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Notch Signaling May Be Involved in the Abnormal Differentiation of Epidermal Keratinocytes in Psoriasis

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Localization of each keratin isoform differs among epidermal layers. Proliferating basal cells synthesize keratin 14 (K14) and suprabasal cells express keratin 10 (K10) in normal skin. Notch signaling is essential for keratinocyte differentiation. Notch1 is expressed in all epidermal layers, Notch2 in the basal cell layer and Notch3 in basal cell and spinous cell layers in normal epidermis. It has been poorly elucidated how localization and expression levels of Notch molecules are related to epidermal molecular markers K10 and K14 in psoriatic skin with abnormal differentiation of epidermal tissue. This study aimed to investigate the relationship between abnormal differentiation of epidermal cells in psoriatic skin and expression of Notch molecules. We investigated keratins (K14 and K10) and Notches (1, 2, 3 and 4) using immunohistochemistry in psoriatic skin (n=30) and normal skin (n=10). In normal skin, K14 and K10 were discretely observed in the basal cell layer and suprabasal layer, respectively. In psoriatic skin, K14 was expressed in the pan epidermal layer while it and K10 were co-expressed in some middle suprabasal layer cells. Notch1, 2, 3, and 4 localized in all epidermal layers in normal skin. In psoriatic skin, Notch1, 2, and 4 mainly localized in suprabasilar layers and Notch3 is lacalized in pan epidermal, suprabasilar, and basilar layers. Protein and mRNA of Notch1, 2, and 3 isoforms decreased in psoriatic epidermis compared with normal epidermis. These data suggest that decrements in these Notch molecules might cause aberrant expression of K10 and K14 leading to anomalous differentiation of the epidermis in psoriatic lesions.

Key words: keratin, Notch, psoriasis, skin, laser microdissection

I. Introduction

Psoriasis is a common, genetically determined, inflammatory and proliferative disease of the skin. Psoriatic skin contains large numbers of immune cells that produce many cytokines, chemokines, and inflammatory molecules. The epidermis divides much faster than normal and has a defective outer layer or barrier, which under normal circumstances protects from infection and dehydration [19]. Psoriatic skin is characterized by increased turnover of epidermal cells, thickened epidermis, abnormal keratinization, and inflammatory cell infiltrates [3].

Epidermis expresses predominantly two pairs of keratin polypeptides. Basal cells express K5 (type II basic keratin of 58 kDa) and K14 (type I acidic keratin polypeptide of 50 kDa). As the basal cells divide and enter the first suprabasal layer, downregulation of K5 and K14 expression and the induction of K1 (type II basic keratin of 67 kDa)

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and K10 (type I acidic keratin polypeptide of 56.5 kDa) expression occurs. Keratin K1 and keratin K10 are only synthesized in differentiating epidermal cells. This synthesis continues until a thickness of 4 to 8 spinous cells is achieved and represents one of the earliest changes indicating the commitment of the cell to terminal differentiation [6, 7, 21, 22]. Many studies have shown that in the majority of patients with psoriasis, suprabasal K1 and K10 are downregulated [2, 21, 24, 26]. Furthermore, the expression levels of K5 and K14 in the basal cell layer are altered in the psoriatic epidermis [9]. Aberrant expressions of keratin molecules suggest that an abnormal differentiation may arise in psoriasis epidermis.

Notch proteins comprise a family of four type 1 transmembrane receptors that include four genes (Notch1, 2, 3, and 4), with abnormalities in organ development and adult homeostasis reported in cases with mutations in these genes [1, 25]. Notch ligands in mammals include Delta-like 1, 3, and 4 and Jagged-1 and -2. Notch signaling results in interaction between two neighboring cells, which leads to successive proteolytic cleavage reactions that liberate the cytoplasmic domain of Notch (NICD, Notch intracellular domain) from the membrane [10–12, 16, 20]. The activation of Notch signaling results in the promotion of growth arrest and onset of differentiation. Notch signaling functions as a molecular switch that controls the transition of cells between skin layers during the epidermal differentiation process [16]. In addition, previous studies showed the involvement of Notch signaling in keratinocyte differentiation in embryos of mice and rats [5, 25]. The role of Notch signaling remains obscure in the pathogenesis of psoriasis, although an abnormal differentiation of epidermis has been observed. This study aimed to investigate the relationship between abnormal differentiation in psoriatic skin and expression of Notch molecules.

II. Materials and Methods

Patients and skin samples

Skin punch (6 mm) biopsies were obtained from 30 patients who were clinically and pathologically diagnosed as having psoriasis (21 males, 9 females; age range 21–75 years). Ten normal skin specimens were obtained from surgical margins from the abdomen of non-psoriatic individuals (6 males, 4 females; age range 31–85 years). Skin samples were taken from the trunk (n=7), forearms (n=5), upper arms (n=4), femurs (n=6), and lower legs (n=8). Measures of disease burden (BSA: body surface area of involvement) assist in determining the severity of disease. This study was approved by the Tokai University Hospital ethical committee (Authorization number: 10-114), and written informed consent was obtained from all individuals.

Immunohistochemistry

Human skin specimens were fixed in 10% buffered formalin, dehydrated through successively more concentrated ethanol solutions, and finally embedded in paraffin. Tissue sections 4-μm thick were prepared for hematoxylin and eosin staining and immunohistochemistry (IHC). For IHC, specimens were dewaxed and rehydrated before staining. Endogenous peroxidase was inactivated with methanol containing 0.3% hydrogen peroxidase for 30 min at room temperature. For immunostaining of Notch1, Notch2, Notch3, and Notch4, sections were heated in a microwave for 5 min in 10 mmol/L citrate buffer (pH 6.0). Rabbit anti-human Notch1 antibody (Neomarkers Inc, Fremont, CA, USA) was used at a 1:100 dilution. Rabbit anti-human Notch2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a 1:100 dilution. Rabbit anti-human Notch3 antibody (Santa Cruz Biotechnology) was used at a 1:50 dilution, and rabbit anti-human Notch4 antibody (Santa Cruz Biotechnology) was used at a 1:100 dilution. After incubation overnight at 4°C and washing thoroughly with 0.01 M PBS, secondary antibody was performed by using anti-rabbit. Following washing with 0.01 M PBS, the signal was amplified with DAKO ENVISION+Kit (DAKO cytomation, Glostrup, Denmark) according to the manufacturer's recommendations. Horseradish peroxidase activity was visualized with 3'3-diaminobenzidinetetrahydrochloride. The sections were lightly counterstained with hematoxylin. Immunohistochemical specificity of all the antibodies was confirmed by non-immune immunoglobulins or normal serum as negative controls.

Anti-human K10 and K14 monoclonal antibodies were used at a 1:100 dilution (Abcam, Cambridge, UK). For double immunostaining of K10 and K14, sections were autoclaved at 121°C in 10 mmol/L citrate buffer (pH 6.0) for 10 min. First, the sections were incubated with K10 monoclonal antibody overnight at 4°C. Following washing with 0.01 M PBS, the signal was amplified with DAKO ENVISION+Kit. After being washed 5 times in 0.01 M PBS, horseradish peroxidase activity was visualized with 3'3-diaminobenzidine tetrahydrochloride. After DAB color development, we preceded with the second phase of double-labeling the tissue. To thoroughly remove the antibodies used in the K10 immuno-labeling process, tissues were immersed in glycine-HCl buffer (pH 2.2) for 2 hr, followed by a 30 min wash in 0.01 M PBS. Sections were then processed for the second labeling with antibodies to K14. The sections were incubated with anti-human K14 monoclonal antibody overnight at 4°C. After 10 washes, the signal was amplified with Histofine simple stain AP (Nichirei, Tokyo, Japan) according to the manufacturer's recommendations. Immunoreactivity was visualized by Fast Blue RR Salt (Sigma Aldrich, St Louis, MO, USA) and naphthol AS-BI phosphate disodium salt (Sigma Aldrich) in 0.05 M propanediol buffer (pH 9.8) without the use of counterstain.

Laser microdissection and real-time reverse transcription polymerase chain reaction (RT-PCR)

Tissue sections (8-μm thick) were prepared from the formalin-fixed paraffin-embedded tissue blocks and

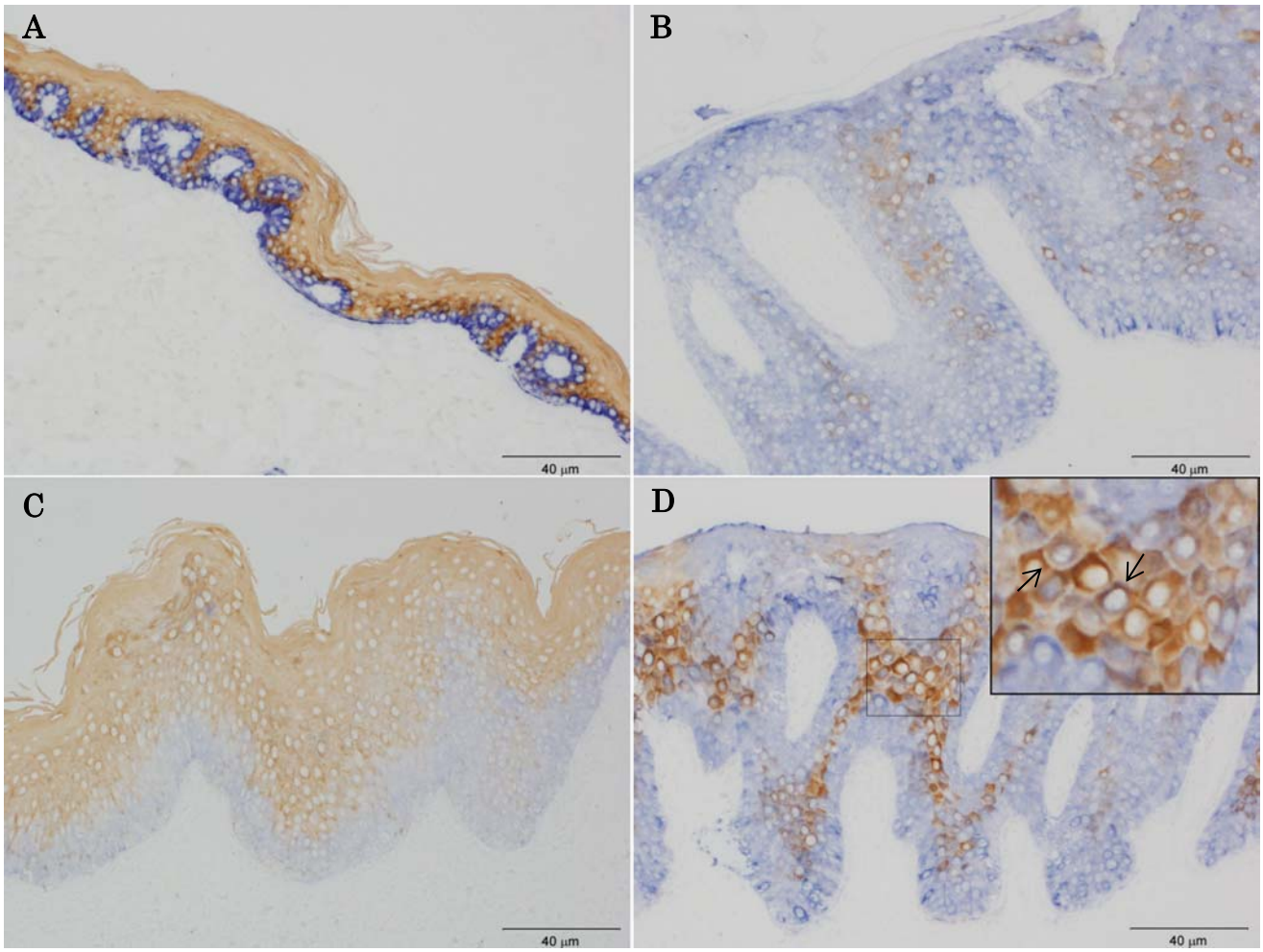

Fig. 1. Double immunostaining for K10 and K14. **A**, normal skin. **B–D**, psoriatic skin. In normal epidermis, K10 (brown) and K14 (violet) localized in spinous layers and basal cell layers, respectively. K14-immunopositive cells were observed in the full thickness of the epidermis (**B**). In only 1 case was K14 localized in the basilar layer (**C**). Co-localization of K14 with K10 was observed in psoriatic skin (**D**, black arrows). (Original magnification ×100).

counterstained with toluidine blue. We used 5 psoriatic skin samples and 5 normal skin samples. For separation of epidermis and dermis in the psoriatic and normal skin, a laser microdissection assay was performed using a laser microdissection system (LM) (Carl Zeiss MicroImaging, Jena, Germany) coupled with real-time reverse transcriptase PCR [13, 17]. Total RNA was extracted from dissected epidermis or dermis skin sections using RNeasy FFPE Kit (QIAGEN, Hilden, Germany). For synthesis of first-strand cDNA, RNA was reverse transcribed by incubation with random primers and a first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions, and Notch1, Notch2, Notch3, Notch4, and GAPDH were quantified using commercially available kits (TaqMan Gene Expression Assays Hs01062014, Hs02341881, Hs01128541, Hs00965889 and Hs99999905_m1, respectively; Applied Biosystems). These primer sets were designed to span one intron to allow identification of genomic contamination. The reaction protocol consisted of the following cycles: 95°C for 15 min, 95°C for 15 sec, and 60°C for 1 min for 50 cycles of PCR amplification on an Opticon 2 System (BioRad, Hercules, CA, USA). All data were analyzed on an Option monitor 3 (BioRad).

Statistical analysis

Values are expressed as the mean±SD. Data were assessed for localization of K14, Notch1, 2, 3, and 4 by using Fisher's exact test. $P<0.001$ was considered significant. With respect to intensity of immunostaining for Notch1, 2, 3, and 4, data were assessed by using the Mann-Whitney *U* test. $P<0.001$ was considered significant. The correlations between the body surface area of the involved skin in psoriasis and the intensity of Notch1, 2, 3, and 4 were analyzed using a Spearman's rank correlation test. $P<0.05$ was considered significant. Data from real time RT-PCR assay were compared by using a paired *t* test. $P<0.05$ was considered significant. Statistical analysis of immuno-

Table 1. *Double immunostaining for K10 and K14 in normal and psoriatic epidermis*

Keratin10	Psoriasis (No.=30)	Normal (No.=5)	Test of significance	P
Localization				
Basilar	0	0		
Suprabasilar	30	5	NA	—
Pan epidermal	0	0		
Negative	0	0		
Keratin14	Psoriasis (No.=30)	Normal (No.=5)		
Localization				
Basilar	1	5		
Suprabasilar	0	0	Fisher exact test	*<0.001
Pan epidermal	29	0		
Negative	0	0		

NA indicates not assessed. *P<0.001.

histochemistry was performed using IBM SPSS Statistics Version 22 (IBM SPSS, Tokyo, Japan).

III. Results

Unlike normal skin, the border between the basal and spinous cell layers in the psoriasis epidermis is obscure. In this study, the epidermis was examined in three categories, the suprabasilar, basilar, and pan-epidermal layers and subjected to immunohistochemical analysis. The pan-epidermal area consisted of the basilar and suprabasilar layers.

Immunohistochemical analysis for K10, 14 and Notch1, 2, 3, 4

Double IHC of K10 and K14 showed that K14 localized in the basal layer and K10 expressed in the suprabasal layers in the normal epidermis (Fig. 1A). In psoriatic lesions, K14 was expressed in the pan epidermal layer (Fig. 1B). In only one case was there basal layer localization (Fig. 1C). K10 was expressed in suprabasal layers in all cases. Interestingly, K14 and K10 co-localized in cells in the middle suprabasal layer in 15 cases (Fig. 1D). The staining intensity of K10 and K14 in psoriatic epidermis was slightly weaker compared with that of normal epidermis (Fig. 1). Results of immunohistochemical analysis for K10 and K14 are summarized in Table 1. We examined the difference in the localization between normal and psoriatic skin. As for Keratin14, there was a significant difference (P<0.001). We compared the proportion of positive areas of K10 and K14 between psoriatic and normal epidermis and found that with both K10 and K14 there were significant differences (positive area of normal epidermis: K10:K14=69%:31%, positive area of psoriatic epidermis: K10:K14=17%:83%).

Results of IHC for Notch1, 2, 3, and 4 are summarized in Table 2. In normal skin, Notch1, 2, 3, and 4 was seen in every layer of the epidermis (Fig. 2A, C, E and G). In psoriatic skin, there was a tendency for Notch3 to be localized in every layer of the epidermis (Fig. 2F), and there was a tendency for Notch1, 2, and 4 to be localized in only the suprabasal layers (Fig. 2B, 2D and 2H). As for significance, there was a significant difference between normal

Table 2. *Localization of Notch1, 2, 3, and 4 in normal and psoriatic epidermis*

Notch1	Psoriasis (No.=30)	Normal (No.=10)
Localization		
Basilar	0	0
Suprabasilar	26*	0
Pan epidermal	4	10
Negative	0	0
Notch2	Psoriasis (No.=30)	Normal (No.=10)
Localization		
Basilar	0	0
Suprabasilar	18	0
Pan epidermal	12	10
Negative	0	0
Notch3	Psoriasis (No.=30)	Normal (No.=10)
Localization		
Basilar	9	0
Suprabasilar	4	0
Pan epidermal	17	10
Negative	0	0
Notch4	Psoriasis (No.=30)	Normal (No.=10)
Localization		
Basilar	0	0
Suprabasilar	27*	0
Pan epidermal	1	10
Negative	2	0

Differences were analyzed using the Fisher exact test, *P<0.001.

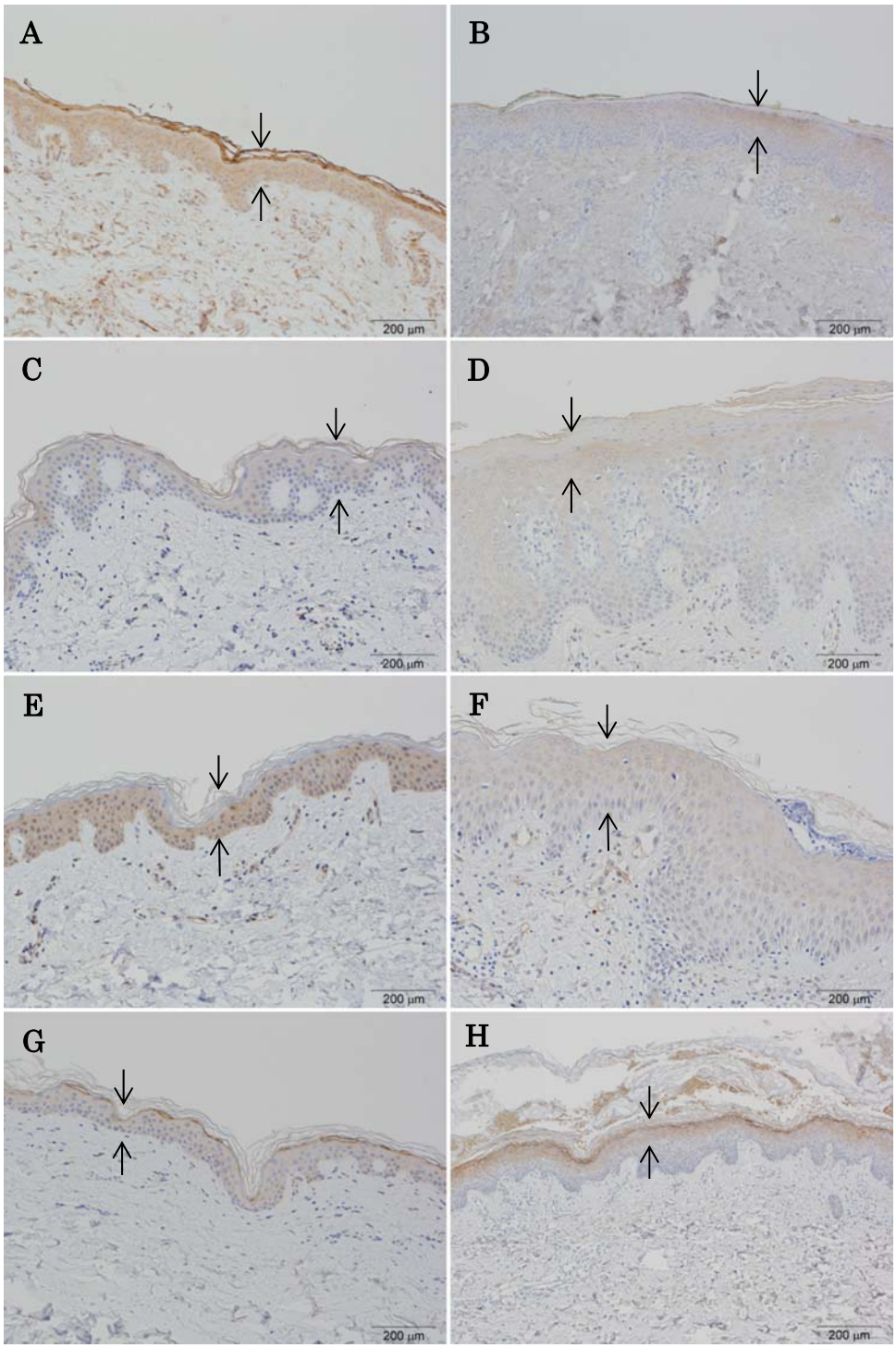

Fig. 2. Immunohistochemical localization of Notch1 (**A, B**), Notch2 (**C, D**), Notch3 (**E, F**), and Notch4 (**G, H**). Representative examples from normal skin (**A, C, E, G**) and psoriatic skin (**B, D, F, H**). (Original magnification ×100). In psoriatic skin, Notch1, 2, and 4 localized in suprabasal layers and Notch3 localized in pan epidermal. Black arrows indicate positive cells.

skin and psoriatic skin in the localization of Notch1 and 4 ($P<0.001$). Based on the results that in normal skin and psoriatic skin there were differences in Notch1 and 4 in the suprabasilar layer, it could be said that aberrant expression of K14 may be the result of this difference. With regard to the pattern of staining, Notch1, 2, and 4 localized in cytoplasm in both normal and psoriatic skin whereas Notch3 was observed in both the cytoplasm and nucleus in 8 non-psoriatic cases and 2 non-psoriatic cases showed cytoplasmic staining. In psoriatic skin, Notch3 staining revealed a

Table 3. *Intensity of immunostaining for Notch1, 2, 3, and 4 in normal and psoriatic epidermis*

Case No	BSA	Age/Sex	Notch1	Notch2	Notch3	Notch4
1	100	51/F	2	1	1	1
2	100	70/F	1	1	1	1
3	80	44/M	2	1	1	1
4	80	30/M	2	1	2	2
5	70	58/M	1	1	1	1
6	70	56/M	1	2	1	1
7	70	33/F	1	2	2	1
8	50	21/F	1	1	1	1
9	40	83/M	1	1	2	1
10	30	35/M	1	1	1	1
11	30	59/M	1	2	2	1
12	30	76/M	2	1	2	2
13	30	71/M	2	1	2	1
14	30	47/M	1	1	1	1
15	20	59/M	1	2	2	1
16	15	60/F	1	1	2	1
17	15	59/F	2	1	2	1
18	15	41/M	1	2	2	1
19	15	64/M	1	1	1	1
20	10	72/M	1	1	1	1
21	10	33/M	1	1	2	1
22	10	39/F	1	2	1	2
23	10	57/M	1	1	2	2
24	10	36/M	1	1	1	1
25	10	29/M	1	1	1	0
26	10	61/M	2	1	1	1
27	10	75/M	1	1	1	1
28	10	34/F	1	1	1	0
29	10	45/F	1	1	1	1
30	5	63/F	2	1	1	1
control No.						
1		96/F	2	2	2	1
2		73/F	1	2	2	1
3		73/F	2	2	1	1
4		84/M	2	2	2	1
5		72/M	2	2	2	1
6		31/M	2	2	1	1
7		76/F	2	2	2	1
8		61/F	2	2	1	2
9		69/M	2	2	2	2
10		77/F	2	2	2	2

0, negative; 1, weakly positive; 2, positive. Moderate to severe form was defined by body surface area (BSA) involvement of not less than 10%.

cytoplasmic and nuclear pattern in 18 cases while 12 cases had a cytoplasmic pattern.

These data demonstrate that the abnormal differentiation of keratinocytes is caused by a decline in Notch expression in the inferior portion of the stratum spinosum.

Expression level of Notch in normal skin and psoriatic skin

The intensity of Notch expression was assessed as negative (0), weakly positive (1), and positive (2). The intensities of Notch1 and 4 were not correlated with the BSA (Notch1: $r=0.15$, $P=0.44$; Notch4: $r=-0.12$, $P=0.55$). But the intensities of Notch2 and 3 were slightly correlated with the BSA (Notch2: $r=-0.21$, $P=0.27$; Notch3: $r=-0.34$, $P=0.07$).

The staining intensity of Notch1, 2, and 3 in psoriatic epidermis was slightly weak compared with that of normal

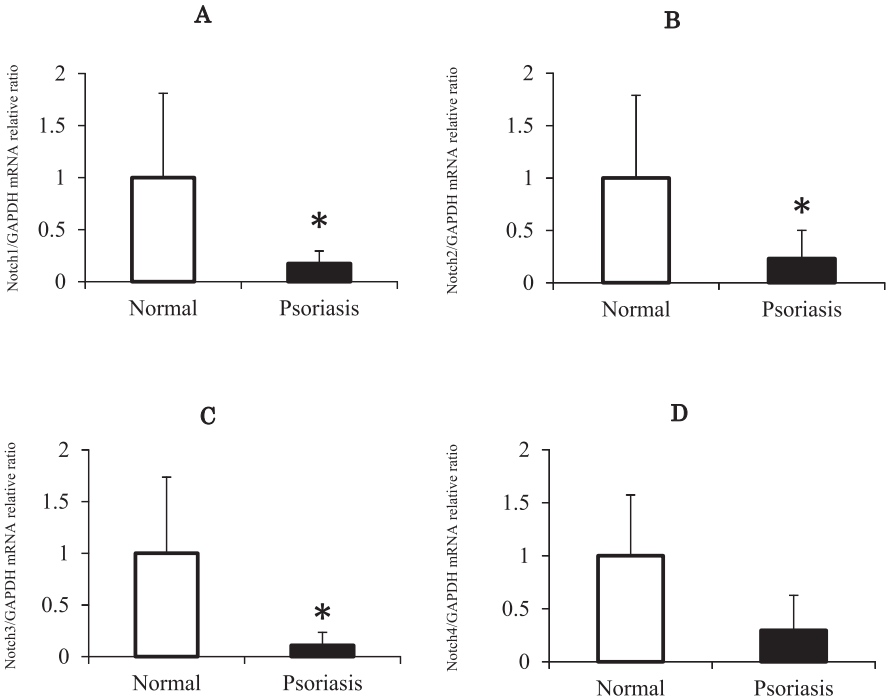

Fig. 3. Quantitative real-time RT-PCR analysis of Notch mRNA in the normal and psoriatic epidermis using laser microdissection. **A**, Notch1; **B**, Notch2; **C**, Notch3; **D**, Notch4. mRNA expressions were analyzed by quantitative RT-PCR. *P<0.05.

epidermis (Table 3). Staining intensity of Notch1 and Notch2 differed significantly between normal and psoriatic skin (Mann-Whitney *U* test, P<0.001). Furthermore, quantitative real-time RT-PCR analysis showed that the expression of Notch1, 2, and 3 in psoriatic skin was significantly decreased in comparison with normal skin (Notch1 was 83% reduced, Notch2 was 77% reduced, and Notch3 was 89% reduced). The expression of Notch4 did not differ significantly between normal and psoriatic skin (Fig. 3).

IV. Discussion

In this study, we showed the aberrant localization of K10 and K14 in psoriatic epidermis by double IHC. In addition, it was clearly demonstrated that the expressions of Notch1, 2, and 3 were decreased in psoriatic epidermis by quantitative real-time RT-PCR analysis. Notch1, 2, 3, and 4 localized in all epidermal layers in normal skin, while in psoriatic skin, Notch1, 2, and 4 mostly localized in the suprabasal layer and Notch3 localized in the pan epidermal, suprabasal and basilar layers. Notch1 and 4 were expressed at low levels in the basilar layer of the psoriatic epidermis. These results suggested that Notch1 and 4 were related to decreased expression of skin differentiation marker K14. Meanwhile, K14 was localized at the pan epidermal layer, although it was stained weakly compared with the normal epidermis. Therefore, it was suggested that Notch3, the expression of which was decreased at the pan-epidermal layer, was potentially the cause of decreased K14 expression. These results suggest that the decrease in some Notch molecules in psoriasis causes an aberrant differentiation of the epidermis.

Blanpain *et al.* found that Notch was activated by ligands and released the Notch intracellular domain (NICD) transfer nucleus, and then the NICD, CSL (CBF1/SU(H)/LAG-1) protein and Mastermind form a transcriptional activation complex that promotes expression of target genes in mouse skin [4]. The NICD plays two roles in driving the transition from basal to spinous cells. NICD interacts with RBP-J (recombination signal-binding protein-J kappa) to drive the expression of Hes1, a canonical target gene. This leads to the downstream induction of spinous layer genes encoding differentiation-specific proteins such as K1 and K10. NICD-RBP-J signaling represses basal gene expression, allowing basal cells to detach from the basal layers. However, this mechanism of repression does not require Hes1 molecules [4]. Taken together, our results demonstrated that the aberrant expression of K10 is independent of the action of Hes1 in psoriatic skin.

Psoriatic skin is characterized by hyperproliferation and aberrant differentiation. Notch1 is required for induction of p21 expression. Increased p21 causes proliferation of keratinocytes to promote cell cycle arrest and helps to initiate terminal differentiation [18]. Taking the above evidence into account, we considered that the decrease in Notch1 and Notch2 expression caused keratinocyte hyperproliferation in psoriatic skin through the induction of the expression of the p21 gene.

Notches are well known for cross-talking with several signaling molecules such as p63. Notch1 is regulated by

p63 in keratinocytes [14]. P63 is expressed in basal keratinocytes, blocking Notch1 dependent growth arrest and differentiation in mouse keratinocytes. Thus, p63 and Notch repress each other's function. In psoriatic skin, p63 expression was increased [14, 15]. Therefore, immunohistochemistry of p63 was performed with normal and psoriatic tissues. Contrary to expectations, there was no difference between p63 immunohistochemical expressions in normal and psoriatic tissues.

The results of this study elucidated that expression of Notch molecules was decreased in the psoriatic epidermis. It was speculated that decreased expression of Notch molecules induced dysfunction of Notch signaling and played an important role in the occurrence of psoriasis. Therefore, activation of Notch signaling with Notch molecules as a target may become a treatment option for psoriasis. To date, it has been reported that some diseases can be treated by activation of Notch signaling. Administration of antibody with the function of activating Notch2 suppressed urine protein excretion and advancement of glomerular sclerosis in mice with nephrotic syndrome and glomerular sclerosis [23]. Meanwhile, drugs such as lithium are used to control mood-activated Notch1 signaling [8]. It is anticipated that lithium or antibody to activate Notch signaling will be used to treat psoriasis.

Taken together, our study demonstrated that keratinocyte differentiation marker molecules were expressed anomalously and also that mRNA of Notch1, 2, and 3 were decreased in the psoriatic epidermis. We speculated that a void in Notch signaling underlies the abnormal differentiation of epidermal keratinocytes seen in psoriasis.

V. Acknowledgments

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