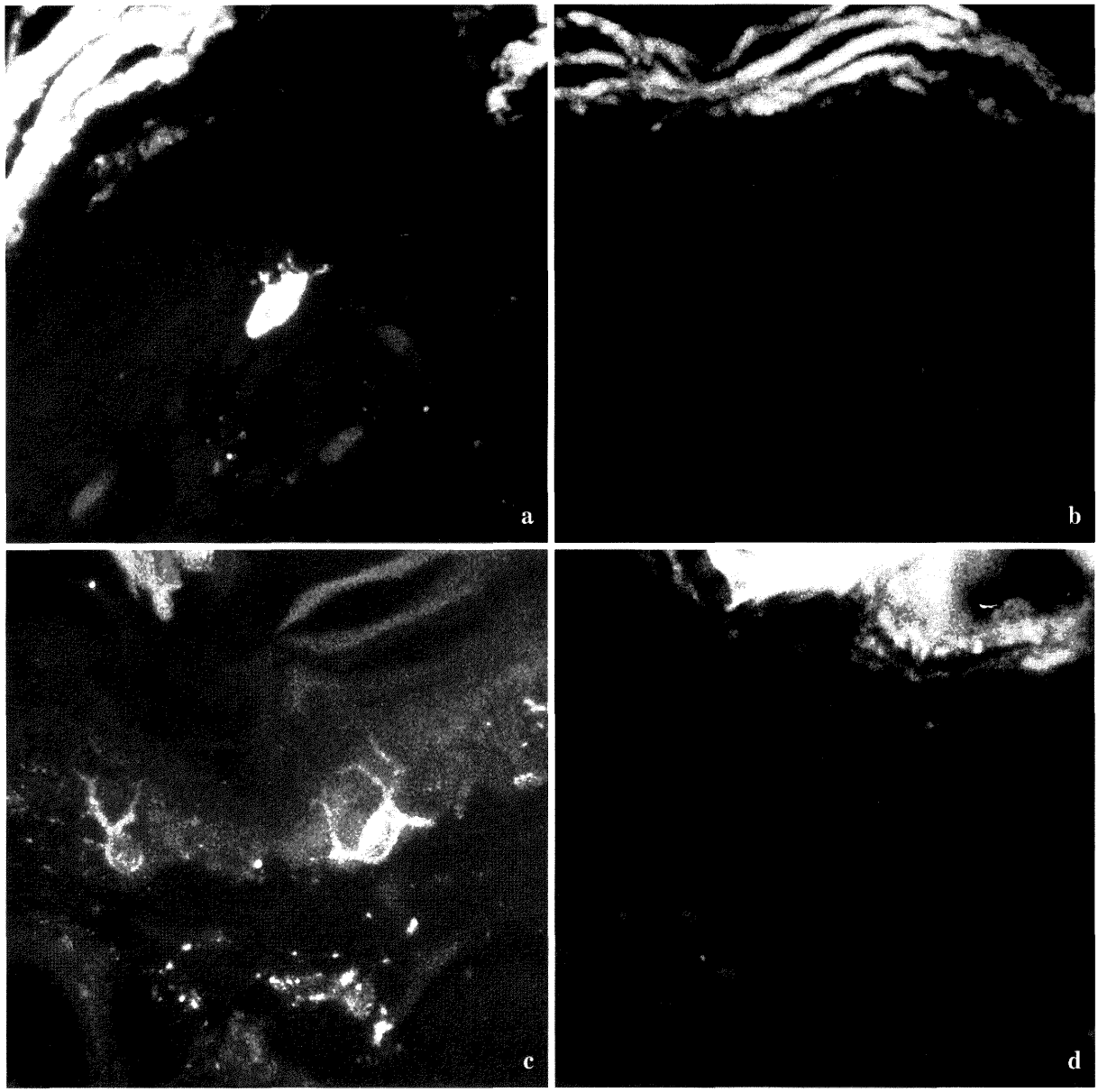

Fig. 5. Tβ4 expression in rat epidermis during development. **a.** In the newborn rat skin, anti-Tβ4 antibody intensely stains a solitary cell with short processes in the epidermis **c.** The dendritic and elongated processes are conspicuous in the Tβ4$^+$ cells in 5-month-old rat skin **b and d.** No specific staining, except for a horny layer, is shown with normal mouse serum. ×850

Recent studies showed that nondifferentiated CD34$^+$ hematopoietic progenitors were derived from peripheral blood and differentiated into resident epidermal LC through the cooperation of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-α presumably released from keratinocytes and melanocytes.[40,41]

The implication of Tβ4 in LC is unknown; however, a series of evidence has suggested that Tβ4 plays an important role in cell movement, extension, or attachment through actin polymerization or depolymerization by the G-actin-sequestering activity of Tβ4.[7,9,42] The involvement of actin is easily presumed in the migration of LC from the circulation to the epidermis. Additionally, actin-filaments in the LC may be implicated in the intimate adhesion of LC to keratinocytes. This intimate adhesion is mediated by E-cadherin and involved in the transfer of antigens from keratinocytes to LC.[43–45] In conclusion, Tβ4 intensely expressed in LC may play a crucial role in

the migration and adhesion of LC to keratinocytes. The roles of Tβ4 in the LC remain to be studied in the future.

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